Microbialites from the Freshwater System of Cuatro Ciénegas, Mexico: Genomic, Molecular Organic, and Stable Isotopic Perspectives

Anthony G. Nitti

University of South Florida

Follow this and additional works at: http://scholarcommons.usf.edu/etd

Part of the American Studies Commons

Scholar Commons Citation

This Thesis is brought to you for free and open access by the Graduate School at Scholar Commons. It has been accepted for inclusion in Graduate Theses and Dissertations by an authorized administrator of Scholar Commons. For more information, please contact scholarcommons@usf.edu.
Microbialites from the Freshwater System of Cuatro Ciéñegas, Mexico:
Genomic, Molecular Organic, and Stable Isotopic Perspectives

by

Anthony G. Nitti

A thesis submitted in partial fulfillment of the requirements for the degree of
Master of Science
College of Marine Science
University of South Florida

Co-Major Professor: Mya Breitbart, Ph.D.
Co-Major Professor: David Hollander, Ph.D.
Lisa Robbins, Ph.D.

Date of Approval:
September 27, 2010

Keywords: Stromatolite, Cyanobacteria, Proteobacteria, Carbonate, Lipid

Copyright © 2010, Anthony G. Nitti
Acknowledgments

I would like to thank the Government of the State of Coahuila, the City of Cuatro Ciénegas, Semarnat, CONANP, Pronatura Noreste and the people of Cuatro Ciénegas, Mexico for welcoming us to their town and allowing us to collect samples in Rio Mesquites. A great deal of thanks must also go to Dawn Goldsmith, Neilan Kuntz, Janet Siefert, and Valeria Souza for assistance in sampling. Tony Greco, from the College of Marine Science Electron Microscopy Laboratory was also extremely helpful in obtaining SEM images. This project was funded by grants from the National Geographic Society and the University of South Florida Internal Awards Program. Additionally, personal financial assistance was provided by the USGS/USF Cooperative Assistantship, the Gulf Oceanographic Trust Fellowship, and the Von Rosenstiel Fellowship. Finally, I am endlessly grateful for the personal friendships and intellectual guidance of members of the Breitbart Microbiology Lab and the Paleoeclimatology, Paleoceanography, and Biogeochemistry Laboratory. Specifically, I would have been unable to complete this work without the help of Camille Daniells, Ana Hoare, and Ethan Goddard.
Table of Contents

List of Tables ........................................................................................................ iii
List of Figures ......................................................................................................... iv
Abstract ................................................................................................................ v
Introduction ............................................................................................................ 1
Materials and Methods .......................................................................................... 9
  Sample Collection ............................................................................................... 9
  DNA Extraction .................................................................................................. 11
  Amplification, Cloning, and rRNA Sequencing .................................................. 11
  Sequence Analysis ............................................................................................. 12
  Lipid Extraction .................................................................................................. 12
  Separation of Compound Classes ..................................................................... 13
  Analysis by GC-MS ........................................................................................... 14
  Stable Isotope Analysis ...................................................................................... 15
  Microscopy ......................................................................................................... 16
  Material Balance ................................................................................................ 16
Results .................................................................................................................... 18
  Bacterial Clone Libraries ................................................................................... 18
  Lipid Extracts ..................................................................................................... 22
    FAME Distribution ........................................................................................... 22
    Alcohol Distribution ......................................................................................... 23
    Hydrocarbon Distribution .............................................................................. 23
  Carbon Isotope Profiles ..................................................................................... 24
  Microbialite Composition .................................................................................. 24
Discussion ............................................................................................................... 25
  Phylogenetic Analysis ....................................................................................... 25
    Uniqueness of 16S Clone Libraries ................................................................... 25
    Phototrophic Community Composition ......................................................... 26
    Non-phototrophic Community Composition ................................................... 28
    Phylogeny Summary ........................................................................................ 30
  Molecular Organic Biomarkers ......................................................................... 30
    Hydrocarbons .................................................................................................. 31
Fatty Acids ................................................. 32
Alcohols – Phytol Degradation ......................... 34
Biomarker Summary ........................................ 36
Carbon Isotope Profiles .................................. 36
Organic Carbon .............................................. 38
Inorganic Carbon (CaCO₃) ................................ 40
Carbonate Accretion ....................................... 40

Summary ........................................................ 44
An Integrated Perspective .................................. 44
Significance .................................................... 45

Conclusion ...................................................... 49

References ...................................................... 51

Appendices ..................................................... 60
Appendix A: Extra Tables .................................. 61
Appendix B: Extra Figures ................................. 66
List of Tables

Table A1: FAME Distribution................................................................. 61
Table A2: Alcohol Distribution............................................................. 62
Table A3: Hydrocarbon Distribution...................................................... 63
Table A4: Archaeal 16S rDNA Clone Identification.................................. 64
Table A5: Eukaryotic 18S rDNA Clone Identification.............................. 64
Table A6: Bacterial 16S rDNA Clone Diversity Results............................ 65
List of Figures

Figure 1: Site Location ........................................................................................................... 3
Figure 2: Layer Isolation ....................................................................................................... 10
Figure 3: Bacterial Community Overlap ............................................................................. 19
Figure 4: Bacterial Community Profile ............................................................................... 21
Figure 5: Profile of Select Biomarkers ............................................................................... 34
Figure 6: Carbon Isotope Profiles ....................................................................................... 37
Figure 7: Carbonate Accretion Model ................................................................................ 43
Figure 8: Summary Model .................................................................................................. 48
Figure B1: Organic $\delta^{15}$N data ..................................................................................... 66
Figure B2: Percent Sequence Identity (Top Blast Hit) ......................................................... 67
Figure B3: Bacterial 16S rDNA Diversity Results (Layer 1) ........................................... 68
Figure B4: Bacterial 16S rDNA Diversity Results (Layer 2) ........................................... 69
Figure B5: Bacterial 16S rDNA Diversity Results (Layer 3) ........................................... 70
Figure B6: Bacterial 16S rDNA Diversity Results (Layer 4) ........................................... 71
Figure B7: Bacterial 16S rDNA Diversity Results (Layer 5) ........................................... 72
Figure B8: Bacterial 16S rDNA Diversity Results (Total Community) ......................... 73
Abstract

Modern microbialites are carbonate-precipitating microbial mats and represent the closest living analogues to ancient stromatolites. These ancient carbonate formations are the oldest fossil evidence of life on Earth; however, our comprehension of their relationship to early earth ecosystems relies heavily on understanding the formation of modern microbialites. Research regarding these formation processes has suggested that chemical constraints of CaCO\(_3\) precipitation vary on sub-millimeter spatial scales within the living microbial community. In an attempt to shed light on the importance of these chemical microenvironments, this study focused on understanding the spatial distribution of the organisms and processes involved in the formation of modern microbialites. This was accomplished by isolating five visually distinct layers from the upper 2 – 3 cm of an actively forming microbialite found in the freshwater system of Cuatro Ciénegas, Mexico. Each layer was analyzed using genomic, molecular organic, and stable isotopic techniques. Bacterial diversity was determined by 16S rRNA gene analyses, lipid biomarker content was detected by GC-MS, and carbon isotope composition of organic matter and CaCO\(_3\) were used as indicators of specific microbial processes. Results of the 16S rRNA gene analysis showed that there is little overlap in the community composition of individual layers. Approximately 90% of the ribotypes identified in the microbialite
were unique to a single layer. Furthermore, the relative accretion of CaCO$_3$ at each layer was used to connect the distribution of organisms and processes with two specific zones of CaCO$_3$ precipitation. The first zone of CaCO$_3$ accretion, which accounted for approximately 55% of total CaCO$_3$ accumulation, is found in the surface two layers of the microbialites and dominated by photoautotrophic cyanobacteria and algae. The second zone of CaCO$_3$ precipitation, found at the interior (layers 4 and 5), is composed primarily of heterotrophic proteobacteria and dominated by sulfate-reducing δ-proteobacteria. The lipid content of the microbialite reflected the community structure as determined by genomics. Numerous photosynthetic biomarkers were detected and decreased in abundance with depth, indicating the important function of heterotrophic degradation. Additionally, the detection of sulfurized phytol compounds in layer 5 highlighted an important mechanism for the preservation of biogenic signatures, and reflected both the abundance of phototrophic organisms and sulfate-reducing bacteria. In combination, these interdisciplinary analyses provided an understanding of microbial community composition and metabolism while indicating the spatial relationship to CaCO$_3$ formation and the preservation of distinct biochemical signatures.
Introduction

Microbialites are organosedimentary mats composed of diverse microbial communities that influence accretion by the trapping of sediment and formation of minerals precipitates (Burne & Moore, 1987). Though rare, microbialites are found in diverse marine and freshwater environments (Awramik & Grey, 2005), often characterized by extreme conditions. For instance, in Sharks Bay, Australia microbialites are forming in hyper-saline water (Burns et al., 2004), Highborne Cay, Bahamas microbialites are found in a high-energy wave-dominated system (Andres & Reid, 2006), and numerous examples can be found from hot spring environments such as those in Yellowstone National Park, U.S.A. (Walter et al., 1972). Additionally, the freshwater system of Cuatro Ciénegas, Mexico, which is the focus of this study, supports the formation of microbialites under extremely low phosphorus conditions (Elser et al., 2005a). Throughout these environments, microbialites develop different structural formations including dendritic thrombolites (Kennard & James, 1986) and oncolites (Dean & Eggleston, 1984), as well as stromatolites which have a laminated morphology and are thought to be analogous to ancient microbialites that are preserved in the rock record (Awramik & Grey, 2005).

Some fossilized stromatolites are thought to be greater than 3.4 billion years old (Allwood et al., 2006), indicating that these ancient carbonate formations are a product of some of the earliest biological communities (Schopf,
Constructing a robust understanding of modern microbialites can enhance our comprehension of the significance of similar communities within the ancient environment as well as their relationship to the evolution of life and ecosystems. While much remains unknown with respect to these ancient stromatolites, great strides have been taken over the past 30 years to describe the processes through which physical, chemical, and biological activity control the formation of modern microbialites. The primary objective of the research done here was to add to this growing understanding of modern microbialite formation by investigating the actively accreting microbialites of Cuatro Ciéñegas, Mexico using an interdisciplinary approach.

Found in the Chihuahuan Desert of Coahuila, Northern Mexico, the Cuatro Ciéñegas Basin (CCB) is a naturally isolated valley (Fig. 1) containing hundreds of permanent lakes, rivers, marshes, and springs as well as ephemerally flooding playas (Minckley & Cole, 1968). The numerous aquatic habitats within this karstic landscape primarily originate from thermal springs (25° – 35° C), and are characteristically hard water environments containing high concentrations of \(\text{SO}_4^{2-}\) and \(\text{NO}_3^-\) (Breitbart et al., 2009; Elser et al., 2005a; Minckley & Cole, 1968). Additionally, the CCB has the lowest phosphorus content reported for continental waters, exerting a selective pressure on the composition of the biological communities and enhancing microbialite formation due to poor grazing efficiency by snails and other eukaryotic predators (Elser et al., 2005a; Elser et al., 2005b).
The physiogeographic isolation of the CCB has led to a remarkably high level of endemism (Stein et al., 2000). In fact, the CCB has the greatest endemic diversity in North America, with more than 70 unique species (Badino et al., 2004). The bacteria, archaea, and viruses of Cuatro Ciénegas are also abundant as well as diverse (Breitbart et al., 2009; Desnues et al., 2008). Notably, the bacterial and viral communities are most closely related to marine phylotypes, even though this region has been isolated from such systems for millions of years (Desnues et al., 2008; Souza et al., 2006). The CCB is a unique location for studying modern microbialite formation due to this hypothesized association with an ancient marine system and the extremely low phosphorus content, which indicates an intriguing similarity to early earth, where phosphorus was much less abundant (Bjerrum & Canfield, 2002).

Figure 1: Site Location
(A) Satellite image of North America, with Cuatro Ciénegas indicated.
(B) Satellite image of the Cuatro Ciénegas valley, with Rio Mesquites indicated.
(C) Actively forming microbialites within Rio Mesquites.
In environments that are conducive to CaCO\textsubscript{3} accretion, such as the CCB, the formation of carbonate deposits is strongly influenced by the microbial community (Casanova et al., 1999). Within modern microbialites, these communities are composed of bacteria, archaea, and often eukaryotes that form vertically stratified layers based on light, oxygen, and nutrient availability (van Gemerden, 1993). Though the microorganisms associated with microbialites from different environments are distinct, there tends to be a conservation of the most dominant groups, at least at the phylum level. The phylogenetic characterizations of bacteria in microbialites from Australia (Allen et al., 2009), the Bahamas (Baumgartner et al., 2009), and Spain (Santos et al., 2009) have all shown an abundance of Alphaproteobacteria, Bacteroidetes, Cyanobacteria, Deltaproteobacteria, and Planctomycetes. Essentially, it is the combined metabolic activity of this characteristic microbial community that alters the chemical and physical composition of the matrix and thus controls the formation of modern microbialites.

Individual metabolic processes utilized by the microorganisms within a microbialite can have discrete effects on the saturation of CaCO\textsubscript{3} and result in its net gain or loss (Visscher & Stolz, 2005). While numerous metabolic processes have the potential to affect CaCO\textsubscript{3} precipitation (Visscher & Stolz, 2005), the general interaction of photoautotrophic and heterotrophic organisms is a key factor in the formation of microbialites (Altermann et al., 2006). The prevalence of photosynthetic metabolism in microbialite communities, primarily by Cyanobacteria, plays an influential role in the formation process. Specifically, the
uptake of CO₂ during photosynthesis increases the local pH through the buffering action of bicarbonate (Visscher & van Gemerden, 1991), and the resulting alkaline state favors the precipitation of CaCO₃. Additionally, Cyanobacteria and other photosynthetic microorganisms produce copious amounts of extracellular polymeric substances (EPS), a complex organic matrix that is the structural backbone of many biofilms (Braissant et al., 2009; De Philippis et al., 2000; Decho et al., 2005; Sutherland, 1999). This carbohydrate-rich material efficiently binds Ca²⁺ restricting the amount available for CaCO₃ precipitation (Kawaguchi & Decho, 2002). However, it is suspected that the subsequent degradation of EPS by heterotrophic organisms releases these bonds, flooding the local environment with Ca²⁺ and thus, indirectly forcing the system into a state where CaCO₃ can precipitate spontaneously (Decho et al., 2005).

Among this heterotrophic community, sulfate reducing bacteria are thought to contribute significantly to CaCO₃ formation, both through the degradation of phototrophic material as well as the production of HCO₃⁻ (Giblin et al., 1990; Visscher et al., 2000; Visscher et al., 1998). In fact, the research on Bahamian stromatolites by Visscher et al. (2000; 1998) gives compelling evidence that sulfate reducing activity is the primary force behind CaCO₃ precipitation. Using SO₄²⁻-coated Ag foil techniques to detect distinct horizons of sulfate reduction and petrographic thin section analysis, they show a direct correlation between zones of elevated sulfate-reducing activity and the formation of lithified micritic laminations (Visscher et al., 2000). The diverse heterotrophic and phototrophic metabolic processes within modern microbialites influence the
immediate chemical environment and thus are essentially responsible for their growth.

This microbial control over CaCO$_3$ formation has been depicted by microelectrode analyses of naturally forming microbialites. Observations at the interface of a microbialite and the ambient water column in Cuatro Ciénegas reveal discrete changes in pH, as well as O$_2$ and Ca$^{2+}$ concentrations with proximity to the microbialite surface (Garcia-Pichel et al., 2004). This demonstrates the ability of the microbial community to control the chemistry of its immediate surroundings, producing what are commonly referred to as chemical microenvironments. Additionally, the processes that drive carbonate precipitation and the formation of these chemical microenvironments are not constant in either time or space. The research in Cuatro Ciénegas (Garcia-Pichel et al., 2004) as well as work in Highborne Cay, Bahamas (Visscher et al., 1998), shows diel fluctuations of chemical microenvironments occurring on a sub-millimeter scale within the microbialite surfaces. In a vertical profile of the top 15 mm of the Highborne Cay microbialites, Visscher et al. (1998) detect significant changes in pH, O$_2$ concentration, and HS$^{-}$ concentration, occurring over a span of 20 hours. 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. Thus, it can be established that the discrete processes relevant to the formation of modern microbialites occur within similar spatial scales.
In addition to influencing chemical microenvironments, organisms within a microbialite also control the isotopic and molecular organic composition of the microbialite matrix. These geochemical signatures contain specific information about the community composition and the interaction of microbial metabolic processes, making them extremely useful for interpreting microbialite formation. The production and subsequent remineralization of organic matter generates isotopic signatures that can be linked to specific enzymatic fractionation pathways or metabolic processes. For example, the examination of $\delta^{13}$C values from CaCO$_3$ crusts in Bahamian stromatolites (Andres et al., 2006) provides insight to the driving force behind aragonite precipitation, showing that heterotrophic processes, rather than photosynthesis, are primarily responsible. In addition, specific molecular organic compounds (lipids) used as indicators of discrete microbial groups have been observed in carbonate matrices as old as 2.7 Ga (Brocks et al., 1999) and thus can allow for the comparison of modern and ancient microbialite systems. This is essential for improving our understanding of ancient microbialites, as genetic material is rarely preserved on geologic time scales (Poinar, 1998).

The general goal of our research was to analyze the bacterial community composition and geochemical signatures in modern, actively accreting microbialites to better understand the processes by which they are forming. However, this goal is complicated by the millimeter-scale variation of these organisms and processes across a depth profile in the microbialite surface. Thus, to observe changes in microbial composition on similar spatial scales as the
previously noted changes in chemical microenvironments, we isolated 5 visually distinct horizons (Fig. 2) from the surface (2.5 cm) of a freshwater microbialite. This work combines genetic, molecular organic, and stable isotopic analyses to facilitate a more complete understanding of how the distribution of organisms relates to microbialite formation and the preservation of biological signatures.

Specifically, we analyze the 16S rRNA gene diversity to characterize the bacterial communities of each of the 5 layers. We also examine the molecular composition of each layer to determine if the lipid content directly reflects the microbial community structure as determined by our genetic analysis, and to better understand how the biomass is degraded, preserved, or altered as it becomes buried with depth. Organic matter $\delta^{13}$C analysis helps us interpret how different carbon cycling processes affect microbialite formation while inorganic $\delta^{13}$C profiles of CaCO$_3$ in each layer enabled us to understand the incorporation of biological signatures into the carbonate matrix. We also look at the relative accretion of CaCO$_3$ within each layer to directly relate the distribution of different organisms and processes to microbialite formation. These interdisciplinary approaches, applied to individual layers within the microbialite provide a unique insight into the formation of modern microbialites and can be used to better understand how specific microbial processes are directly associated with carbonate precipitation.
Materials and Methods

Sample Collection

All samples for this work were collected from Rio Mesquites (Fig. 1) in the CCB during a single sampling trip in July of 2008. Rio Mesquites, the largest river in the CCB, is spring-fed and ranges from 2 to 20 meters across, reaches up to 2.5 meters in depth, and contains many actively forming microbialites (Minckley, 1969). In order to isolate the individual layers, a section of the microbialite surface was removed then cut into slabs (~ 1 cm thick), exposing the vertical face of the sample. A scalpel was then used to separate the visually distinct horizons, resulting in layers 1 through 5 from the surface to the interior (Fig. 2). The 1st layer was approximately 1 – 2 mm thick and was yellowish green in color with a soft string-like texture. Layer 2 was more gelatinous, 2 – 4 mm thick, and had a dark green color. The 3rd layer was bright white with a sandy texture and a thickness of about 5 mm. Layer 4 was grey to tan in color with a varying thickness (~ 5 mm) and a firmer texture than layer 3. Finally, the 5th layer was a dark red and brown color and extremely robust. These 5 layers accounted for approximately the top 2 - 3 cm of microbialites that are greater than 1 meter in height and between 0.5 - 1 meter in diameter.
Figure 2: Layer Isolation
A living microbialite in Rio Mesquites is shown on the left and on the right an image of the exposed vertical profile of the microbialite shows the 5 visually distinct layers.

Samples collected for genomic work were isolated \textit{in situ} into 5 distinct layers (Fig. 2), immediately fixed in RNA-Later (Applied Biosystems/Ambion, Austin, TX, USA) and then stored on ice until return to the University of South Florida, College of Marine Science. Upon return, within 1 week of collection, the RNA-Later was drained off and the samples were frozen (-80 °C) in accordance with the manufacturer's instructions. Samples for molecular organic and isotopic work were placed on ice until return to the lab (within 1 week of collection), at which point they were frozen (-20 °C).
DNA Extraction

Individual layers (1 – 5) were homogenized then approximately 30 mg of sample was extracted using an AllPrep DNA/RNA Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer’s protocol for samples stored in RNA-Later (Applied Biosystems/Ambion). Purified DNA extracts were eluted in sterile H₂O (50 µl) and stored at -20 °C until further analysis.

Amplification, Cloning, and rRNA Gene Sequencing

The bacterial 16S gene was amplified using the Bact-27F (5’- AGA GTT TGA TCM TGG CTC AG -3’) and 1492R (5’- ACG GCT ACC TTG TTA CGA CTT -3’) primer set (Weisburg et al., 1991) acquired from Integrated DNA Technologies (Coralville, IA, USA). All PCR mixtures had a total volume of 50 µl and contained 5 µl of target DNA, 1X REDTaq PCR buffer (10.0 mM Tris-HCl [pH 8.3], 50.0 mM KCl, 1.1 mM MgCl₂, 0.01% gelatin; Sigma-Aldrich, St. Louis, MO, USA), 0.25 mM each deoxynucleoside triphosphate, 1 µM of each primer, and 1 U REDTaq DNA polymerase (Sigma-Aldrich). The PCR conditions contained a 5-minute denaturation step at 95 °C, 30 cycles of (1 minute at 94 °C, 1 minute at 65 °C, and 2 minutes at 72 °C), and a final elongation step of 10 minutes at 72 °C.

Bacterial 16S PCR products were cloned using the TOPO TA cloning kit according to the manufacturer’s instructions (Invitrogen Corp., Carlsbad, CA, USA). Clones were grown on Luria-Bertani (LB) plates with ampicillin (50 µg ml⁻¹) and X-gal (20 µg ml⁻¹) (5-bromo-4-chloroindoly β-D-galactopyranoside in dimethyl formamide). White colonies were further screened by PCR with the primer set
M13F (5’- GTA AAA CGA CGG CCA GT -3’) and M13R (5’- CAG GAA ACA GCT ATG AC -3’) to reaffirm that the insert was taken up. Colonies with inserts were then grown in glycerol stocks (40 ml LB, 10 ml 50% glycerol, 50 μl ampicillin (50 mg ml⁻¹)) and used for sequencing. All gene sequencing was done through Beckman Coulter Genomics, formerly Agencourt Biosciences Corporation (Beverly, MA, USA).

**Sequence Analysis**

Raw sequences were trimmed of all vector sequence and any low quality ends using Sequencher (GeneCodes, Ann Arbor, MI, USA). FastGroupII (http://biome.sdsu.edu/fastgroup/) was used to dereplicate the trimmed 16S sequence libraries, grouping those sequences determined to be a single ribotype (≥97% PSI with gaps) and removing those that were less than 300 base pairs in length (Yu et al. 2006). BLASTN (http://www.ncbi.nlm.nih.gov/) was then used to compare individual ribotypes to previously described gene sequences found in the GenBank database.

**Lipid Extraction**

Approximately 4 – 8 grams of each sample (layer 1 – 5) was homogenized in a pre-cleaned mortar with pestle. Samples were mixed with diatomaceous earth (2:1 DE to sample) then placed in a 22 ml stainless-steel extraction vessel. Total extractable lipids were then obtained by an Accelerated Solvent Extraction system (Dionex ASE 200, Sunnyvale, CA, USA). The ASE method utilized a 5
minute preheating step to bring the sample to an extraction temperature of 120 °C followed by a 10 minute static phase with an extraction cell pressure of 1500 PSI. The extraction program was performed three times; first with dichloromethane (DCM), followed by DCM/methanol (1:1 v/v), and finally with methanol only. Following each extraction phase the cell was flushed with 60% of the total volume and purged for 2 minutes with N₂. The three extracts from each sample were combined, blown down to dryness under a gentle stream of N₂, and then re-dissolved in DCM/methanol (1:1). Elemental sulfur was removed from each sample with activated copper turnings (~5 g; 24hr).

**Separation of Compound Class**

Total lipid extracts were blown down to dryness under a gentle stream of N₂ then re-dissolved in a 0.5N KOH in methanol solution (10 ml). Samples were heated (70 °C) for 2 hrs in a well-sealed 40 ml vial. After saponification, vials were allowed to cool to room temperature prior to opening to avoid any loss of sample. Neutral and acidic compounds were separated by liquid-liquid extractions 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 of DCM/methanol (1:1 v/v). The hydrocarbon fraction was immediately ready for analysis by GC-MS after the volume was reduced under a gentle stream of N₂.
The alcohol fraction was first converted to trimethylsilyl (TMS) derivatives by reducing the sample to dryness, re-dissolving in a solution of bis(trimethylsilyl)-trifluoroacetamide (BSTFA; 50 µl) mixed with pyridine (50 µl) and DCM (10 µl) and heating to 70 °C for 30 minutes.

The fatty acids were converted to methyl ester derivatives by reducing to dryness with N\textsubscript{2} and re-dissolving in methanolic HCl (10 ml). The solution was heated to 60 °C for 3 hours then allowed to cool prior to opening the vial and reducing to dryness. Methyl ester derivatives were re-dissolved in hexane and then separated into polar and apolar fractions on an activated silica column conditioned with hexane. Non-polar compounds were eluted with hexane (7 ml) followed by the fatty acid methyl esters (FAME), which were eluted with 7 ml hexane/ethyl acetate (95:5 v/v). The FAME fraction was then reduced to dryness and re-dissolved in hexane.

**Analysis by GC-MS**

Hydrocarbon, FAME, and TMS-alcohol fractions were analyzed by GC-MS using a Varian CP-3800 GC equipped with a VF-5ms capillary column (Varian Inc., Palo Alto, CA, USA) and linked to a Varian 320-MS operating at 70-eV ionization energy. The GC-MS was fitted with a Varian CP-8400 Autosampler. For hydrocarbon analysis, the injector temperature was held constant at 240 °C while the oven was kept at 50 °C for 2 minutes then gradually raised to 310 °C at a rate of 8 °C minute\textsuperscript{-1}. A constant flow (1 ml minute\textsuperscript{-1}) of helium was used as a carrier gas. For the FAME fraction, the injector temperature was held at 280 °C
while the oven was kept at 50 °C for 1 minute then ramped to 120 °C at a rate of 30 °C minute\(^{-1}\) where it was held for 5 minutes. The oven temperature was then raised to 320 °C at a rate of 8 °C minute\(^{-1}\) where it was again held constant for 10 minutes. A constant flow (2 ml min\(^{-1}\)) of helium was used as a carrier gas. The TMS derivatives of polar alcohol lipids were analyzed with an injector temperature of 250 °C. The oven was kept at 60 °C for 2 minutes then ramped to 250 °C at a rate of 10 °C minute\(^{-1}\) and then to 320 °C at 3°C minute\(^{-1}\). The temperature was then held at 320 °C for 30 minutes. A constant flow (1.5 ml minute\(^{-1}\)) of helium was used as a carrier gas. All compounds were identified by comparison of mass spectra to known compounds found in the literature. Quantification of identified compounds was done by comparison of total ion peaks to those of similar calibration standards.

**Stable Isotope Analysis**

Samples were prepared for isotopic analysis of organic matter by treatment with a mild acid solution (0.5 N HCl) until all carbonate was neutralized. Remaining organic matter was then filtered onto a pre-combusted 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 δ\(^{13}\)C analysis. Solid phase carbonate minerals were prepared for isotopic analysis by grinding samples to a fine powder and drying at low temperature. All isotopic analyses were conducted at the University of South Florida Paleoceanography, Paleoclimatology and Biogeochemistry Laboratory.
A 50 μg aliquot of each carbonate mineral sample was measured for inorganic δ^{13}C using a ThermoFinnigan Delta Plus XL dual-inlet mass spectrometer with an attached Kiel III carbonate preparation device. Isotopic analyses of organic samples were performed using a continuous flow Finnigan Mat Delta Plus isotope ratio mass spectrometer coupled to a Carlo Erba elemental analyzer (EA). Samples were introduced via an autosampler into the combustion furnace of the EA set at 1050°C. Flash combustion converted all carbon in the sample to pure CO₂, which was eluted off a gas chromatograph column and carried by a stream of helium gas to the mass spectrometer, where the ^{13}C abundance was measured based on mass to charge ratios.

**Microscopy**

Scanning electron microscopy (SEM) was completed at the University of South Florida College of Marine Science Electron Microscopy Laboratory, using 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.

**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 are 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 (TC) and total inorganic carbon (TIC) analysis by coulometric titration. Total organic carbon was determined as the difference between TC and TIC.
Results

Bacterial clone libraries

Bacterial 16S rRNA gene libraries were constructed for each of the 5 layers of the microbialite and contained between 62 and 91 clones each. In total, 399 bacterial clones were analyzed from the Cuatro Cié negas microbialite. Approximately 75% of the bacterial clones recovered from the microbialite were less than 97% identical to the most similar sequences found in GenBank, suggesting a high abundance of novel species. Dereplication of the total bacterial clone library (layer 1 – layer 5) with the FastGroupII application (Yu et al., 2006) revealed little overlap in community composition between the individual layers of the microbialite (Fig. 3). This process compared the sequences based on nucleotide similarity, and combined the 399 quality 16S clones into 261 distinct ribotypes. Overlap in community composition was determined as ribotypes that contain sequences from multiple layers. This process revealed that layer 1 and layer 2 have the greatest overlap with 11 ribotypes containing sequences from both layers. Layer 1 did not share a single ribotype with any other layer, while layer 2 shared only two with layer 3. The interior layers (3, 4, and 5) were more similar to one another than to the surface two layers, as there is a total of 12 ribotypes that contained sequences shared between some combination of layers 3, 4, and 5. However, the majority (90%) of ribotypes from the Cuatro Cié negas microbialite clone library contained sequences from only a single layer.
Figure 3: Bacterial Community Overlap
Phylogenetic overlap between layers as determined by a FastGroupII dereplication analysis of the entire bacterial community. Numbers represent the total number of individual ribotypes from a given layer(s).

In addition to comparing the community overlap within the Cuatro Ciénegas microbialite, these sequences were combined with 16S sequences from a Highborne Cay, Bahamas study (Havemann & Foster, 2008) and analyzed together. Dereplication (FastGroupII) of this sequence set revealed that only a single ribotype contained sequences from both the Cuatro Ciénegas and Highborne Cay clone libraries. That ribotype, containing clones from both environments, was composed of sequences that were determined to be most similar to the Cyanobacterial order Pleurocapsales.

Within the Cuatro Ciénegas 16S rDNA clone libraries Cyanobacteria, Bacteroidetes, Proteobacteria, Nitrospiraceae, and Gemmatamonadetes comprised some of the major phylogenetic groups, with disparate distribution throughout the 5 layers (Fig. 4). The microbialite surface was characterized by the overwhelming dominance of phototrophic organisms, comprising
approximately 65% of the total bacterial 16S clones obtained from layer 1 and nearly 50% from layer 2. Cyanobacteria made up the largest portion of the surface community in the Cuatro Ciénechas microbialite bacterial clone library (layer 1 >50%; layer 2 ~30%). Additionally, 11% of the 16S clones in layer 1 had top BLAST hits to chloroplast rRNA gene sequences of eukaryotic algae (diatoms). Sequences with top BLAST hits to α-proteobacteria accounted for approximately 10%, 20%, and 25% of clones in layer 1, 2, and 3 respectively, many of which grouped closely with organisms in the family Rhodospirillaceae, the purple non-sulfur (PNS) bacteria. Following the Cyanobacteria, Bacteroidetes was the second most abundant phylum identified in the layer 1 and layer 2 clone libraries, accounting for 18% and 23%, respectively. The 3rd through 5th layers were primarily characterized by a dominance of the proteobacteria which make up about 45 – 50% of clones, including organisms from the α, β, δ, and γ sub-phyla. The clone libraries of the interior layers (3 – 5) also included numerous groups that were poorly represented (<5% of total clones).

A phylogenetic comparison of the abundant Cyanobacteria sequences with those of cultured Cyanobacteria obtained from GenBank, showed that the Cuatro Ciénechas clones fall into three separate orders. Nearly 60% of the sequences grouped with the order of filamentous Cyanobacteria, Oscillatoriales, primarily in the Pseudanabaena and Leptolyngbya genera. Another 20% of the Cyanobacterial clones were most similar to the Pleurocapsales, and 10% to the Nostocales orders of coccoid Cyanobacteria. Additionally, 10% of the sequences did not appear to group with any specific phylogenetic group of Cyanobacteria.
Figure 4: Bacterial Community Profile
Distribution of bacterial 16S clones for each individual layer. Color-coded pie pieces represent the most similar phylogenetically described BLAST hits in the GenBank database.
Lipid Extracts

The total extractable lipids from the Cuatro Ciéñegas microbialite accounted for 0.03 - 0.68% (Wt% Dry Material) of the individual layers. The distribution of compound classes was characterized by the dominance of fatty acids (analyzed as FAMEs), followed by the polar alcohol compounds including aliphatic and polycyclic constituents (analyzed as TMS derivatives) and a relatively small pool of apolar hydrocarbons. The total organic carbon (TOC) content of each individual layer was used to standardize lipid abundance. Overall there was a decrease in lipid content relative to TOC with depth, however layer 4 exhibited a high lipid concentration, comparable to that of layer 1. The source of this anomaly was not determined.

FAME Distribution

Saturated straight chain fatty acids were the most abundant compounds in all 5 layers, accounting for approximately 50% of the FAMEs analyzed. The saturated C16:0 FAME was the single most dominant compound within every layer’s fatty acid profile (75 – 900 µg g\(^{-1}\) TOC). Monounsaturated fatty acids, primarily C16:1 and C18:1 isomers, were the second most abundant group of compounds, showing an overall decrease in concentration with depth (Fig. 5A). A similar reduction of polyunsaturated C18 and C20 FAMEs is observed between the microbialites surface and interior (Fig. 5B). Branched chain fatty acids comprised a significant but smaller proportion of the total FAMEs in the top layer (343 µg g\(^{-1}\) TOC), however the relative concentration of these compounds increased with depth.
Alcohol Distribution

The normal chain alkanols were the overwhelmingly dominant compounds in the alcohol fraction. Sterols, including cholesterol, ergostanol, stigmasterol, and β-sitosterol accounted for between 15 to 100 µg g\(^{-1}\) TOC in each layer. Phytol ((2E,7R,11R)-3,7,11,15-tetramethyl-2-hexadecen-1-ol) was found in every layer, but most abundant in the 1\(^{st}\) layer at 95 µg g\(^{-1}\) TOC.

Hydrocarbon Distribution

The hydrocarbon fraction displayed a bi-modal distribution of saturated straight chain compounds ranging in length from 16 to 33 carbon atoms. Monounsaturated n-C17:1 and n-C19:1 were also observed in the hydrocarbon profile, with C19:1 becoming the most prominent compound in layer 4 (37 µg g\(^{-1}\) TOC). A series of mid chain methyl-branched alkanes ranging from 18 to 20 carbon atoms were also abundant in the microbialite lipid extracts. Mono-methyl (6-methyl, 7-methyl, and 3-methyl) heptadecanes were found throughout. Additionally, phytene, a derivative of the isoprenoid phytol was detected in layer 1 through layer 4, with the highest concentration (28 µg g\(^{-1}\) TOC) in layer 3. Two C20 isoprenoid thiophene isomers, 3-methyl-2-(3,7,11-trimethyldecyl)-thiophene and 3-(4,8,12-trimethyltridecyl)-thiophene were observed in layer 5. Also present in the hydrocarbon extracts was the apolar hopanoid hop-22(29)-ene (diploptene).
Carbon Isotope Profiles

The stable isotopic composition of organic carbon ($\delta^{13}C$) was significantly depleted in $^{13}C$ within every layer of the Cuatro Ciénegas microbialite. Values ranged from approximately -12 to -23‰ between the five layers (Fig. 6). The $\delta^{13}C$ values of inorganic (CaCO$_3$) carbon (Fig. 6) followed a similar trend to that of the organic pool, however they were significantly more enriched (-1.06 to +3.02‰).

Microbialite Composition

The results of the material balance analysis can be observed in Figure 7A. Briefly, CaCO$_3$ accounted for 35% to 90% (by weight) of the microbialite matrix at every layer, with increasing relative abundance at depth. Alternately, organic matter and water constituted a decreasing proportion of the matrix with depth. 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 occur 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%.
Discussion

This project was very unique in that it combined genomic, molecular organic, and stable isotopic strategies in the analysis of discrete layers within a single microbialite. The layer-specific approach of this project enables us to interpret how the discrete microbial communities within a single microbialite are spatially distributed and how they are connected by the production and decomposition of organic material. The results of this work indicate that there are distinct differences between individual layers of the Cuatro Ciénegas microbialite, both in community composition and geochemical signatures. There was a tremendous amount of information obtained through these diverse analytical approaches, and the major trends in these data lead to a better understanding of microbialite formation, determined by the accretion of CaCO₃ in spatially distinct layers.

Phylogenetic Analysis

Uniqueness of 16S clone libraries

Dereplication of the Cuatro Ciénegas 16S sequences supports the notion that while they are structurally continuous, the individual layers of the microbialite represent phylogenetically distinct communities. The small amount of overlap in community composition between individual layers, as shown in Figure 3, is striking when considering that these 5 layers were isolated from a portion of the
microbialite no more than 3 cm thick. Furthermore, these results contest any concern that the sample isolation methods may have resulted in any significant cross-contamination of material between individual layers.

Comparison of the bacterial 16S clone library to sequences within the GenBank database reveals a high level of similarity to other calcareous microbial mat systems. In particular, the surface shows the greatest similarity to other microbialite communities, as approximately 33% of the clones in layer 1 and layer 2 have a top BLAST hit to sequences recovered from such environments. Top BLAST hits of the Cuatro Ciénegas clones display a wide geographical distribution, but in general there is a high frequency of hits to Sharks Bay Australia (Allen et al., 2009), Yellowstone hot springs (Fouke et al., 2003), and Highborne Cay Bahamas stromatolites (Havemann & Foster, 2008). Other environments with a high frequency of similarity to the Cuatro Ciénegas clones are soil, caves, and karstic limestone systems. The similarity of Cuatro Ciénegas clones to bacteria from other microbialite systems may be an indicator of a unique community that is common to carbonate accreting environments.

Phototrophic Community Composition

Eukaryotic algae, largely diatoms, are abundant in the microbialite surface as shown through hits to chloroplast rRNA gene sequences from the layer 1 clone library (Fig. 4). This was confirmed through a eukaryotic 18S genes analysis and the visual observation (by SEM) of numerous pennate diatoms on the microbialite surface. As displayed though 16S analysis, Cyanobacteria make
up the greatest portion of clones in the layer 1 and layer 2 libraries. This is in agreement with previous work (Breitbart et al., 2009), where a metagenomic analysis of bulk microbialite material (layers were not separated) from Rio Mesquites revealed that 74% of the total sequences recovered were from Cyanobacteria. Among the Cyanobacteria, there is an abundance of clones that group most closely with the order Oscillatoriales. These filamentous Cyanobacteria are common in carbonate precipitating biofilms and observed ubiquitously in modern microbialites (Golubic, 1976; Myshrall et al., 2010). Additionally, the sharing of a Cyanobacterial ribotype through the dereplication of Cuatro Ciénegas and Highborne Cay, Bahamas clones (Havemann & Foster, 2008) reveals some similarity in the Cyanobacteria of these individual communities. This is of significant interest because, Foster and colleagues suggested that Pleurocapsales and other endolithic Cyanobacteria are some of the primary Cyanobacterial groups involved in the formation and growth of stromatolites (Foster et al., 2009). The commonality of Cyanobacteria species found within the Cuatro Ciénegas microbialite to other carbonate precipitating microbial mat environments is a potential indication the role that these specific organisms play in the formation of modern microbialites.

In addition to Cyanobacteria and eukaryotic algae, layers 1 through 3 contain a group of phototrophic $\alpha$-proteobacterial (Fig. 4). The majority of $\alpha$-proteobacterial clones group most closely with species that are physiologically categorized as phototrophic purple non-sulfur (PNS) bacteria. It has been suggested that these 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 & Frigaard, 2006). The presence of such alternating \( \text{O}_2 \) conditions within the surface three layers of the microbialite could be expected, as was observed through chemical microprobe analyses by both Visscher et al. (1998) during a diel cycle study of Bahamian stromatolites and Garcia-Pichel et al. (2004) during a similar study on Cuatro Ciénegas microbialites. The abundance of these organisms suggests that the surface community is dominantly phototrophic and composed of both oxygenic (e.g. Cyanobacteria) and anoxygenic (e.g. PNS bacteria) bacteria.

**Non-phototrophic Community Composition**

Though the surface community is primarily phototrophic in nature, there are many clones most similar to Bacteroidetes, a group of heterotrophic organisms often found in other epilithic biofilms and well adapted for such environments (Bruckner et al., 2008). Their presence in the microbialite surface community provides a possible mechanism for the initial cycling of photoautotrophically produced organic carbon, as they have been described as efficient degraders of EPS and carbohydrates (Bauer et al., 2006; Kirchman, 2002). The ability of Bacteroidetes organisms to efficiently degrade polymeric substances, such as those compounds found in EPS, may also play an important role in \( \text{CaCO}_3 \) formation through the release of EPS bound \( \text{Ca}^{2+} \). Additionally, this initial decomposition of the complex organic matrix likely provides metabolic substrates for heterotrophic organisms found in the interior layers.
Proteobacteria dominate the microbialite interior (layers 3, 4, and 5), as shown by 16S analysis (Fig. 4). The overwhelming diversity of these organisms cannot be overstated, and is probably best described by Kersters et al. (2006) when noted that the Proteobacteria account for more than 40% of all prokaryotic genera, show extreme metabolic diversity, and are of great ecological importance because they play key roles in the carbon, sulfur and nitrogen cycles. Despite this extreme diversity the physiological function of some of these organisms can be resolved. As previously stated the α-proteobacteria, which dominate the 3rd layer, group closely with PNS bacteria and likely utilize a form of anoxygenic photosynthesis. The δ-proteobacteria make up the next largest portion of the layer 3 clone library and are the most abundant single bacterial group in layer 4, accounting for approximately 25% of the clones. The δ-proteobacteria sub-phylum is dominated by anaerobic sulfate-reducing organisms, which account for 75% of the described species (Kersters et al., 2006). 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-reducing activity likely occurs throughout the microbialite, evidence here suggests that it is the dominant process in layer 4.

Nitrospira bacteria, a group of nitrite oxidizers, are the second most abundant organisms in the 4th layer of the microbialite, accounting for about 20% of the clone library. The prevalence of nitrite oxidizing bacteria in this zone is somewhat surprising since the dominant sulfate-reducing bacteria, though
capable of growth under aerobic conditions (Jonkers et al., 2005), typically only
thrive in systems substantially depleted in O\textsubscript{2} (Cypionka, 2000). However, it has
been suggested that bacteria of the genus Nitrospira are competitive under low
nitrite and oxygen conditions (Altmann et al., 2003; Daims et al., 2001; Schramm
et al., 1999). Though the exact metabolic activity of the Nitrospira organisms is
not known in this case, it is likely that they are functioning under extremely low
O\textsubscript{2} to anoxic conditions.

**Phylogeny Summary**

Clone libraries from the 5 distinct layers provide insight into the spatial
distribution of the living microbial community. The individual layers of the Cuatro
Ciénegas microbialite contain discrete bacterial communities, transitioning from
one that is primarily phototrophic at the surface to a heterotrophic community at
depth. Additionally, while the phylogenetic identity of bacteria does not
necessarily reveal their metabolic activity, some interpretations of physiological
roles are possible, especially if supported by geochemical evidence.

**Molecular Organic Biomarkers**

Analyzing the lipid composition of the microbialite provides a dual purpose
in this study. First, the distribution of compounds in each individual layer is
complimentary to the 16S rRNA gene analysis, providing an understanding of the
community composition through specific biomarker abundances. Second,
oberving the relative change in biomarker distribution from layer 1 through layer
5 allows us to interpret the processes through which these microbial communities recycle themselves, as seen through the selective preservation or degradation of individual compounds. Additionally, among the total microbial biomass within the microbialite these lipid components typically have the greatest potential for long-term preservation (Brocks & Banfield, 2009), making them useful for the interpretation of ancient systems.

**Hydrocarbons**

With respect to the community composition, individual hydrocarbons are helpful in identifying specific types of organisms, while the distribution of alkanes reveals more about the overall input of organic material. The shorter chain alkanes (\(n\text{-C}_{16}\) through \(n\text{-C}_{22}\)) are typically derived from a bacterial biomass, while the longer (\(n\text{-C}_{27}\) through \(n\text{-C}_{33}\)) alkanes with an odd-over-even chain length preference are common leaf waxes of higher plants (Eglinton & Hamilton, 1967), indicating some allochthonous deposition of organic matter. The overwhelming dominance of heptadecane (\(n\text{-C}_{17}\)) in layer 1 strongly supports the observed abundance of Cyanobacterial clones in the 16S gene library, as it is a commonly observed component of filamentous Cyanobacteria (Thiel et al., 1997; Winters et al., 1969). Furthermore, the mid-chain methyl-branch hydrocarbons, ranging from 18 to 20 carbon atoms and dominated by 7-methyl-heptadecane, have been used as key indicators of Cyanobacteria in microbial mats (Koster et al., 1999; Shiea et al., 1990; Thiel et al., 1997). The hydrocarbon profile of layer 2 presents an enigma as the abundance of heptadecane (relative to TOC) falls off
drastically, while the clone library continues to display a dominance of Cyanobacteria (Fig. 4). It is possible that this is a function of an increase in the non-lipid component of TOC as a result of abundant EPS production, however this would suggest that there should be a similar decrease in the concentration of other lipid components as well. As heptadecane is the only hydrocarbon to display such a considerable decrease, it is likely that there is a different, unknown force controlling this observation. Thus, the series of mid-chain methyl-branched hydrocarbons provide a more dependable link to the abundance of Cyanobacterial biomass. The only hopanoid identified in the hydrocarbon extracts, diploptene, is found in all layers. While this compound can originate from higher plants such as ferns, it is prevalent in numerous genera of bacteria including Cyanobacteria (Prahl et al., 1992; Rohmer et al., 1984).

**Fatty Acids**

Compounds common in photosynthetic bacteria and eukaryotes, along with numerous general bacterial biomarkers, dominated the fatty acids profile. The abundance of monounsaturated 16:1(n-7) and 18:1(n-9) in the surface correlates well with the genomic analysis as these compounds have primarily been attributed to Cyanobacteria, though not exclusively (Buhring et al., 2009). Polyunsaturated fatty acids, 18:3(n–3) and 18:2(n–6) common in chlorophytes (green microalgae) and 20:5(n-3) and 20:4(n-6) found predominately in diatoms (Boschker & Middelburg, 2002; Harwood & Guschina, 2009), were observed at high concentrations (400 µg g⁻¹ TOC). These unsaturated fatty acids are among
the most abundant compounds in the surface layers, but are drastically reduced at the interior (Fig. 5), indicating that the heterotrophic community is able to efficiently degrade this material.

The change in community composition through the microbialite layers, as determined by the 16S analysis, is further supported by the observed changes in lipid content from layer 1 to layer 5. Figure 5C shows the relative increase in the ratio of 10 Methyl-16:0, a common fatty acid of sulfate-reducing bacteria (Taylor & Parkes, 1983), compared to 16:1 (n-5), a fatty acid ubiquitous to bacteria in general (Vestal & White, 1989). The peak in this ratio occurring in layer 4 coincides with the dominance of δ-proteobacteria within the 16S rRNA gene library, further suggesting the prevalence of sulfate reduction at the microbialite’s interior. The relative increase in this sulfate-reducing bacteria biomarker in association with the decrease of 7-methyl-heptadecane relative to total bacterial alkanes (n-C\textsubscript{16} through n-C\textsubscript{22}) (Fig. 5C) reflects the shift in the bacterial regimes, from an aerobic photoautotrophically dominated community to one that is composed of anaerobic sulfate-reducing organisms. Along with the reduction of unsaturated FAME concentrations (Fig. 5), this observation highlights that the photoautotrophic biomass produced at the surface is readily degraded and recycled by the successive generations of bacteria.
Figure 5: Profile of Select Biomarkers

Both (A) monounsaturated fatty acids found commonly in Cyanobacteria and (B) polyunsaturated fatty acids common in eukaryotic algae decrease in concentration between layer 1 and Layer 5. Polyunsaturated fatty acid concentrations (B) are shown as the sum of 18:3(n–3), 18:2(n–6), 20:5(n-3), and 20:4(n-6). Panel (C) depicts the change in microbial community composition with the ratio of 7 methyl-heptadecane to the sum of (C16 – C22) alkanes (filled squares) decreasing with depth and the ratio of 10 methyl-hexadecanoic acid to 16:1(n-5) (open circles) increasing down to layer 4. These changing ratios indicate a community shift from Cyanobacteria to sulfate-reducing bacteria with depth.

Alcohols - Phytol Degradation

Phytol, which is a component of chlorophyll-a, the photosynthetic pigment of plants, Cyanobacteria, and algae (Bauer et al., 1993; Volkman & Maxwell, 1986), is particularly useful in the identification of phototrophic organisms and additional physiological processes occurring at depth. A common component of recent sediments (Grossi et al., 1998; Rontani et al., 1996), it has been speculated that phytol is the major source of other isoprenoids of 20 or fewer
carbon atoms in geological samples (Rontani & Volkman, 2003). Numerous isoprenoids, including hydrocarbons, fatty acids, and alcohols, found among the Cuatro Ciéñegas microbialite lipid extracts, can be linked to the degradation of phytol and thus the cycling of photoautotrophically produced biomass.

The abundance of phytol in the 1st layer is likely a direct reflection of the numerous phototrophic Cyanobacteria and eukaryotic microalgae, and the release of phytol from intact chlorophyll compounds during senescence of these organisms (Jeffrey & Hallergraeff, 1987). However, more interesting than the presence of phytol itself, is the observation of numerous intermediate metabolites that reflect specific degradation pathways. Sulfate-reducing bacteria are able to efficiently degrade phytol (Rontani et al., 1999), producing isomeric phytadienes and phytenes as metabolites (Grossi et al., 1998). Phytene was observed throughout the Cuatro Ciéñegas microbialite with the highest concentrations occurring in Layer 3. This is in accordance with the dominance of sulfate-reducing activity at the microbialite interior, while the presence of isoprenoid thiophenes detected in the 5th layer further substantiates this concept. These organic sulfur compounds are produced through the reaction of inorganic sulfur (H₂S) with phytol (De Graaf et al., 1992; Fukushima et al., 1992). Thus, the observation of isoprenoid thiophenes is indicative of both the dominant phototrophic community at the microbialite surface as well as a sulfate-reducing community within, which is necessary to produce sufficient amounts of H₂S for this reaction to occur.
Biomarker Summary

In general, the variety of biomarkers found throughout the microbialite layers supports the observation of community composition as determined by 16S analysis. Additionally, this analysis reveals that the molecular composition of organic matter drastically changes between layer 1 and layer 5, with the majority of the photoautotrophic signature being degraded (Fig. 5). However, while many of the straight chain and unsaturated compounds are preferentially degraded (e.g. n-C$_{17}$, unsaturated fatty acids, etc.) the preservation of some compounds within the layer 5 extracts, such as sulfurized derivatives of phytol and some mid-chain methyl-branched hydrocarbons 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) and one that likely leads to increased preservation of distinctive microbial biomarkers (Brocks & Banfield, 2009). Furthermore, the detection of isoprenoid thiophenes in cretaceous deposits (Sinninghe DamstÈ et al., 1989) identifies the stability of such compounds on geologic timescales.

Carbon Isotope Profiles

Previous work (Breitbart et al., 2009) has discussed the isotopic signatures of the Rio Mesquites waters ($\delta^{13}$C, $\delta^{15}$N, $\delta^{34}$S) and the process by which these signatures are incorporated into the microbialite matrix. In general, it was noted that the stable isotopic composition in the CCB waters is consistent with a system that is derived from the chemical weathering of ancient marine
limestone, of which the region is formed. While the previous analysis by Breitbart and colleagues allowed for interpreting the enzymatic processes responsible for the isotopic signatures of the microbialite matrix, the layer specific examination applied here builds on that knowledge by providing a spatial context for understanding these processes.

Figure 6: Carbon Isotope Profiles
Organic (open squares) and inorganic (filled squares) carbon isotope values in the 5 layers of the microbialite.
**Organic Carbon**

The profile of organic $\delta^{13}$C values (Fig. 6) supports the hypothesis that this system is dominated by photoautotrophic carbon fixation, primarily by Cyanobacteria and eukaryotic algae. The $\delta^{13}$C value in the 1st layer (-18.2‰) is typical of photosynthetic carbon fractionation patterns observed in both Cyanobacteria (Calder & Parker, 1973; Pardue *et al.*, 1976) and diatoms (Thompson & Calvert, 1994), when considering the $\delta^{13}$C value of the ambient DIC is approximately +4‰ (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 driving this observed trend. In hypersaline Cyanobacterial mats, Wieland *et al.* (2008) observed a peak concentration of EPS at a depth of 2 – 4 mm, corresponding to the 2nd layer in the CCB microbialite. An abundance of EPS, which is 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), is a possible factor. A second possibility is that this shift is caused by a change in the carbon substrate used for photosynthesis by the organisms at this position in 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 & 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 from CO₂ utilization towards more $^{13}$C enriched HCO₃⁻ (Emrich et al., 1970; Mook et al., 1974) as a substrate for photosynthesis. The $\delta^{13}$C values observed in the 2nd layer of the Cuatro Ciénergas microbialite could easily be produced through the effect of high EPS production, causing both a change in the molecular composition of organic matter (increased EPS) as well as a shift in the primary carbon substrate for photosynthesis.

The steady decrease of organic matter $\delta^{13}$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 a biomass with a $\delta^{13}$C composition that is enriched in $^{13}$C relative to its substrate (ŠantRůčková et al., 2000). Thus, assuming that the abundant heterotrophic bacteria at depth are utilizing the buried photoautotrophic biomass as a carbon substrate, we can conclude that the increase of heterotrophic biomass does not produce this carbon isotope trend. Rather, the driving force behind the observed $^{13}$C depletion with depth (Fig. 6) is likely the continual remineralization of buried organic matter that is rich in $^{13}$C, such as the carbohydrates of EPS. Microbial decomposition, by the heterotrophic community, results in the TOC pool gradually becoming more depleted in $^{13}$C as these enriched component are removed (Benner et al., 1987). Additionally, organic matter remineralization also affects the isotopic composition of the dissolved inorganic carbon (DIC) pool at depth.
Inorganic Carbon (CaCO$_3$)

While the stable isotopic composition of organic material provides insight to the nutrient cycling processes within the microbialite community, the observation of CaCO$_3$ $\delta^{13}$C allows us to interpret how these organic signatures are incorporated into the inorganic carbonate matrix. The similarity in $\delta^{13}$C patterns between organic and inorganic carbon from layer 1 through layer 5 (Fig. 6) suggest a direct link between these two carbon pools. As previously shown by Breitbart et al. (2009) the $\delta^{13}$C of CaCO$_3$ in the Cuatro Ciénegas microbialites is depleted in $^{13}$C relative to the water column DIC, indicating the incorporation of photoautotrophically derived carbon into the inorganic matrix. Through our layer-specific analyses we are able to observe that the $\delta^{13}$C of CaCO$_3$ becomes increasingly depleted at depth (Fig. 6). This observation highlights the process of organic matter remineralization by heterotrophic organisms, forcing a negative shift in the localized DIC-$\delta^{13}$C with depth and the subsequent incorporation of this isotopic signature into the CaCO$_3$ matrix.

Carbonate Accretion

A primary goal of this research is to link the spatial distribution of bacteria and metabolic processes in the Cuatro Ciénegas microbialites to the precipitation of CaCO$_3$. The mass distribution of the primary components of the microbialite matrix (Fig. 7A) provides a better understanding of how the composition changes with depth in the microbialite and how specific organisms and processes are related to those changes. The decrease in OM with depth is indicative of
decomposition by heterotrophic bacteria. Calcium carbonate, as expected, accounts for a high proportion of the total mass, increasing from approximately 35% in layer 1 to 90% (by weight) in layer 5 (Fig. 7A). This is important as it shows that CaCO$_3$ accumulates throughout the 5 layers. Imaging the individual layers with SEM, also reveals this increase in CaCO$_3$ with depth (Fig. 7B), and a graphical illustration more easily depicts how the 5$^{th}$ layer is composed of the accretion of individual generations of CaCO$_3$. Furthermore, the relative contribution of individual CaCO$_3$ generations to the total accumulation (layer 5) can be calculated (Fig. 7C) using the results of the mass balance analysis. 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 (layers 1 and 2) produce approximately 50% of the total CaCO$_3$ (by weight), likely a result of both in situ precipitation and accumulation through the deposition and trapping of allochthonous grains. The second zone of carbonate accretion is observed in the 4$^{th}$ and 5$^{th}$ layers, where approximately 20% of the total CaCO$_3$ is added in each. These distinct zones of precipitation correspond to specific processes that influence precipitation, with the first zone falling within the photoautotrophically dominated portion of the mat and the second zone associated with the abundance of sulfate-reducing heterotrophic organisms.

Understanding the relative contribution of the individual generations of CaCO$_3$ precipitates is important for quantifying the incorporation of biogenic isotopic signatures into the inorganic carbonate matrix. The differences between CaCO$_3$-$\delta^{13}$C values from any one layer of the microbialite to the next layer (Fig.
6) can be accounted for by the newly precipitated generations of CaCO₃. Combining the mass balance analysis, which gave an understanding of the relative mass of CaCO₃ added at each layer, with the CaCO₃-δ¹³C data we are able determine the approximate δ¹³C value of the carbonate being added at each layer (Fig. 7D). This reveals that the δ¹³C values of carbonate grains precipitated at layer 4 and 5 are extremely depleted (-7‰ to -8‰) compared to the ambient DIC of +4‰ (Breitbart et al., 2009). The drastic depletion in CaCO₃-δ¹³C precipitates suggests that up to 50% of the carbon in the DIC pool of these interior layers (4 and 5) is composed of remineralized photoautotrophic biomass. While the calculations do not take into account the processes of CaCO₃ dissolution and reprecipitation, these estimations do predict a significant incorporation of biologically fractionated carbon into the inorganic matrix. This isotopic signature can be preserved on geologic time-scales and used in identifying a biogenic source of ancient carbonates.
Figure 7: Carbonate Accretion Model
(A) Mass balance of organic matter (green), CaCO$_3$ (grey) and water (blue) across the 5 layers. (B) A series of SEM images with an interpretational depiction of separate generations of CaCO$_3$ precipitated at each layer. (C) The mass contribution of each CaCO$_3$ generation relative to the total accumulation in layer 5, with the grey boxes highlighting the 2 primary zones of CaCO$_3$ precipitation. (D) The calculated $\delta^{13}$C of individual generations of CaCO$_3$. 
Summary

An Integrated Perspective

One utility of incorporating these interdisciplinary studies into a single project is that we can construct a interpretive model of how a microbialite forms (Fig. 8), while creating a better understanding of how specific microorganisms contribute to the suite of molecular organic compounds, isotopic signatures, and the precipitation of CaCO₃. The results of this work show that the Cuatro Ciénegas microbialite supports a diverse microbial community with differing composition from the surface to the interior. The general distribution of bacterial groups can be used to interpret the primary forces driving the accretion of CaCO₃. As determined in the 16S gene analysis, the microbial community transitions from one that is dominated at the surface by Cyanobacteria and other phototrophic organisms to a primarily heterotrophic community composed of abundant proteobacteria at depth (Fig. 8A).

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 in this study we were able to show that approximately 50% of the carbonate accumulation occurs within a zone (layers 1 and 2) that is overwhelmingly dominated by these organisms (Fig. 8). Additionally, in a recent study by Bosak et al. (2007) it was determined that anoxygenic photosynthesis increases carbonate precipitation. In combination, the diverse photosynthetic
pathways occurring in the Cuatro Ciénegas microbialite community undoubtedly promote the accretion of CaCO₃. Furthermore, the degradation of photoautotrophic material by the heterotrophic community is also thought to be significant to the formation of microbialites. This process occurs throughout the Cuatro Ciénegas microbialites, with Bacteroidetes-like organisms contributing significantly to the surface community. Anaerobic heterotrophic decomposition also plays an important role in CaCO₃ precipitation at depth. Specifically, sulfate reduction is one of the physiological pathways thought to significantly contribute to the lithification of microbial mats. The prevalence of sulfate reduction in the Cuatro Ciénegas microbialite community is well supported through the observation of not only an abundance of sulfate-reducing δ-proteobacteria (layer 4), but also the presence of sulfurized organic compounds (thiophenes) in the 5th layer. The coupling of this process to the second zone of CaCO₃ precipitation (Fig. 8) suggests that sulfate reduction is vital to the formation of the Cuatro Ciénegas microbialites. In addition to facilitating CaCO₃ precipitation, the anaerobic heterotrophic organisms produce distinct ¹³C depleted CaCO₃-δ¹³C signatures in the 4th and 5th layers of the microbialite (Fig. 8D), due to remineralization of organic matter.

**Significance**

Compared to the previous work by Breitbart *et al.* (2009), which analyzed bulk material of microbialites from Rio Mesquites, the layer-specific approach of this study confirmed many of the same results while adding a spatial context to
our understanding. Much like the metagenomic analysis (Breitbart et al., 2009) the 16S rRNA gene analysis showed a diverse bacterial population while also revealing little overlap in the communities of individual layers. Additionally, Breitbart and colleagues (2009) discovered many genes for the production and utilization of EPS while results of this project further suggest that EPS is involved in the formation of distinct isotopic signatures and contributes to the precipitation of CaCO₃ through heterotrophic decomposition. These complimentary studies produce a broad base of knowledge with respect to the microbialites of Cuatro Ciéñegas. Together they produce a detailed portrayal of the diverse aerobic and anaerobic, phototrophic and heterotrophic organisms and processes responsible for the formation of the CCB microbialites and the preservation of distinct geochemical signatures.

Beyond understanding the formation of modern freshwater microbialites, this work has also provided the opportunity to consider the integrated molecular 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. The selective degradation, preservation, and alteration of distinctive biomarkers throughout the microbialite results 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.
Figure 8: Summary Model
An integrated model of the Cuatro Ciéneegas microbialite showing how (A) the general distribution of photoautotrophic and heterotrophic communities affect (B) the dominant metabolic processes occurring in the Cuatro Ciéneegas microbialites. Panel (C) depicts the 2 primary zones of CaCO₃ precipitation that are associate with specific metabolic processes (indicated by the solid arrows) and (D) shows the gradual depletion of ¹³C in CaCO₃ generations with depth, a product of heterotrophic remineralization of organic matter (indicated by the dashed arrow)
Conclusion

Many previous studies have looked at the diversity of organisms and geochemical signatures in modern microbialites, however this study was one of the first to apply genomic, molecular organic, and both organic and inorganic isotopic analyses simultaneously to examine discrete horizons within an actively accreting microbialite. This unique approach demonstrates the utility of micro-scale analyses when examining the complex community structure of microbial mat systems. Additionally, this work confirms that the complex microbial communities and metabolic processes, which essentially control CaCO$_3$ precipitation, are distinct between the individual layers of the microbialite. The results of this work contribute to the current understanding of microbialite formation by showing that the spatial distribution of bacteria is directly tied to the distribution of CaCO$_3$ precipitation and results in the preservation of distinct geochemical signatures.

In Cuatro Ciénegas the microbialite is composed of a diverse bacterial community with dominant populations of Cyanobacteria and photoautotrophic eukaryotes, anoxygenic phototrophic bacteria, and heterotrophic sulfate-reducing bacteria. This bacterial composition was found to be similar to that of other carbonate forming microbial mats, supporting the concept that distinct microbial assemblages are influential to the formation of microbialites. The photoautotrophic organisms account for the majority of the surface community,
producing a significant amount of biomass that can be identified through both molecular organic and isotopic ($\delta^{13}$C) signatures. This organic matrix, including extracellular polymeric substances, becomes the substrate for heterotrophic decomposition at depth, supporting an abundant sulfate-reducing community that efficiently degrades the photoautotrophic biomass and produces a molecular signature of its own. Through the process of photoautotrophic production, heterotrophic decomposition, and remineralization of organic matter a distinctively biogenic signature is incorporated into the inorganic CaCO$_3$ matrix. The photoautotrophic and heterotrophic communities both contribute to the precipitation of CaCO$_3$, and as previously suggested the tight coupling of these populations is vital to the formation of modern microbialites in Cuatro Ciénegas.
References


variable salinity meltwater ponds of McMurdo Ice Shelf, Antarctica. *Environmental Microbiology*, 7, 519 - 529.


Appendices
Appendix A: Extra Tables

Table A1: FAME Distribution
Concentration of FAME compounds detected in the microbialite lipid extracts.
* indicates an unidentified branching position
Concentration (µg g⁻¹ TOC)

<table>
<thead>
<tr>
<th></th>
<th>Layer 1</th>
<th>Layer 2</th>
<th>Layer 3</th>
<th>Layer 4</th>
<th>Layer 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C₁₂:₀</td>
<td>34.3</td>
<td>22.7</td>
<td>17.8</td>
<td>31.9</td>
<td>20.3</td>
</tr>
<tr>
<td>C₁₃:₀</td>
<td>20.2</td>
<td>18.1</td>
<td>14.5</td>
<td>24.6</td>
<td>16.2</td>
</tr>
<tr>
<td>C₁₄:₀</td>
<td>232.7</td>
<td>72.6</td>
<td>50.7</td>
<td>141.9</td>
<td>23.7</td>
</tr>
<tr>
<td>C₁₅:₀</td>
<td>47.6</td>
<td>32.6</td>
<td>20.7</td>
<td>44.8</td>
<td>16.3</td>
</tr>
<tr>
<td>C₁₆:₀</td>
<td>880.8</td>
<td>363.2</td>
<td>232.1</td>
<td>565.0</td>
<td>78.5</td>
</tr>
<tr>
<td>C₁₇:₀</td>
<td>27.6</td>
<td>17.9</td>
<td>15.5</td>
<td>28.3</td>
<td>11.4</td>
</tr>
<tr>
<td>C₁₈:₀</td>
<td>120.9</td>
<td>56.5</td>
<td>54.9</td>
<td>95.2</td>
<td>20.9</td>
</tr>
<tr>
<td>C₂₁:₀</td>
<td>23.1</td>
<td>22.6</td>
<td>20.4</td>
<td>29.7</td>
<td>21.6</td>
</tr>
<tr>
<td>C₂₂:₀</td>
<td>15.7</td>
<td>24.0</td>
<td>18.5</td>
<td>42.6</td>
<td>17.1</td>
</tr>
<tr>
<td>C₂₆:₀</td>
<td>4.5</td>
<td>16.0</td>
<td>12.9</td>
<td>33.8</td>
<td>9.9</td>
</tr>
<tr>
<td>Unsaturated</td>
<td>L₁</td>
<td>L₂</td>
<td>L₃</td>
<td>L₄</td>
<td>L₅</td>
</tr>
<tr>
<td>C₁₄:₁, n-3</td>
<td>16.5</td>
<td>14.7</td>
<td>12.3</td>
<td>18.4</td>
<td>-</td>
</tr>
<tr>
<td>C₁₆:₁, n-9</td>
<td>9.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C₁₈:₁, n-9</td>
<td>47.4</td>
<td>32.2</td>
<td>9.9</td>
<td>33.9</td>
<td>3.3</td>
</tr>
<tr>
<td>C₁₈:₁, n-7</td>
<td>322.6</td>
<td>91.4</td>
<td>41.0</td>
<td>106.4</td>
<td>4.8</td>
</tr>
<tr>
<td>C₁₈:₁, n-5</td>
<td>42.4</td>
<td>16.8</td>
<td>10.2</td>
<td>10.5</td>
<td>8.8</td>
</tr>
<tr>
<td>C₁₈:₁, n-5</td>
<td>226.5</td>
<td>121.3</td>
<td>5.1</td>
<td>105.4</td>
<td>10.6</td>
</tr>
<tr>
<td>C₁₈:₁, n-9</td>
<td>22.1</td>
<td>17.0</td>
<td>1.1</td>
<td>13.4</td>
<td>8.5</td>
</tr>
<tr>
<td>C₁₈:₂, n-6</td>
<td>115.9</td>
<td>50.1</td>
<td>11.7</td>
<td>33.5</td>
<td>8.8</td>
</tr>
<tr>
<td>C₁₈:₂, n-x</td>
<td>14.9</td>
<td>14.4</td>
<td>0.4</td>
<td>16.0</td>
<td>8.8</td>
</tr>
<tr>
<td>C₁ₘ:₃, n-6</td>
<td>14.7</td>
<td>10.0</td>
<td>8.2</td>
<td>14.8</td>
<td>-</td>
</tr>
<tr>
<td>C₁ₘ:₃, n-3</td>
<td>203.4</td>
<td>92.1</td>
<td>41.5</td>
<td>112.5</td>
<td>11.0</td>
</tr>
<tr>
<td>C₁ₘ:₄, n-3</td>
<td>19.6</td>
<td>10.6</td>
<td>8.4</td>
<td>13.2</td>
<td>-</td>
</tr>
<tr>
<td>C₁₉:₁, n-9</td>
<td>28.4</td>
<td>34.2</td>
<td>25.3</td>
<td>54.2</td>
<td>7.1</td>
</tr>
<tr>
<td>C₂₀:₁, n-9</td>
<td>38.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C₂₀:₄, n-6</td>
<td>28.7</td>
<td>24.6</td>
<td>20.1</td>
<td>30.8</td>
<td>-</td>
</tr>
<tr>
<td>C₂₀:₅, n-3</td>
<td>40.5</td>
<td>24.6</td>
<td>20.1</td>
<td>30.9</td>
<td>-</td>
</tr>
<tr>
<td>Branched</td>
<td>L₁</td>
<td>L₂</td>
<td>L₃</td>
<td>L₄</td>
<td>L₅</td>
</tr>
<tr>
<td>10-Me-12:₀</td>
<td>18.3</td>
<td>16.8</td>
<td>14.1</td>
<td>21.8</td>
<td>16.3</td>
</tr>
<tr>
<td>3,7,11-Me-12:₀</td>
<td>15.7</td>
<td>13.9</td>
<td>11.7</td>
<td>18.5</td>
<td>13.4</td>
</tr>
<tr>
<td>12-Me-13:₀</td>
<td>25.5</td>
<td>17.4</td>
<td>14.5</td>
<td>22.7</td>
<td>15.1</td>
</tr>
<tr>
<td>13-Me-14:₀</td>
<td>142.1</td>
<td>56.7</td>
<td>45.9</td>
<td>69.0</td>
<td>23.2</td>
</tr>
<tr>
<td>12-Me-14:₀</td>
<td>30.9</td>
<td>22.9</td>
<td>20.7</td>
<td>36.7</td>
<td>17.0</td>
</tr>
<tr>
<td>5,9,13-Me-14:₀</td>
<td>-</td>
<td>10.3</td>
<td>8.7</td>
<td>14.0</td>
<td>9.3</td>
</tr>
<tr>
<td>10-Me-15:₀</td>
<td>39.8</td>
<td>14.9</td>
<td>12.7</td>
<td>17.6</td>
<td>5.9</td>
</tr>
<tr>
<td>*-Me-16:₀</td>
<td>21.3</td>
<td>16.4</td>
<td>18.2</td>
<td>28.5</td>
<td>17.0</td>
</tr>
<tr>
<td>*-Me-16:₀</td>
<td>11.2</td>
<td>12.7</td>
<td>9.3</td>
<td>13.8</td>
<td>12.3</td>
</tr>
<tr>
<td>15-Me-16:₀</td>
<td>41.4</td>
<td>23.4</td>
<td>21.8</td>
<td>31.3</td>
<td>12.3</td>
</tr>
<tr>
<td>14-Me-16:₀</td>
<td>16.2</td>
<td>12.4</td>
<td>13.5</td>
<td>21.3</td>
<td>10.8</td>
</tr>
<tr>
<td>3-Me-18:₀</td>
<td>19.0</td>
<td>17.7</td>
<td>28.5</td>
<td>38.9</td>
<td>-</td>
</tr>
<tr>
<td>17-Me-18:₀</td>
<td>9.6</td>
<td>6.6</td>
<td>6.2</td>
<td>10.5</td>
<td>3.7</td>
</tr>
<tr>
<td>TOTAL</td>
<td>3104</td>
<td>1461</td>
<td>981</td>
<td>1999</td>
<td>467</td>
</tr>
</tbody>
</table>
### Table A2: Alcohol Distribution

Concentration of alcohol compounds detected in the microbialite lipid extracts.

<table>
<thead>
<tr>
<th>Saturated</th>
<th>Layer 1</th>
<th>Layer 2</th>
<th>Layer 3</th>
<th>Layer 4</th>
<th>Layer 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_{10:0}</td>
<td>26.8</td>
<td>8.3</td>
<td>3.1</td>
<td>7.5</td>
<td>-</td>
</tr>
<tr>
<td>C_{12:0}</td>
<td>5.8</td>
<td>-</td>
<td>3.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C_{14:0}</td>
<td>37.6</td>
<td>12.9</td>
<td>7.0</td>
<td>13.9</td>
<td>-</td>
</tr>
<tr>
<td>C_{15:0}</td>
<td>4.3</td>
<td>3.3</td>
<td>5.1</td>
<td>16.8</td>
<td>2.8</td>
</tr>
<tr>
<td>C_{16:0}</td>
<td>11.5</td>
<td>4.4</td>
<td>7.6</td>
<td>14.7</td>
<td>-</td>
</tr>
<tr>
<td>C_{17:0}</td>
<td>4.0</td>
<td>4.6</td>
<td>2.6</td>
<td>9.9</td>
<td>-</td>
</tr>
<tr>
<td>C_{18:0}</td>
<td>580.7</td>
<td>259.0</td>
<td>141.3</td>
<td>242.2</td>
<td>16.9</td>
</tr>
<tr>
<td>C_{19:0}</td>
<td>99.7</td>
<td>-</td>
<td>-</td>
<td>3.0</td>
<td>1.9</td>
</tr>
<tr>
<td>C_{20:0}</td>
<td>-</td>
<td>10.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C_{22:0}</td>
<td>8.4</td>
<td>6.4</td>
<td>5.5</td>
<td>-</td>
<td>4.1</td>
</tr>
<tr>
<td>C_{24:0}</td>
<td>-</td>
<td>-</td>
<td>3.1</td>
<td>-</td>
<td>3.0</td>
</tr>
<tr>
<td>C_{26:0}</td>
<td>5.3</td>
<td>6.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Unsaturated</th>
<th>L1</th>
<th>L2</th>
<th>L3</th>
<th>L4</th>
<th>L5</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_{14:1, n-12}</td>
<td>7.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C_{20:1, n-17}</td>
<td>7.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Isoprenoid</th>
<th>L1</th>
<th>L2</th>
<th>L3</th>
<th>L4</th>
<th>L5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phyt-2-one</td>
<td>6.7</td>
<td>4.6</td>
<td>5.7</td>
<td>12.7</td>
<td>1.8</td>
</tr>
<tr>
<td>Phytol</td>
<td>94.5</td>
<td>30.3</td>
<td>20.7</td>
<td>93.2</td>
<td>3.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sterols</th>
<th>L1</th>
<th>L2</th>
<th>L3</th>
<th>L4</th>
<th>L5</th>
</tr>
</thead>
<tbody>
<tr>
<td>cholesterol</td>
<td>30.8</td>
<td>15.9</td>
<td>11.1</td>
<td>16.3</td>
<td>4.1</td>
</tr>
<tr>
<td>ergostanol</td>
<td>11.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>stigmasterol</td>
<td>45.2</td>
<td>18.1</td>
<td>21.5</td>
<td>25.1</td>
<td>4.4</td>
</tr>
<tr>
<td>(\beta)-sitosterol</td>
<td>21.7</td>
<td>11.3</td>
<td>11.5</td>
<td>16.9</td>
<td>6.1</td>
</tr>
</tbody>
</table>

| TOTAL       | 1454| 864 | 378 | 917 | 119 |
## Table A3: Hydrocarbon Distribution
Concentration of hydrocarbon compounds detected in the microbialite lipid extracts.

<table>
<thead>
<tr>
<th>n-Chain</th>
<th>Concentration (μg g⁻¹ TOC)</th>
<th>Layer 1</th>
<th>Layer 2</th>
<th>Layer 3</th>
<th>Layer 4</th>
<th>Layer 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-C&lt;sub&gt;16&lt;/sub&gt;</td>
<td>2.0</td>
<td>2.1</td>
<td>2.1</td>
<td>2.8</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>n-C&lt;sub&gt;17&lt;/sub&gt;</td>
<td>22.8</td>
<td>3.8</td>
<td>19.3</td>
<td>19.2</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>n-C&lt;sub&gt;18&lt;/sub&gt;</td>
<td>2.1</td>
<td>2.3</td>
<td>3.2</td>
<td>8.6</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>n-C&lt;sub&gt;19&lt;/sub&gt;</td>
<td>2.3</td>
<td>2.6</td>
<td>3.9</td>
<td>9.9</td>
<td>8.2</td>
<td></td>
</tr>
<tr>
<td>n-C&lt;sub&gt;20&lt;/sub&gt;</td>
<td>2.3</td>
<td>2.5</td>
<td>3.0</td>
<td>5.8</td>
<td>7.4</td>
<td></td>
</tr>
<tr>
<td>n-C&lt;sub&gt;21&lt;/sub&gt;</td>
<td>2.5</td>
<td>2.7</td>
<td>2.9</td>
<td>3.6</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>n-C&lt;sub&gt;22&lt;/sub&gt;</td>
<td>2.7</td>
<td>2.7</td>
<td>2.8</td>
<td>3.6</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>n-C&lt;sub&gt;23&lt;/sub&gt;</td>
<td>3.4</td>
<td>3.2</td>
<td>3.3</td>
<td>4.0</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>n-C&lt;sub&gt;24&lt;/sub&gt;</td>
<td>3.4</td>
<td>3.1</td>
<td>3.1</td>
<td>3.7</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>n-C&lt;sub&gt;25&lt;/sub&gt;</td>
<td>4.3</td>
<td>3.6</td>
<td>4.3</td>
<td>4.4</td>
<td>4.4</td>
<td></td>
</tr>
<tr>
<td>n-C&lt;sub&gt;26&lt;/sub&gt;</td>
<td>4.0</td>
<td>3.5</td>
<td>3.4</td>
<td>4.1</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>n-C&lt;sub&gt;27&lt;/sub&gt;</td>
<td>4.7</td>
<td>4.1</td>
<td>5.0</td>
<td>5.0</td>
<td>5.1</td>
<td></td>
</tr>
<tr>
<td>n-C&lt;sub&gt;28&lt;/sub&gt;</td>
<td>4.5</td>
<td>3.7</td>
<td>3.9</td>
<td>4.6</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>n-C&lt;sub&gt;29&lt;/sub&gt;</td>
<td>5.1</td>
<td>4.4</td>
<td>6.7</td>
<td>5.8</td>
<td>5.4</td>
<td></td>
</tr>
<tr>
<td>n-C&lt;sub&gt;30&lt;/sub&gt;</td>
<td>4.6</td>
<td>3.9</td>
<td>4.5</td>
<td>5.1</td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td>n-C&lt;sub&gt;31&lt;/sub&gt;</td>
<td>5.1</td>
<td>4.6</td>
<td>8.5</td>
<td>6.9</td>
<td>6.3</td>
<td></td>
</tr>
<tr>
<td>n-C&lt;sub&gt;32&lt;/sub&gt;</td>
<td>4.4</td>
<td>4.0</td>
<td>3.9</td>
<td>4.8</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>n-C&lt;sub&gt;33&lt;/sub&gt;</td>
<td>4.2</td>
<td>4.4</td>
<td>6.4</td>
<td>6.3</td>
<td>5.2</td>
<td></td>
</tr>
</tbody>
</table>

### Unsaturated

<table>
<thead>
<tr>
<th>n-Chain</th>
<th>Concentration (μg g⁻¹ TOC)</th>
<th>Layer 1</th>
<th>Layer 2</th>
<th>Layer 3</th>
<th>Layer 4</th>
<th>Layer 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-C&lt;sub&gt;17:1&lt;/sub&gt;</td>
<td>2.9</td>
<td>-</td>
<td>2.7</td>
<td>7.1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>n-C&lt;sub&gt;19:1&lt;/sub&gt;</td>
<td>8.1</td>
<td>5.0</td>
<td>20.6</td>
<td>37.2</td>
<td>3.7</td>
<td></td>
</tr>
</tbody>
</table>

### Branched*

<table>
<thead>
<tr>
<th>Chain</th>
<th>Concentration (μg g⁻¹ TOC)</th>
<th>Layer 1</th>
<th>Layer 2</th>
<th>Layer 3</th>
<th>Layer 4</th>
<th>Layer 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-Me-17</td>
<td>9.3</td>
<td>2.7</td>
<td>3.6</td>
<td>4.5</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>4-Me-17</td>
<td>3.9</td>
<td>-</td>
<td>2.1</td>
<td>2.8</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>3-Me-17</td>
<td>3.3</td>
<td>2.5</td>
<td>2.2</td>
<td>3.1</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>7-Me-18</td>
<td>2.2</td>
<td>2.8</td>
<td>2.0</td>
<td>2.8</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>6-Me-18</td>
<td>2.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>4-Me-18</td>
<td>4.6</td>
<td>2.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>2-Me-18</td>
<td>2.4</td>
<td>2.6</td>
<td>2.0</td>
<td>3.0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>3-Me-18</td>
<td>2.4</td>
<td>3.1</td>
<td>2.4</td>
<td>4.2</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>3,4-Me-17</td>
<td>2.1</td>
<td>2.4</td>
<td>-</td>
<td>3.1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>2,3-Me-17</td>
<td>2.0</td>
<td>2.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>5-Me-19</td>
<td>2.1</td>
<td>2.5</td>
<td>2.4</td>
<td>7.4</td>
<td>2.7</td>
<td></td>
</tr>
</tbody>
</table>

### Isoprenoid

<table>
<thead>
<tr>
<th>Chain</th>
<th>Concentration (μg g⁻¹ TOC)</th>
<th>Layer 1</th>
<th>Layer 2</th>
<th>Layer 3</th>
<th>Layer 4</th>
<th>Layer 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phyt-2-ene</td>
<td>9.5</td>
<td>3.4</td>
<td>27.9</td>
<td>12.9</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Thiophene A</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Thiophene B</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

### Hopanoids

<table>
<thead>
<tr>
<th>Chain</th>
<th>Concentration (μg g⁻¹ TOC)</th>
<th>Layer 1</th>
<th>Layer 2</th>
<th>Layer 3</th>
<th>Layer 4</th>
<th>Layer 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diploptene</td>
<td>7.4</td>
<td>8.9</td>
<td>17.8</td>
<td>12.0</td>
<td>4.5</td>
<td></td>
</tr>
</tbody>
</table>

**TOTAL** | 175 | 120 | 195 | 245 | 105
### Table A4: Archaeal 16S rDNA Clone Identification
Phylogenetic distribution of Archaeal clones based on top BLAST hits.

<table>
<thead>
<tr>
<th>Layer</th>
<th>Unidentified Crenarchaeota</th>
<th>Marine Group 1 Crenarchaeota</th>
<th>Uncultured</th>
<th># of Clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Layer 1</td>
<td>51%</td>
<td>-</td>
<td>49%</td>
<td>39</td>
</tr>
<tr>
<td>Layer 2</td>
<td>19%</td>
<td>4%</td>
<td>77%</td>
<td>52</td>
</tr>
<tr>
<td>Layer 3</td>
<td>6%</td>
<td>51%</td>
<td>43%</td>
<td>51</td>
</tr>
<tr>
<td>Layer 4</td>
<td>33%</td>
<td>13%</td>
<td>54%</td>
<td>48</td>
</tr>
<tr>
<td>Layer 5</td>
<td>36%</td>
<td>18%</td>
<td>46%</td>
<td>61</td>
</tr>
</tbody>
</table>

### Table A5: Eukaryotic 18S rDNA Clone Identification
Phylogenetic distribution of eukaryote clones based on top BLAST hits.

<table>
<thead>
<tr>
<th>Layer</th>
<th>Diatoms &amp; other algae</th>
<th>Protists(^a)</th>
<th>Copepods &amp; Ostracods</th>
<th>Nematodes</th>
<th>Fungi</th>
<th>Land Plants</th>
<th>Uncultured</th>
<th># of Clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Layer 1</td>
<td>34%</td>
<td>18%</td>
<td>20%</td>
<td>29%</td>
<td>2%</td>
<td>-</td>
<td>2%</td>
<td>58</td>
</tr>
<tr>
<td>Layer 2</td>
<td>10%</td>
<td>3%</td>
<td>-</td>
<td>76%</td>
<td>-</td>
<td>9%</td>
<td>2%</td>
<td>56</td>
</tr>
<tr>
<td>Layer 3</td>
<td>19%</td>
<td>17%</td>
<td>-</td>
<td>62%</td>
<td>-</td>
<td>-</td>
<td>2%</td>
<td>53</td>
</tr>
<tr>
<td>Layer 4</td>
<td>21%</td>
<td>75%</td>
<td>-</td>
<td>2%</td>
<td>2%</td>
<td>-</td>
<td>-</td>
<td>53</td>
</tr>
<tr>
<td>Layer 5</td>
<td>44%</td>
<td>24%</td>
<td>12%</td>
<td>-</td>
<td>20%</td>
<td>-</td>
<td>-</td>
<td>25</td>
</tr>
</tbody>
</table>
Table A6: Bacterial 16S rDNA Clone Diversity Results

Expected ribotype abundance and Shannon-Wiener diversity calculated by the FastGroupII application.

<table>
<thead>
<tr>
<th></th>
<th>Chao-1 (ribotypes)</th>
<th>Shannon-Wiener (nats)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Layer 1</td>
<td>180</td>
<td>3.71</td>
</tr>
<tr>
<td>Layer 2</td>
<td>199</td>
<td>3.54</td>
</tr>
<tr>
<td>Layer 3</td>
<td>645</td>
<td>4.10</td>
</tr>
<tr>
<td>Layer 4</td>
<td>236</td>
<td>3.86</td>
</tr>
<tr>
<td>Layer 5</td>
<td>310</td>
<td>4.12</td>
</tr>
<tr>
<td>Total Community</td>
<td>1035</td>
<td>5.33</td>
</tr>
</tbody>
</table>
Appendix B: Extra Figures

Figure B1: Organic $\delta^{15}\text{N}$ data
Isotopic composition of nitrogen in organic material from each layer. Data collected in the same analysis as organic carbon isotopic composition.
Figure B2: Percent Sequence Identity (Top Blast Hit)
The percent identity distribution of bacterial, archaeal, and eukaryotic clones compared to the most similar sequences in the GenBank database. Values of ≥97% indicate they are from the same species, 92 – 96% indicates they are from a single genus, and values lower than 92% indicate novel organisms.
Figure B3: Bacterial 16S rDNA Diversity Results (Layer 1)
The rank-abundance and rarefaction curves showing the relative ribotype abundance and richness, as calculated by FastGroupII.
Figure B4: Bacterial 16S rDNA Diversity Results (Layer 2)
The rank-abundance and rarefaction curves showing the relative ribotype abundance and richness, as calculated by FastGroupII.
Figure B5: Bacterial 16S rDNA Diversity Results (Layer 3)
The rank-abundance and rarefaction curves showing the relative ribotype abundance and richness, as calculated by FastGroupII.
Figure B6: Bacterial 16S rDNA Diversity Results (Layer 4)
The rank-abundance and rarefaction curves showing the relative ribotype abundance and richness, as calculated by FastGroupII.
Figure B7: Bacterial 16S rDNA Diversity Results (Layer 5)
The rank-abundance and rarefaction curves showing the relative ribotype abundance and richness, as calculated by FastGroupII.
Figure B8: Bacterial 16S rDNA Diversity Results (Total Community)
The rank-abundance and rarefaction curves showing the relative ribotype abundance and richness, as calculated by FastGroupII.