Spatially Resolved Genomic, Stable Isotopic, and Lipid Analyses of a Modern Freshwater Microbialite from Cuatro Ciénegas, Mexico

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Abstract

Microbialites are biologically mediated carbonate deposits found in diverse environments worldwide. To explore the organisms and processes involved in microbialite formation, this study integrated genomic, lipid, and both organic and inorganic stable isotopic analyses to examine five discrete depth horizons spanning the surface 25 mm of a modern freshwater microbialite from Cuatro Ciénegas, Mexico. Distinct bacterial communities and geochemical signatures were observed in each microbialite layer. Photoautotrophic organisms accounted for approximately 65% of the sequences in the surface community and produced biomass with distinctive lipid biomarker and isotopic ($\delta^{13}\text{C}$) signatures. This photoautotrophic biomass was efficiently degraded in the deeper layers by heterotrophic organisms, primarily sulfate-reducing proteobacteria. Two spatially distinct zones of carbonate precipitation were observed within the microbialite, with the first zone corresponding to the phototroph-dominated portion of the microbialite and the second zone associated with the presence of sulfate-reducing heterotrophs. The coupling of photoautotrophic production, heterotrophic decomposition, and remineralization of organic matter led to the incorporation of a characteristic biogenic signature into the inorganic CaCO_3 matrix. Overall, spatially resolved multidisciplinary analyses of the microbialite enabled correlations to be made between the distribution of specific organisms, precipitation of carbonate, and preservation of unique lipid and isotopic geochemical signatures. These findings are critical for understanding the formation of modern microbialites and have implications for the interpretation of ancient microbialite records. **Key Words:** Microbial ecology—Microbe-mineral interactions—Microbial mats—Stromatolites—Genomics. *Astrobiology* 12, 685–698.

1. Introduction

MICROBIALITES are organosedimentary mats composed of microorganisms that influence accretion through the trapping of sediment and formation of mineral precipitates (Burne and Moore, 1987). The abundance of these carbonate structures in the geological record and presence of fossilized examples that are over 3 billion years old (Grotzinger and Knoll, 1999) suggest that microbialite research is an important avenue for examining the evolution of life and ecosystems (Foster and Green, 2011). Although significantly rarer on today's Earth, actively accreting microbialites can still be found in a diverse range of aquatic environments (Baumgartner *et al.*, 2009a).

There has been an abundance of research describing how physical, chemical, and biological factors contribute to the formation of modern microbialites. These formation

processes are strongly influenced by the microbial community, composed of bacteria, archaea, and often eukaryotes in vertically stratified layers based on light, oxygen, and nutrient availability (Krumbein, 1983; van Gemerden, 1993; Casanova *et al.*, 1999). The metabolic activity of this community of diverse organisms is essential to the development of microbialites in that it alters the chemical and physical composition of the matrix and produces microenvironments that control the saturation of CaCO_3 (Poinar, 1998; Visscher and Stolz, 2005).

The processes responsible for carbonate precipitation are not constant in either space or time. Microelectrode analyses of microbialite surface chemistry have demonstrated the presence of discrete chemical microenvironments with differing pH, O_2 , and Ca^{2+} concentrations near the microbialite surface (Garcia-Pichel *et al.*, 2004). Research in both Cuatro Ciénegas, Mexico (Garcia-Pichel *et al.*, 2004) and in

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Highborne Cay, Bahamas (Visscher *et al.*, 1998) has shown diel fluctuations of chemical microenvironments that occur within millimeter-scale increments on the microbialite surfaces. These chemical parameters are directly affected by the distribution of organisms and metabolic processes within the microbialite and can influence CaCO_3 precipitation and dissolution. It can be inferred that discrete processes relevant to the formation of modern microbialites occur within similar spatial scales. Numerous molecular studies on the microbial diversity of actively forming microbialites (Burns *et al.*, 2004; Papineau *et al.*, 2005; Allen *et al.*, 2009; Baumgartner *et al.*, 2009a, 2009b; Breitbart *et al.*, 2009; Goh *et al.*, 2009; Santos *et al.*, 2010) have revealed diverse bacterial communities, including Alphaproteobacteria, Bacteroidetes, Cyanobacteria, Deltaproteobacteria, and Planctomycetes, yet few have addressed spatial scales that can resolve community changes with depth in the microbialite and determine the dominant community members present in zones of carbonate precipitation.

This study combines genomic, lipid, and both organic and inorganic stable isotopic analyses to examine discrete depth horizons within a modern microbialite from Río Mesquites, a large spring-fed river in the Cuatro Ciénegas Basin, Mexico (Minckley, 1969). The layer-specific approach elucidates the spatial distribution of specific microbial communities within the microbialite and demonstrates how these microbes are connected through the production and decomposition of organic material. The results indicate distinct differences between individual layers of the Cuatro Ciénegas microbialite, both in community composition and geochemical signatures, which are correlated with specific zones of CaCO_3 accretion. The integrated approach utilized in this study enables linkages to be made between modern and ancient microbialite systems, since isotopic and lipid markers can be preserved over much longer timescales than genomic material.

2. Materials and Methods

2.1. Sample collection

Microbialite samples were collected from Río Mesquites (26°55'21.13"N 102°06'31.37"W) in Cuatro Ciénegas, Mexico, in July 2008. The Cuatro Ciénegas Basin is a naturally isolated valley containing hundreds of permanent lakes, rivers, and marshes that originate from thermal springs (25–35°C), as well as ephemerally flooding playas (Minckley and Cole, 1968). The numerous aquatic habitats within this karstic landscape are characteristically hard-water environments and contain high levels of SO_4^{2-} and NO_3^- , with the lowest reported phosphorus concentrations in any continental waters (Minckley and Cole, 1968; Elser *et al.*, 2005; Breitbart *et al.*, 2009). Río Mesquites is the largest spring-fed river in the Cuatro Ciénegas Basin, ranging from 2 to 20 m across and reaching up to 2.5 m in depth (Minckley, 1969).

Microbialites in Río Mesquites have smooth surfaces and are typically greater than 1 m in height and approximately 0.5 m wide (Fig. 1A). To isolate individual microbialite layers, a section of the surface from a single microbialite was removed and cut into slabs (~1 cm thick) such that the vertical face of the sample was exposed. A scalpel was then used to separate the visually distinct horizons, which resulted in layers 1 through 5 from the surface toward the

interior (Fig. 1A). A homogenate of each layer, integrating over the horizontal distance of the cross-sectional slab, was produced and used for subsequent analyses. The first layer was approximately 1–2 mm thick and was yellowish green in color with a soft stringlike texture. Layer 2 was more gelatinous, 2–4 mm thick, and had a dark green color. The third layer was bright white with a sandy texture and a thickness of ~5 mm. Layer 4 was gray to tan in color with a varying thickness (~5 mm) and a firmer texture than layer 3. Finally, the fifth layer was a dark red and brown and extremely dense. In total, these five layers accounted for the top 2–3 cm of the microbialite.

Samples collected for genomic work were immediately preserved in RNAlater (Applied Biosystems/Ambion, Austin, TX, USA). After collection, all samples were stored on ice until they were returned to the laboratory (within 1 week of collection). Upon return to the laboratory, RNAlater was removed from the samples for genomic work, and they were frozen at –80°C. Samples collected for lipid and stable isotope analyses were frozen at –20°C.

2.2. Genomic analysis of bacterial community composition

Nucleic acids were extracted from 30 mg samples from the homogenate of each individual layer (1–5) with the AllPrep DNA/RNA Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol for samples stored in RNAlater. The bacterial 16S rRNA gene was amplified with Bact-27F (5'-AGA GTT TGA TCM TGG CTC AG -3') and 1492R (5'-ACG GCT ACC TTG TTA CGA CTT -3') primers (Weisburg *et al.*, 1991). All PCR mixtures had a total volume of 50 μL and contained 5 μL of target DNA, 1 $\times$ REDTaq PCR buffer [10.0 mM Tris-HCl (pH 8.3), 50.0 mM KCl, 1.1 mM MgCl_2 , 0.01% gelatin; Sigma-Aldrich, St. Louis, MO, USA], 0.25 mM of each deoxynucleoside triphosphate, 1 μM of each primer, and 1 U REDTaq DNA polymerase (Sigma-Aldrich). PCR conditions contained 5 min of denaturation at 95°C, 30 cycles of [1 min at 94°C, 1 min at 65°C (–0.5°C per cycle), and 2 min at 72°C], and a final elongation step of 10 min at 72°C. All PCR reactions were run in triplicate, and products from each layer were pooled before cloning. PCR products were cloned with the TOPO TA cloning kit according to the manufacturer's instructions (Invitrogen Corp., Carlsbad, CA, USA). Transformants were screened by PCR with the M13F (5'-GTA AAA CGA CGG CCA GT -3') and M13R (5'-CAG GAA ACA GCT ATG AC -3') primers to verify presence of an insert, and approximately 400 clones were sequenced by Beckman Coulter Genomics (Beverly, MA, USA).

Raw sequences were trimmed of vector sequence and low-quality data with Sequencher version 4.7 (GeneCodes, Ann Arbor, MI, USA). FastGroupII (<http://biome.sdsu.edu/fastgroup>) was used to dereplicate the trimmed sequences by grouping sequences with $\geq 97\%$ sequence identity with gaps into a single operational taxonomic unit (OTU), remove sequences less than 300 base pairs in length, and calculate standard diversity indices (Yu *et al.*, 2006). Dereplicated 16S rDNA sequences were analyzed with the Ribosomal Database Project (RDP) Classifier tool (Wang *et al.*, 2007) to determine taxonomic identity at the class level. Sequences with bootstrap confidence values of less than 50% were reported as unclassified. BLASTN (<http://blast.ncbi.nlm.nih.gov/>)

FIG. 1. Physically and phylogenetically distinct layers within the Río Mesquites microbialite. **(A)** Modern microbialites within Río Mesquites, with an exposed vertical profile of the microbialite showing the five visually distinct layers analyzed in this study. **(B)** Phylogenetic overlap between layers as determined by a FastGroupII dereplication analysis of bacterial 16S rDNA sequences. Numbers represent the number of OTUs from a given layer(s).

Blast.cgi) was also used to compare the sequences against GenBank (Altschul *et al.*, 1997) to identify similarities between the microbialite sequences and those previously reported from other environmental surveys. All bacterial 16S rRNA gene sequences obtained in this study have been deposited to GenBank (accession numbers: HQ882850–HQ883265).

2.3. Lipid analyses

Subsamples of each layer (1–5) were homogenized and extracted with an Accelerated Solvent Extraction system (Dionex ASE 200, Sunnyvale, CA, USA). The ASE extraction program utilized elevated temperature (120°C) and pressure (1500 psi) and was performed three times: first with dichloromethane (DCM), followed by DCM/methanol (1:1 v/v), and finally with methanol only. After the three extracts from each sample were combined, elemental sulfur was removed with activated copper turnings (~5 g; 24 h), and the total lipid extracts were stored at –20°C.

Total lipid extracts were saponified by heating (70°C) for 2 h in a well-sealed vial with 10 mL of 0.5 N KOH in methanol. After saponification, neutral and acidic compounds were separated by liquid-liquid extractions (3×) at a pH of 7 and 2, respectively. Neutral compounds were separated into polar and apolar fractions on an activated silica gel column preconditioned with hexane. Hydrocarbons were eluted first with hexane (~12 mL), followed by the ketone compounds eluted in 10 mL hexane/DCM (6:4 v/v). Finally, the alcohol fraction was eluted in 10 mL DCM/methanol (1:1 v/v). After the volume was reduced under a gentle stream of N₂, the

hydrocarbon fraction was immediately ready for analysis by gas chromatography–mass spectrometry. The alcohol fraction was processed further and converted to trimethylsilyl (TMS) derivatives by reducing the sample to dryness, redissolving in a solution of bis(trimethylsilyl)-trifluoroacetamide (BSTFA; 50 µL) mixed with pyridine (50 µL) and DCM (10 µL), and heating at 70°C for 30 min. The acidic fraction was heated (60°C) for 3 h in methanolic HCl (10 mL), which converted fatty acids to methyl esters. Methyl ester derivatives were redissolved in hexane and separated into polar and apolar fractions on an activated silica column conditioned with hexane. Nonpolar compounds were eluted with hexane (7 mL), and the fatty acid methyl esters (FAMES) were subsequently eluted with 7 mL hexane/ethyl acetate (95:5 v/v). The FAME fraction was then reduced to dryness and redissolved in hexane.

Hydrocarbon, FAME, and TMS-alcohol fractions were analyzed by gas chromatography–mass spectrometry with a Varian CP-3800 gas chromatograph equipped with a VF-5ms capillary column (Varian Inc., Palo Alto, CA, USA), linked to a Varian 320-MS operating at 70-eV ionization energy. The gas chromatograph–mass spectrometer was also fitted with a Varian CP-8400 autosampler. Chromatographic conditions were adjusted for hydrocarbons (2 min at 50°C; 50°C→310°C at 8°C/min), FAMES (1 min at 50°C; 50°C→120°C at 30°C/min; 5 min at 120°C; 120°C→320°C at 8°C/min; 10 min at 320°C), and TMS-alcohols (2 min at 60°C; 60°C→250°C at 10°C/min; 250°C→320°C at 3°C/min; 30 min at 320°C) to optimize compound isolation. A constant flow of helium was used as a carrier gas for all analyses. The concentration of identified compounds was determined with

external calibration standards. Calibration curves were constructed by measuring the total ion peak of a standard solution injected at three known concentrations. All lipid analyses were run in duplicate.

2.4. Stable isotope analyses

Samples for isotopic analysis of organic matter were prepared by treatment with a mild acid solution (0.5 N HCl) until all CaCO_3 was removed. Remaining organic matter was filtered onto a precombusted 0.7 μm GF/F glass fiber filter, rinsed with deionized water, dried at a low temperature (60°C), and subsequently packed into tin capsules for $\delta^{13}\text{C}$ analysis. Analyses were performed with a continuous flow, Finnigan Mat Delta Plus isotope ratio mass spectrometer coupled to a Carlo Erba elemental analyzer. Samples were introduced via an autosampler into the combustion furnace of the elemental analyzer set at 1050°C. Solid phase carbonate minerals were prepared for isotopic analysis by grinding samples to a fine powder and drying at a low temperature. A 50 μg aliquot of each carbonate mineral sample was measured for inorganic $\delta^{13}\text{C}$ with a Thermo-Finnigan Delta Plus XL dual-inlet mass spectrometer with an attached Kiel III carbonate preparation device. Natural abundances of stable carbon isotopes are expressed as per mil (‰) units in delta notation with respect to the Pee Dee Belemnite (PDB) standard. Standard deviation was determined to be ± 0.11 (‰ PDB) for organic carbon and ± 0.2 (‰ PDB) for inorganic carbon measurements. Duplicate reactions were conducted for all isotopic analyses at the University of South Florida Paleocyanography, Paleoclimatology and Biogeochemistry Laboratory.

2.5. Microscopy

Scanning electron microscopy (SEM) was completed at the University of South Florida College of Marine Science Electron Microscopy Laboratory, with a Hitachi S-3500N variable pressure scanning electron microscope. Samples were prepared for microscopic analysis by drying at a low temperature (60°C) then coating with a thin layer of AuPd in a sputter coater.

2.6. Material balance

The physical composition of individual layers of the microbialite was determined by analyzing the relative mass distribution of water, carbonate, and organic material, the sum of which is assumed to account for the entire microbialite matrix. A sample of each layer was weighed, dried, and then weighed a second time to determine the water content. The dried material was then ground to a fine powder, and subsamples were used for total carbon and total inorganic carbon analysis by coulometric titration. Total organic carbon (TOC) was determined as the difference between total carbon and total inorganic carbon.

3. Results

3.1. Genomic analysis of bacterial community composition

A total of 399 high-quality bacterial 16S rDNA sequences (between 62 and 91 sequences from each layer) was obtained from the Cuatro Ciénegas microbialite. Dereplication of these

sequences with FastGroupII (Yu *et al.*, 2006) revealed 261 distinct OTUs, and these dereplicated sequences were used to calculate Chao1 (Chao, 1984) and Shannon-Wiener (Shannon and Weaver, 1963) diversity indices (Supplementary Table S1; Supplementary Data are available online at www.liebertonline.com/ast). Chao1 minimum estimates of richness were between 180 OTUs (layer 1) and 645 OTUs (layer 3) in each layer, with a total of 1035 predicted OTUs for the combined layers. Based on the Shannon-Wiener Index, layer 2 was the least diverse [3.54 natural digits (nats)], while layers 3 and 5 had the highest diversity (4.10 and 4.12 nats, respectively). Predicted Shannon-Wiener diversity for the community as a whole (5.33 nats) was much higher than for any individual layer.

Overall, there was very little overlap in the bacterial sequences detected in the individual layers of the microbialite (Fig. 1B). Layers 1 and 2 had the greatest overlap, with 11 OTUs found in both of these layers. Layer 1 did not share a single OTU with any other layer, while layer 2 shared only two OTUs with layer 3. The deeper layers (3, 4, and 5) were more similar to one another than to the surface two layers, as there was a total of 12 OTUs shared between some combination of layers 3, 4, and 5. The majority (90%) of OTUs were unique to an individual layer, which demonstrates that distinct bacteria dominated the community in each microbialite layer.

As determined through the RDP Classifier, phototrophic organisms dominated the microbialite surface (Fig. 2). Cyanobacteria accounted for more than 50% of the sequences in layer 1 and approximately 30% in layer 2. Nearly 60% of the cyanobacterial sequences grouped with the order of filamentous cyanobacteria, Oscillatoriales, primarily in the *Pseudanabaena* and *Leptolyngbya* genera. Another 20% of the cyanobacterial sequences were most similar to Pleurocapsales, and 10% to Nostocales. Alphaproteobacteria accounted for approximately 15–25% of the sequences in layers 1–3, with nearly 50% of these sequences grouping with purple nonsulfur (PNS) bacteria in the family *Rhodospirillaceae*. Additionally, 8% of the sequences from layer 1 were from chloroplast rRNA gene sequences of eukaryotic algae (diatoms). Following the Cyanobacteria, Bacteroidetes was the second most abundant class identified in layers 1 and 2, and accounted for 19% and 24% of the sequences, respectively.

The deeper layers of the microbialite (layers 3–5) contained few photoautotrophic sequences and were dominated by proteobacteria (45–50% of clones), including organisms from the Alpha-, Beta-, Delta-, and Gamma classes (Fig. 2). Alphaproteobacteria were the most abundant organisms in layer 3 and layer 5, while Deltaproteobacteria dominated layer 4. Nitrite-oxidizing bacteria (*Nitrospira*) were also abundant in layer 4 and comprised approximately 20% of the sequences. A high proportion of unclassified clones (15–30%) was detected in layers 3–5, in addition to a variety of heterotrophic bacterial groups (*i.e.*, Gemmatimonadetes, Acidobacteria, Clostridia, etc.).

3.2. Lipid composition

Total lipids extracted from the Cuatro Ciénegas microbialite accounted for 0.03–0.68% of the dry material weight in each of the individual layers. The TOC content of each individual layer was used to standardize lipid abundance.

FIG. 2. Phylogenetic classifications for bacterial 16S rDNA sequences from each microbialite layer as determined by comparison to the RDP. A 50% bootstrap value at the class level was used as a cutoff for identification. *Eukaryotic (diatom) sequences were detected through amplification of chloroplast 16S sequences.

Overall, a decrease in lipid content relative to TOC was observed with depth; however, layer 4 exhibited the second-highest concentration of total lipids relative to TOC ($72 \text{ mg g}^{-1} \text{ TOC}$). All identifiable hydrocarbons, FAMES, and alcohols are shown in Supplementary Figs. S1, S2, and S3, respectively.

Hydrocarbon distribution. The relatively small pool of hydrocarbons ($100\text{--}250 \text{ } \mu\text{g g}^{-1} \text{ TOC}$) displayed a bimodal distribution, with saturated straight-chain compounds ranging in length from 16 to 33 carbon atoms (Supplementary Fig. S1). Monounsaturated *n*-C19:1 was also observed in the hydrocarbon profile and was the most prominent compound in layer 4 ($37 \text{ } \mu\text{g g}^{-1} \text{ TOC}$). A series of midchain methyl-branched alkanes, ranging from 18 to 19 carbon atoms, were also abundant in the microbialite lipid extracts. The predominant branched hydrocarbon, 7-methyl heptadecane (Supplementary Fig. S1), was found in every layer yet decreased in concentration with depth (Fig. 3C). Phytene, a derivative of the isoprenoid phytol, was detected in layers 1 through 4, with the highest concentration ($28 \text{ } \mu\text{g g}^{-1} \text{ TOC}$) in layer 3. Two C20 isoprenoid thiophene isomers, 3-methyl-2-(3,7,11-trimethyldodecyl)-thiophene and 3-(4,8,12-trimethyltridecyl)-thiophene, were observed only in layer 5 (Supplementary Fig. S1). The apolar hopanoid, diploptene, was also present in the hydrocarbon extracts from each layer.

FAME distribution. Fatty acids, analyzed as FAMES, were the most abundant compound class in the microbialite extracts ($450\text{--}3100 \text{ } \mu\text{g g}^{-1} \text{ TOC}$). Saturated, straight-chain fatty acids dominated each of the five layers and accounted for approximately 50% of the FAMES analyzed. The satu-

rated C16:0 FAME (Supplementary Fig. S2) was the single most abundant compound detected in each layer's fatty acid profile ($75\text{--}900 \text{ } \mu\text{g g}^{-1} \text{ TOC}$). Monounsaturated fatty acids, primarily C16:1 and C18:1 isomers, were the second most abundant group of compounds and showed an overall decrease in concentration with depth (Fig. 3A). Similar reductions of polyunsaturated C18 and C20 FAMES (Supplementary Fig. S2) were observed from the surface to the deeper layers of the microbialite (Fig. 3B). Branched-chain fatty acids comprised a significant, but smaller, proportion of the total FAMES in the top layer ($343 \text{ } \mu\text{g g}^{-1} \text{ TOC}$). However, the percentage of fatty acids identified as branched compounds increased from 10% to 33% (by mass) between layers 1 and 5.

Alcohol distribution. Identifiable alcohols included both aliphatic and polycyclic constituents (analyzed as TMS derivatives) and accounted for $120\text{--}1500 \text{ } \mu\text{g g}^{-1} \text{ TOC}$. Normal chain alkanols were the most abundant compounds within the alcohol fraction (50–65% of measurable alcohols in every layer), and the highest compound concentration was observed for C_{18:0} (Supplementary Fig. S3). Sterols, including cholesterol, ergostanol, stigmasterol, and β -sitosterol, accounted for $15\text{--}100 \text{ } \mu\text{g g}^{-1} \text{ TOC}$ in each layer. Phytol ((2E,7R,11R)-3,7,11,15-tetramethyl-2-hexadecen-1-ol) was found in every layer but was most abundant in layer 1 at $95 \text{ } \mu\text{g g}^{-1} \text{ TOC}$.

3.3. Carbon isotope profiles

The stable isotopic composition of organic carbon ($\delta^{13}\text{C}$) was significantly depleted in ^{13}C within each layer of the Cuatro Ciénegas microbialite. Values ranged from

FIG. 3. Profile of select biomarkers. Concentrations of (A) monounsaturated fatty acids ($C_{16:1}, n-7 + C_{18:1}, n-9$) commonly found in cyanobacteria and (B) polyunsaturated fatty acids ($C_{18:3}, n-3 + C_{18:2}, n-6 + C_{20:5}, n-3 + C_{20:4}, n-6$) common in eukaryotic algae. (C) Filled squares represent the ratio of a cyanobacterial biomarker (7 Me- C_{17}) to the sum of common bacterial alkanes ($C_{16}-C_{22}$), and open circles show the ratio of a biomarker of sulfate-reducing bacteria (10Me-16:0) to the common bacterial fatty acid $C_{16:1}, n-5$. FAMES, fatty acid methyl esters; TOC, total organic carbon.

approximately -12‰ to -23‰ between the five layers (Fig. 4). An overall decrease in the $\delta^{13}\text{C}$ value was observed from the surface to deeper layers (layer 1 to layer 5), with the exception of a peak in layer 2. The $\delta^{13}\text{C}$ values of inorganic (CaCO_3) carbon (Fig. 4) followed a similar trend to that of the organic pool; however, they were significantly more enriched (-1.06‰ to $+3.02\text{‰}$ PDB). Calculation of the $\delta^{13}\text{C}$ values of individual carbonate generations (based on mass distribution of CaCO_3 additions) revealed that layers 4 and 5 contain mineral precipitates that are highly depleted of ^{13}C

(Table 1, Fig. 5D). Furthermore, the local (by layer) $\delta^{13}\text{C}$ values of dissolved inorganic carbon (DIC) were calculated to be similarly depleted in ^{13}C and contain a high percentage of remineralized organic carbon (Table 1).

3.4. Microbialite composition

X-ray diffraction demonstrated that the microbialite was pure calcite (data not shown). Material balance analyses revealed that CaCO_3 accounted for 35–90% (by weight) of the microbialite matrix in each layer, with increasing relative abundance in deeper layers (Fig. 5A). This trend was also evident from the SEM, where CaCO_3 density increased with depth (Fig. 5B). In contrast, organic matter and water constituted a decreasing proportion of the matrix with depth (Fig. 5A). Organic matter made up 6% (by weight) at the surface and less than 3% in layer 5. Through this analysis, the formation of CaCO_3 was determined to have occurred in two primary zones, with the first zone (layers 1 and 2) accounting for approximately 55% of the total carbonate accumulation by mass and the second zone (layers 4 and 5) responsible for 40% (Fig. 5C).

4. Discussion

4.1. Genomic analysis of bacterial community composition

Despite structural continuity, the individual layers of the microbialite contained phylogenetically distinct bacterial communities. Even though only a limited amount of sequencing was performed, the small overlap in community composition between individual layers (Fig. 1B) is striking when considering that these five layers were isolated from a section of the microbialite no more than 3 cm thick (Fig. 1A). Although deeper sequencing would undoubtedly reveal more overlap in the communities, the available sequences demonstrate that the individual layers are dominated by distinct bacteria. These results support previous studies

FIG. 4. Carbon isotope profiles. Organic (open squares) and inorganic (filled squares) carbon isotope values in the five layers of the microbialite. OM, organic matter; PDB, Pee Dee Belemnite.

TABLE 1. CaCO_3 MASS BALANCE AND ISOTOPIC CALCULATIONS

	CaCO_3 abundance (wt %)	$\delta^{13}\text{C}$ of cumulative CaCO_3 (‰)	$\delta^{13}\text{C}$ of discrete CaCO_3 generations (‰) ^a	$\delta^{13}\text{C}$ of local DIC (‰) ^b	Remineralized OM content in DIC ^c
Layer 1	35.5%	2.20	2.20	-0.18	20.9%
Layer 2	48.8%	3.02	5.21	2.83	5.9%
Layer 3	54.2%	2.92	2.05	-0.33	21.6%
Layer 4	70.9%	0.37	-7.90	-10.28	71.4%
Layer 5	88.5%	-1.06	-6.85	-9.23	66.2%

Measured values of relative carbonate accumulation and CaCO_3 $\delta^{13}\text{C}$ from each layer of the microbialite were used to calculate the $\delta^{13}\text{C}$ composition of discrete generations of CaCO_3 and the $\delta^{13}\text{C}$ composition of the dissolved inorganic carbon pool from which those discrete generations precipitated. These measured and calculated values provide a basis for understanding the amount of remineralized organic carbon that is incorporated into the carbonate matrix, preserving a distinct biogenic signature.

^aCalculated with the equation: $C_n = [(A_n * B_n) - (A_{n-1} * B_{n-1})] \div (A_n - A_{n-1})$ —where n is the layer of interest, A is the CaCO_3 abundance, B is the measured $\delta^{13}\text{C}$ value, and C is the calculated $\delta^{13}\text{C}$ value of the discrete CaCO_3 generation.

^bCalculated using the equation of Emrich *et al.* (1970), assuming an average temperature of 30°C.

^cCalculated assuming an ambient DIC- $\delta^{13}\text{C}$ value of +4‰ (Breitbart *et al.*, 2009) and a remineralized organic matter $\delta^{13}\text{C}$ value of -16‰. OM, organic matter.

showing that microbialites contain diverse groups of bacteria (Burns *et al.*, 2004; Papineau *et al.*, 2005; Allen *et al.*, 2009; Baumgartner *et al.*, 2009a, 2009b; Breitbart *et al.*, 2009; Goh *et al.*, 2009; Santos *et al.*, 2010), and further demonstrate that the bacterial communities are highly structured spatially within the microbialite. Overall, the bacterial community

transitions from primarily phototrophic at the microbialite surface to a heterotrophic community at depth (Fig. 2). The presence of discrete bacterial communities on millimeter scales supports the importance of localized metabolic processes, which produce chemical microenvironments that can promote microbialite accretion. Future work should examine

FIG. 5. Carbonate accretion model. (A) Mass balance of organic matter (green), CaCO_3 (gray), and water (blue) across the five layers. (B) Series of scanning electron microscope images depicting separate generations of CaCO_3 precipitated at each layer (left) and cartoon schematic demonstrating the addition of new CaCO_3 in each layer. The schematic does not reflect the actual spatial distribution of the newly precipitated carbonate but is provided to demonstrate the idea of successive generations of carbonate precipitation in each layer, assuming no dissolution. (C) The mass contribution of each CaCO_3 generation relative to the total accumulation in layer 5, with the gray boxes highlighting the two primary zones of CaCO_3 precipitation. (D) The calculated $\delta^{13}\text{C}$ values for individual generations of CaCO_3 and the corresponding color they represent in the cartoon portion of (B).

the extent of horizontal variability in the microbialite bacterial community as well as the degree of seasonal variability.

Many of the top BLAST hits of the Cuatro Ciénegas sequences were to bacteria identified in other CaCO₃-depositing environments, including Shark Bay (Papineau *et al.*, 2005), Highborne Cay (Havemann and Foster, 2008; Baumgartner *et al.*, 2009b), and Yellowstone National Park (Fouke *et al.*, 2003). In particular, the surface shows the greatest similarity to other microbialite communities, as approximately 33% of the sequences from layers 1 and 2 had a top BLAST hit to sequences recovered from such environments. The most abundant phylogenetic groups observed in the Cuatro Ciénegas clone library, including Cyanobacteria, Bacteroidetes, Alphaproteobacteria, and Deltaproteobacteria, are similar to the groups described in microbialites from Shark Bay, Australia (Papineau *et al.*, 2005); Highborne Cay, Bahamas (Havemann and Foster, 2008; Baumgartner *et al.*, 2009b); and Ruidera Pools, Spain (Santos *et al.*, 2010). Additionally, these microbial groups can all be linked to specific metabolic processes that are known to be integral to CaCO₃ accumulation. The identification of similar communities in these contrasting systems implies that the biological component of microbialite formation is particularly important and supports the idea that it is possible to identify a core community of bacteria conserved in modern microbialite systems worldwide (Foster and Green, 2011).

Phototrophic community composition. The abundance of photoautotrophs, especially cyanobacteria, in the surface of the microbialite (layers 1 and 2) based on 16S rDNA analyses agrees with a previous metagenomic survey from this site. Breitbart *et al.* (2009) determined that 74% of the identifiable metagenomic sequences obtained from bulk Río Mesquites microbialite material (layers not separated) originated from cyanobacteria. Many of the cyanobacteria identified in the current study grouped most closely with the Oscillatoriales, an order of filamentous cyanobacteria that are common in carbonate-precipitating biofilms and observed ubiquitously in modern microbialites (Golubic, 1976; Myshrall *et al.*, 2010; Santos *et al.*, 2010; Foster and Green, 2011). Photosynthetic metabolism plays an influential role in microbialite formation, and cyanobacteria are dominant in a wide range of microbialite ecosystems (Foster and Green, 2011). The uptake of CO₂ increases the local pH through the buffering action of bicarbonate (Visscher and van Gernerden, 1991), and the resulting alkaline state favors the precipitation of CaCO₃. Bacterial-specific 16S rDNA PCR also amplified diatom chloroplasts, which indicates the presence of eukaryotic algae (primarily class Bacillariophyceae) in layer 1 (Fig. 2). Further confirmation was provided by the observation of numerous pennate diatoms on the microbialite surface by SEM (Supplementary Fig. S4).

The majority of the alphaproteobacterial clones in layers 1 through 3 grouped most closely with species that are physiologically categorized as phototrophic PNS bacteria. It has been suggested that PNS bacteria, which have the ability to function as either aerobic chemoheterotrophs or anaerobic photoheterotrophs, grow naturally under microaerophilic or alternating oxic and anoxic conditions (Bryant and Frigaard, 2006). The presence of such alternating O₂ conditions is likely within the three surface layers of the microbialite, as this has been observed through chemical microprobe analyses by

Visscher *et al.* (1998) during a diel cycle study of Bahamian stromatolites and by Garcia-Pichel *et al.* (2004) during a similar study on Cuatro Ciénegas microbialites. The abundance of these organisms suggests that the surface community was dominantly phototrophic and composed of both oxygenic (*i.e.*, cyanobacteria) and anoxygenic (*i.e.*, PNS) bacteria.

Nonphototrophic community composition. Although layers 1 and 2 were predominantly phototrophic in nature, many sequences were most similar to Bacteroidetes, a group of heterotrophic organisms often found in other epilithic biofilms (Bruckner *et al.*, 2008). Bacteroidetes are known to efficiently degrade extracellular polymeric substances (EPS) and carbohydrates (Kirchman, 2002; Bauer *et al.*, 2006) that form the structural backbone of many biofilms (Sutherland, 1999; De Philippis *et al.*, 2000; Decho *et al.*, 2005; Baumgartner *et al.*, 2009b). In other microbialite systems, the presence and degradation of EPS have been shown to play an important role in CaCO₃ precipitation (Kawaguchi and Decho, 2002; Decho *et al.*, 2005). Additionally, this initial decomposition of the complex organic matrix by organisms such as the Bacteroidetes likely provides metabolic substrates for heterotrophic organisms found in the deeper microbialite layers.

Proteobacteria dominated the deeper layers of the microbialite (layers 3, 4, and 5) based on 16S rDNA analysis (Fig. 2). Organisms within the phylum Proteobacteria have a wide range of metabolic capabilities and are of great ecological importance due to their key roles in the carbon, sulfur, and nitrogen cycles (Kersters *et al.*, 2006). Despite this extreme diversity, the physiological function of some of these organisms can be resolved. Alphaproteobacteria, which dominated the third layer, grouped closely with PNS bacteria and likely perform a type of anoxygenic photosynthesis. Deltaproteobacteria comprised the second largest portion of layer 3 and were the most abundant bacterial group in layer 4, accounting for approximately 25% of the sequences. A high proportion (75% of the described species) of organisms within the class Deltaproteobacteria are anaerobic sulfate-reducing bacteria (Kersters *et al.*, 2006). Sulfate-reducing bacteria are known to contribute significantly to CaCO₃ formation (Lyons *et al.*, 1984), and research on Bahamian stromatolites by Visscher *et al.* (1998, 2000) provides compelling evidence that sulfate reduction is a primary force behind CaCO₃ precipitation. The abundance of sulfate-reducing organisms in the Cuatro Ciénegas microbialites is supported by the prior observation of numerous genes related to sulfate reduction within the microbialite metagenome (Breitbart *et al.*, 2009). While some sulfate reduction likely occurs throughout the microbialite, the concentration of sulfate-reducing bacteria in layer 4 suggests that this may be a dominant process in the deeper layers of the microbialite.

4.2. Lipid analyses

Changes in the hydrocarbon, fatty acid, and alcohol composition from layers 1 through 5 complement the 16S rDNA analyses and provide substantial evidence for a shift from a phototrophic community in the surface layers to a heterotrophic, sulfate-reducing community in the deeper layers. Incorporating lipid analyses is advantageous because of all the organic components within the microbialite; lipids typically have the greatest potential for long-term

preservation (Brocks and Banfield, 2009), which makes them useful for the interpretation of ancient systems where nucleic acids are no longer present.

Hydrocarbons. Heptadecane is a commonly observed component of filamentous cyanobacteria (Winters *et al.*, 1969; Thiel *et al.*, 1997). The overwhelming dominance of heptadecane ($n\text{-C}_{17}$) in layer 1 (Supplementary Fig. S1) strongly supports the abundance of cyanobacterial 16S rDNA sequences in this layer. Furthermore, the midchain methyl-branched hydrocarbons (Supplementary Fig. S1) dominated by 7-methyl-heptadecane have been used as key indicators of cyanobacteria in microbial mats (Shiea *et al.*, 1990; Thiel *et al.*, 1997; Koster *et al.*, 1999) and provide another link to the abundance of cyanobacterial sequences. The reduced relative concentration of the cyanobacterial biomarker 7-methyl-heptadecane relative to total bacterial alkanes ($n\text{-C}_{16}$ through $n\text{-C}_{22}$) with depth provides further molecular evidence of the changes occurring within the microbial community (Fig. 3C).

Fatty acids. Fatty acid biomarkers (Supplementary Fig. S2) also document the decrease in phototrophs and increase in sulfate-reducing bacteria with depth in the microbialite. The decrease of monounsaturated 16:1($n\text{-7}$) and 18:1($n\text{-9}$) concentrations with depth (Fig. 3A) correlates well with the genomic analysis, as these compounds have primarily, though not exclusively, been attributed to cyanobacteria (Buhning *et al.*, 2009). Polyunsaturated fatty acids, 18:3($n\text{-3}$) and 18:2($n\text{-6}$), which are common in chlorophytes (green microalgae), and 20:5($n\text{-3}$) and 20:4($n\text{-6}$), which are found predominantly in diatoms (Boschker and Middelburg, 2002; Harwood and Guschina, 2009), were among the most abundant compounds in the surface layers ($400\text{ }\mu\text{g g}^{-1}\text{ TOC}$). The concentrations of these algal biomarkers were drastically reduced in the deeper layers (Fig. 3B).

Figure 3C shows the relative increase with depth in the ratio of 10 methyl-16:0, a common fatty acid of sulfate-reducing bacteria (Taylor and Parkes, 1983), compared to 16:1 ($n\text{-5}$), a fatty acid ubiquitous to all bacteria (Vestal and White, 1989). The peak in this ratio occurred in layer 4, which coincides with the dominance of Deltaproteobacteria in the 16S rDNA sequences and provides further evidence for the prevalence of sulfate reduction in the deeper layers of the microbialite. The relative increase in this biomarker of sulfate-reducing bacteria in association with the decrease of 7-methyl-heptadecane relative to total bacterial alkanes (Fig. 3C) reflects the shift in the bacterial regime from an aerobic photoautotrophically dominated community to one that contains anaerobic sulfate-reducing organisms. Along with the reduction of unsaturated FAME concentrations (Fig. 3A, 3B), this observation indicates that the photoautotrophic biomass produced at the surface is readily degraded and recycled by the successive generations of bacteria in the deeper layers of the microbialite.

Alcohols—phytol degradation. Numerous isoprenoids found within the microbialite lipid extracts can be linked to the degradation of phytol, a component of the chlorophyll *a* molecule. As a constituent of the photosynthetic pigment of plants, cyanobacteria, and algae (Volkman and Maxwell, 1986; Bauer *et al.*, 1993), phytol and its derivatives can be used as indicators of photoautotrophic organisms and spe-

cific metabolic processes involved in the degradation of their biomass. Phytol is a common component of recent sediments (Rontani *et al.*, 1996; Grossi *et al.*, 1998), and it has been speculated that it is the major source of other isoprenoids of 20 or fewer carbon atoms in geological samples (Rontani and Volkman, 2003). The large quantity of phytol in the first layer (Supplementary Fig. S3) is likely a direct reflection of the numerous phototrophic cyanobacteria and eukaryotic algae in this layer and the release of phytol from intact chlorophyll compounds during senescence of these organisms (Jeffrey and Hallergaef, 1987).

More informative than the presence of phytol itself is the observation of intermediate metabolites that reflect specific phytol degradation pathways. Sulfate-reducing bacteria can efficiently degrade phytol (Rontani *et al.*, 1999), producing isomeric phytadienes and phytenes as metabolites (Grossi *et al.*, 1998). Phytene (Supplementary Fig. S1) was observed throughout the Cuatro Ciénegas microbialite with the highest concentrations occurring in layer 3. This coincides with the increase of sulfate-reducing bacteria in the deeper layers of the microbialite. In addition, isoprenoid thiophenes, which are organic sulfur compounds produced through the reaction of inorganic sulfur (H_2S) with phytol (De Graaf *et al.*, 1992; Fukushima *et al.*, 1992), were detected in the fifth layer.

4.3. Carbon isotope profiles

Organic carbon. Organic matter $\delta^{13}\text{C}$ values (Fig. 4) support the hypothesis that the Cuatro Ciénegas microbialite system is based upon photoautotrophic carbon fixation, primarily by cyanobacteria and eukaryotic algae. The $\delta^{13}\text{C}$ value in the first layer (-18.2‰) is typical of photosynthetic carbon fractionation patterns observed in both cyanobacteria (Calder and Parker, 1973; Pardue *et al.*, 1976) and diatoms (Thompson and Calvert, 1994), given that the $\delta^{13}\text{C}$ value of the ambient DIC in Río Mesquites is 4.1‰ (Breitbart *et al.*, 2009). The relatively ^{13}C -enriched organic carbon value seen in layer 2 (-12‰) is not typical of most photoautotrophically produced material; however, a number of possible factors may be influencing this observed trend. In hypersaline cyanobacterial mats, Wieland *et al.* (2008) observed a peak concentration of EPS at a depth of 2–4 mm, which corresponds to the second layer in this study. Since EPS are composed of a high proportion of carbohydrates (Klock *et al.*, 2007) that are typically enriched in ^{13}C relative to total cellular carbon (Deines, 1980), an increase in EPS in layer 2 could explain the ^{13}C enrichment. Future work should examine the concentration and composition of EPS in the Cuatro Ciénegas microbialites in order to evaluate the feasibility of this explanation. A second possibility is that this shift is caused by a change in the carbon substrate used for photosynthesis by the organisms in this layer of the microbialite. Cyanobacteria, which are the most abundant photoautotrophs in layer 2, are notorious for having highly variable ^{13}C fractionation during photosynthesis, primarily constrained by CO_2 concentrations (Calder and Parker, 1973). High rates of photosynthesis within a dense microbial community can result in local CO_2 concentrations becoming depleted faster than they are replenished. The result of this process, which was clearly shown by Staal *et al.* (2007), is that the thickening of a natural microbial mat can cause a shift away from CO_2 utilization toward the assimilation of more

^{13}C -enriched HCO_3^- (Emrich *et al.*, 1970; Mook *et al.*, 1974) as a substrate for photosynthesis.

The steady decrease of organic matter $\delta^{13}\text{C}$ values from -19‰ to -23‰ between layers 3 and 5 could reflect the addition of organic matter with a low ^{13}C content at depth; however, heterotrophic communities typically will produce biomass with a $\delta^{13}\text{C}$ composition enriched in ^{13}C relative to its substrate (Šantrůčková *et al.*, 2000). Assuming that heterotrophic bacteria at depth are utilizing buried photoautotrophic biomass as a carbon substrate, we can conclude that an increase in heterotrophic biomass did not produce this carbon isotope trend. Rather, the main process influencing the observed ^{13}C depletion with depth (Fig. 4) is likely the continuous remineralization of buried organic matter that is rich in ^{13}C , such as the carbohydrates of EPS. Remineralization of organic carbon by the heterotrophic community results in the TOC pool gradually becoming more depleted in ^{13}C , as the enriched components are preferentially removed (Benner *et al.*, 1987). Furthermore, remineralization processes change the ^{13}C composition of the local DIC pool.

Inorganic carbon (CaCO_3). While the stable isotopic composition of organic material provides insight into the nutrient cycling processes within the microbialite community, examination of CaCO_3 $\delta^{13}\text{C}$ allows for interpretation of how these processes incorporate signatures into the inorganic carbonate matrix. The similarity in $\delta^{13}\text{C}$ patterns between organic and inorganic carbon from layer 1 through layer 5 (Fig. 4) suggests a direct link between these two carbon pools. As previously shown by Breitbart *et al.* (2009),

the $\delta^{13}\text{C}$ of CaCO_3 in the Cuatro Ciénegas microbialites is depleted in ^{13}C relative to the water column DIC. The layer-specific analysis in the present study demonstrated that the $\delta^{13}\text{C}$ of CaCO_3 becomes increasingly depleted with depth in the microbialite (Fig. 4). Remineralization of photoautotrophically derived carbon causes a negative shift in the localized DIC- $\delta^{13}\text{C}$ values with depth. Subsequently, this negative signature is incorporated into the solid phase as CaCO_3 precipitates in the deeper layers. These results emphasize the importance of organic matter remineralization by heterotrophic organisms (Andres *et al.*, 2006; Breitbart *et al.*, 2009) and reveal that the link between organic carbon and inorganic (CaCO_3) pools is the dissolved carbon phase.

4.4. Distribution of carbonate precipitation

The mass distribution of the primary components of the microbialite matrix (Fig. 5A) provides a better understanding of how the microbialite composition changes between layers and how specific organisms and processes are linked to the precipitation of CaCO_3 . The decrease in organic matter with depth (Fig. 5A) is indicative of decomposition by heterotrophic bacteria. Calcium carbonate accounts for a high proportion of the total mass, increasing from approximately 35% (by weight) in layer 1 to 90% in layer 5 (Fig. 5A). This finding demonstrates that CaCO_3 accumulates throughout the five layers, although not continuously. Visualization of the individual layers with SEM also revealed this same increase in CaCO_3 with depth, and a graphical illustration better depicts how the fifth layer is composed of the accretion of many

FIG. 6. An integrated schematic of the Cuatro Ciénegas microbialite. (A) General distribution of bacteria in the microbialite, showing a transition from a primarily photoautotrophic community (green) at the surface to a heterotrophic community (red) at depth. The gray area indicates spatial or temporal variability in the data, as well as members of the bacterial community that could not be identified. (B) Dominant metabolic processes likely occurring at each layer of the Cuatro Ciénegas microbialites. (C) Two primary zones of CaCO_3 precipitation that are associated with specific metabolic processes (indicated by the solid arrows). (D) Gradual depletion of ^{13}C in CaCO_3 generations with depth, a product of heterotrophic remineralization of organic matter (indicated by the dashed arrow).

individual generations of CaCO_3 (Fig. 5B). Furthermore, the relative contribution of individual CaCO_3 generations to the total accumulation (layer 5) can be calculated (Fig. 5C) from the results of the mass balance analysis (Table 1). This reveals that the majority of CaCO_3 forms in two spatially distinct zones. Relative to the total accumulation of CaCO_3 , the top two layers of the microbialite (corresponding to the photo-synthetic zone) produce approximately 55% of the CaCO_3 (by weight). The second zone of carbonate accretion, where approximately 40% of the total CaCO_3 is added, occurs in the fourth and fifth layers where sulfate-reducing organisms are present.

Differences between CaCO_3 - $\delta^{13}\text{C}$ values from any one layer of the microbialite to the next layer (Fig. 4) can be accounted for by the newly precipitated generations of CaCO_3 . Combining the mass balance analysis, which gives an understanding of the relative mass of CaCO_3 added at each layer, with the CaCO_3 - $\delta^{13}\text{C}$ data allows the approximate $\delta^{13}\text{C}$ value of the carbonate being added at each layer to be calculated (Fig. 5D). Based on this analysis, the $\delta^{13}\text{C}$ values of carbonate minerals precipitated at layers 4 and 5 are strongly depleted (-7‰ to -8‰) compared to the ambient DIC of $+4\text{‰}$ (Breitbart *et al.*, 2009). The depletion in $\delta^{13}\text{C}$ of mineral precipitates is thought to be a function of organic matter remineralization (Breitbart *et al.*, 2009). The results of this study show that a significant portion of the DIC pool, as much as 70% in layer 4, is composed of remineralized photoautotrophically derived carbon (Table 1). These calculations do not take into account the processes of CaCO_3 dissolution and reprecipitation, since no evidence for diagenetic alteration was observed. However, these estimates predict a significant incorporation of biologically fractionated carbon into the inorganic matrix. Such isotopic signatures can be preserved on geological timescales and used to identify a biogenic source of ancient carbonates.

5. Conclusions

The integrated genomic, lipid, and isotopic analyses performed in this study produced a detailed portrait of the diverse phototrophic and heterotrophic organisms and aerobic and anaerobic processes responsible for microbialite formation and the preservation of distinct geochemical signatures. Based on these collective data, a schematic model describing the formation of the Cuatro Ciénegas microbialites can be created (Fig. 6), which likely extends to other systems.

The presence of oxygenic photosynthesis in microbialites, specifically by cyanobacteria, is well documented (Burns *et al.*, 2004; Jahnke *et al.*, 2004; Jungblut *et al.*, 2005), and this study demonstrated that approximately 50% of the carbonate accumulation in the Cuatro Ciénegas microbialite occurs within a zone that is overwhelmingly dominated by these organisms (Fig. 6). The data also suggest that anoxygenic photosynthesis occurs in this system, where it may also increase carbonate precipitation (Bosak *et al.*, 2007). Furthermore, the degradation of photoautotrophic material by the heterotrophic community is thought to be significant to the formation of microbialites. This process occurs throughout the Cuatro Ciénegas microbialites, with Bacteroidetes-like organisms that are known to degrade EPS contributing significantly to the surface community. Anaerobic heterotrophic

decomposition also plays an important role in CaCO_3 precipitation in deeper layers. Specifically, the prevalence of sulfate reduction in the Cuatro Ciénegas microbialite community is well supported through the observation of an abundance of sulfate-reducing Deltaproteobacteria (layer 4), as well as the presence of sulfurized organic compounds (thiophenes) in the fifth layer. The coupling of this process to the second zone of CaCO_3 precipitation suggests that sulfate reduction is vital to the formation of the Cuatro Ciénegas microbialites. The anaerobic heterotrophic organisms also produce distinct ^{13}C -depleted CaCO_3 - $\delta^{13}\text{C}$ signatures in the fourth and fifth layers of the microbialite (Fig. 6D) due to remineralization of organic matter and the subsequent incorporation of this carbon into CaCO_3 .

Beyond understanding the formation of modern freshwater microbialites, this work also provides the opportunity to consider the integrated lipid and isotopic signatures that are a result of the sum of these organisms and processes. Understanding these signatures is a key step to forming a conceptual bridge between modern and ancient microbialite communities, since genomic material is rarely preserved over geologically relevant timescales. Selective degradation, preservation, and alteration of distinctive biomarkers throughout the microbialite result in the lipid composition of layer 5, which can be interpreted as a molecular fingerprint of the community as a whole. Though many details about the community are removed through heterotrophic degradation, this research demonstrates that a portion of the microbialite's organic matrix survives the initial stages of decomposition and has potential utility for the interpretation of ancient systems. While many of the straight-chain and unsaturated compounds were preferentially degraded (*e.g.*, $n\text{-C}_{17}$, unsaturated fatty acids), the preservation of some compounds, such as sulfurized derivatives of phytol and some midchain methyl-branched hydrocarbons, within layer 5 indicates their possible utility as distinctive biomarkers. Organic matter sulfurization has been described as a key process within the early stages of diagenesis (Hebting *et al.*, 2006), one that likely leads to increased preservation of these microbial biomarkers (Brocks and Banfield, 2009). Furthermore, the detection of isoprenoid thiophenes in cretaceous deposits (Sinninghe Damsté *et al.*, 1989) signifies the stability of such compounds on geological timescales.

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No competing financial interests exist.

Abbreviations

DCM, dichloromethane; DIC, dissolved inorganic carbon; EPS, extracellular polymeric substances; FAMES, fatty acid methyl esters; nats, natural digits; OTU, operational taxonomic unit; PDB, Pee Dee Belemnite; PNS, purple nonsulfur; RDP, Ribosomal Database Project; SEM, scanning electron microscopy; TMS, trimethylsilyl; TOC, total organic carbon.

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