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Metagenomic and stable isotopic analyses of modern freshwater microbialites in Cuatro Ciénegas, Mexico

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Summary

Ancient biologically mediated sedimentary carbonate deposits, including stromatolites and other microbialites, provide insight into environmental conditions on early Earth. The primary limitation to interpreting these records is our lack of understanding regarding microbial processes and the preservation of geochemical signatures in contemporary microbialite systems. Using a combination of metagenomic sequencing and isotopic analyses, this study describes the identity, metabolic potential and chemical processes of microbial communities from living microbialites from Cuatro Ciénegas, Mexico. Metagenomic sequencing revealed a diverse, redox-dependent microbial community associated with the microbialites. The microbialite community is distinct from other marine and freshwater microbial communities, and demonstrates extensive environmental adaptation. The microbialite metagenomes contain a large number of genes involved in the production of exopolymeric substances and the formation of biofilms, creating a complex, spatially structured environment. In addition to the spatial complexity of the biofilm, microbial activity is tightly controlled by sensory and regulatory systems, which allow for coordination of autotrophic and heterotrophic processes. Isotopic measurements of the intracrystalline organic matter demonstrate the importance of heterotrophic respiration of photoautotrophic biomass in the precipitation of calcium carbonate. The genomic and stable isotopic data presented here significantly enhance our evolving knowledge of contemporary biomineralization processes, and are directly applicable to studies of ancient microbialites.

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Introduction

Stromatolites dominated life on early Earth, and fossilized stromatolites represent the oldest macroscopic evidence of life on this planet (Awramik, 1992; Allwood *et al*., 2006). Geochemical signatures (the fingerprints of biological and chemical processes) preserved within biologically mediated sedimentary carbonate deposits, including stromatolites and other microbialites, have been used to reconstruct the interactions between chemical processes and biological evolution over the past 3.5 billion years (Schidlowski *et al*., 1983; Schopf, 1983; Schopf *et al*., 1983; Schidlowski, 1985; 2000; Awramik, 1992; Brocks *et al*., 1999). The ability to accurately reconstruct chemical and biological processes using geochemical signatures preserved in ancient microbialites relies on our understanding of how microorganisms and chemical processes lead to the precipitation of modern microbialites (Schidlowski, 1985; 2000; Thompson and Ferris, 1990; Dupraz and Visscher, 2005).

In the fossil record, the abundance of stromatolites abruptly declined with the appearance of multicellular organisms (Grotzinger and Knoll, 1999), and in modern times, living microbialites are only found within a few select locations. One of these locations is Cuatro Ciénegas, Mexico (Fig. 1). The Cuatro Ciénegas Basin (CCB) is a system of several hundred springs, pools and streams in the Chihuahuan desert of Coahuila, Northern Mexico (Minckley, 1969). In the CCB, geothermal waters are associated with a major north-south fault that bisects the basin. The pools of Cuatro Ciénegas exhibit the lowest phosphorus content reported in continental waters, which

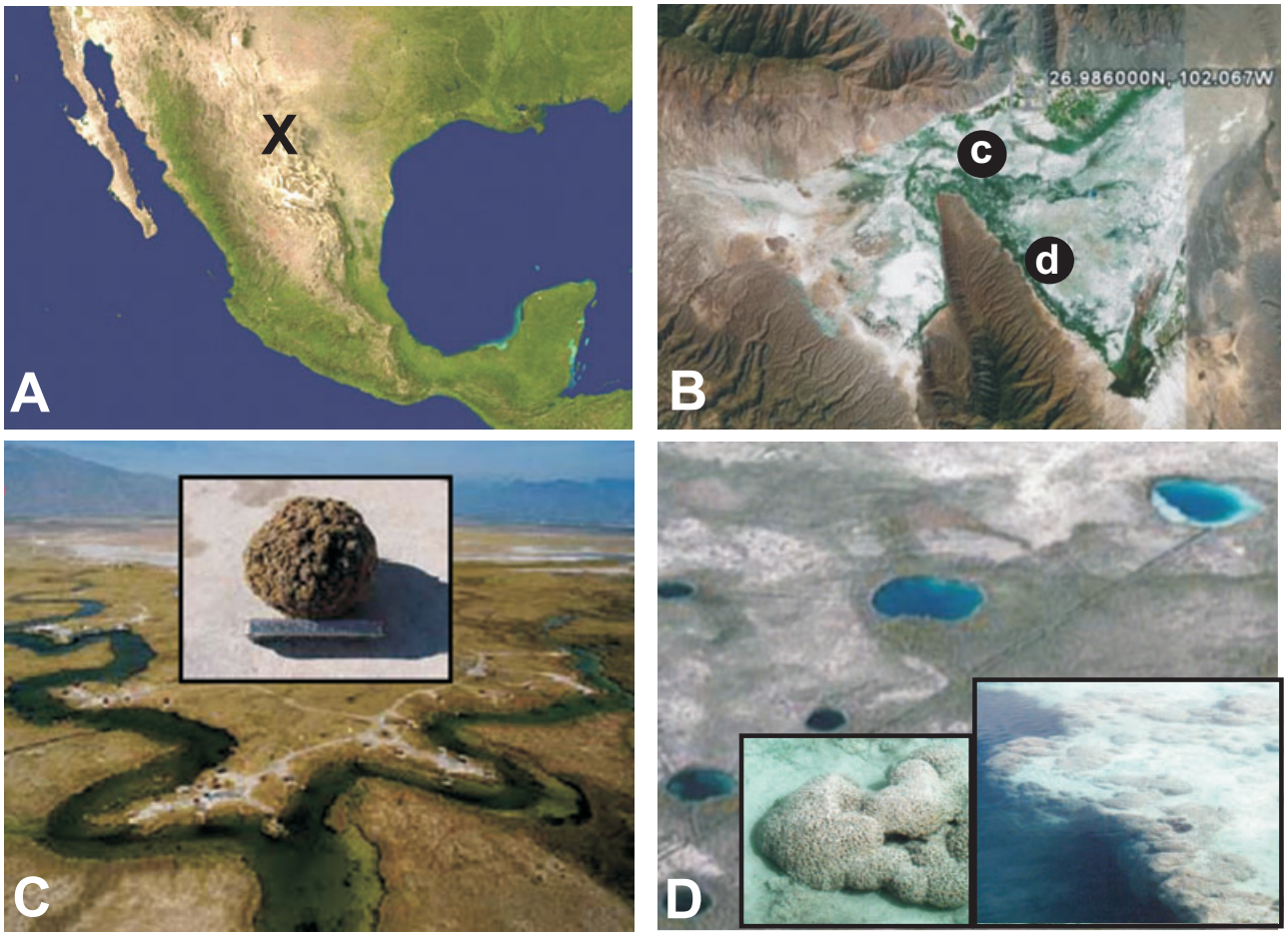


Fig. 1. Site overview.
A. Map of Mexico, with Cuatro Ciénegas indicated.
B. Google Earth image of the Cuatro Ciénegas Valley. Locations of Rios Mesquites (c) and Pozas Azules II (d) indicated on image.
C. Aerial photo of Rios Mesquites, inset shows oncolite.
D. Aerial photo of Pozas Azules II, inset shows shelf-like thrombolites. Aerial photos from Badino and colleagues (2004).

exerts selective pressure on the structure and function of the local biological communities (Elser *et al*., 2005a,b). Microbialites thrive in this region because stoichiometric nutrient constraints prevent snails and other eukaryotes from effectively grazing the microbialites (Elser *et al*., 2005a).

The CCB has the highest level of endemic biodiversity in North America, which is likely a result of its geographic isolation (Stein *et al*., 2000). The region supports more than 70 endemic species of aquatic invertebrates and vertebrates (Badino *et al*., 2004). Recent studies have documented high abundance and diversity of *Bacteria*, *Archaea* and viruses in the pools of Cuatro Ciénegas (Souza *et al*., 2006; Desnues *et al*., 2008). A marine origin for the Cuatro Ciénegas bacteria and viruses was identified, and the viruses followed the trend of the macrofauna in their endemicity (Souza *et al*., 2006; Desnues *et al*., 2008).

Studies of modern microbialites from various systems have documented the existence of extremely novel and diverse microbial communities (Burns *et al*., 2004; Lopez-Garcia *et al*., 2005; Papineau *et al*., 2005; Hagele *et al*., 2006; Souza *et al*., 2006; Desnues *et al*., 2008), as well as a coupling between chemical processes, microorganisms and carbonate mineral precipitation (Krumbein *et al*., 1977; 1979; Vasconcelos *et al*., 1995; Vasconcelos and McKenzie, 1997; Visscher *et al*., 1998; 2000; Reid *et al*., 2000; Dupraz and Visscher, 2005). These processes have been examined extensively in the stromatolites of Highborne Cay, Bahamas, where research has indicated the key microbial groups involved in microbialite development are cyanobacteria, aerobic heterotrophic bacteria, sulfate-reducing bacteria, sulfide-oxidizing bacteria and fermentative bacteria (Visscher and Stolz, 2005). These studies also indicate that biofilms created through the production of extracellular polymeric substances (EPS) generate

steep chemical gradients within the microbialites that undergo large diel fluctuations (Dupraz and Visscher, 2005; Visscher and Stolz, 2005).

Here we examine the microbial processes occurring in two morphologically distinct living microbialites from Cuatro Ciénegas, Mexico using a combination of metagenomic sequencing and isotopic analyses. Metagenomic sequencing allows for identification of the microbial community present in the microbialites and description of their metabolic potential (e.g. Edwards *et al*., 2006). Stable isotopic analysis of geochemical signatures in intracrystalline organic matter can provide valuable information about the chemical and biological processes occurring at, or adjacent to, the site of carbonate precipitation (Des Marais *et al*., 1989; Engel and Macko, 1993; Hayes, 1993; Farrimond *et al*., 2000; Jahnke *et al*., 2001; 2004; Ingalls *et al*., 2003). Examining the pathways present in the microbialite metagenomes coupled with performing stable isotope analyses is a powerful strategy for determining the interplay between microorganisms and chemical processes that influence carbonate precipitation. This study indicates that there is a diverse, redox-dependent microbial community associated with the microbialites, the coordination of which creates chemical microenvironments suitable for carbonate precipitation.

Results and discussion

Metagenomic sequencing and stable isotopic analyses were combined to analyse the composition, metabolic potential and chemical processes of the microbial communities from the modern freshwater microbialites of Cuatro Ciénegas, Mexico. Overall, the results demonstrate that the microbialite communities are highly distinctive in taxa and function. The microbialites are composed of a diverse community of microbes that coexist in an intricate matrix of exopolymeric substances (EPS). The microbial communities demonstrate extensive adaptation to the environment of Cuatro Ciénegas, which is low in phosphorus but high in nitrate and sulfate. Complex sensory and regulatory systems control microbial activity to allow for coordination of autotrophic and heterotrophic processes, creating conditions suitable for the precipitation of calcium carbonate. The diversity and metabolic potential of the microbial communities, as well as the isotopic signatures that these processes impart to the carbonate minerals, are discussed below.

Community members

The taxonomic composition of the microbial communities were determined from the microbialite metagenomic sequences based on BLAST similarities to the SEED database with $E < 0.1$ and then generating a taxonomic profile from the hits (Fig. 2) (Overbeek *et al*., 2005). It is important to note that the metagenomic sequences only represent a partial sampling of the total community, and therefore reveal information about the more abundant community members and metabolisms. Therefore, complete characterization of the entire microbial community is not possible, and rare community members may have not been sampled. The number of sequences recognized from each taxonomic group will be dependent on abundance in the sample, genome size and the available sequences in the database for comparison.

The taxonomic classification was based on 58 593 sequences in the Pozas Azules II metagenome and 30 030 sequences in the Rio Mesquites metagenome with significant similarities to the SEED database. Both samples were dominated by *Bacteria* (87% of the assignable sequences in Pozas Azules II and 95% of the assignable sequences in Rio Mesquites). Heterotrophic bacteria (69%) dominated the metagenomic sequences from Pozas Azules II, while the Rio Mesquites metagenome was dominated by cyanobacteria (74%). Among the heterotrophic bacteria at Pozas Azules II, *Alphaproteobacteria*, *Gammaproteobacteria*, and *Planctomycetes* were the most common. The most common cyanobacterial sequences in both microbialite metagenomes were *Nostocales* and *Chroococcales*. Genes for cell wall components of Gram-negative bacteria (outer membrane proteins), Gram-positive bacteria (teichoic acid, lipoteichoic acid, teichuronic acid synthesis) and *Mycobacteria* (mycolic acid synthesis) were identified in both microbialite metagenomes.

Comparison against the SEED database identified 1061 and 203 archaeal sequences from the Pozas Azules II and Rio Mesquites metagenomes respectively. In both microbialite metagenomes, > 86% of the archaeal sequences were similar to *Euryarchaeota*, although sequences similar to *Crenarchaeota* were also observed. Both metagenomes also contained several genes for archaeal isoprenoid lipids, further supporting the presence of *Archaea* in the microbialites.

The SEED analysis identified 6232 and 1335 sequences in Pozas Azules II and Rio Mesquites respectively, which were similar to eukaryotic sequences. Of these, the majority were similar to *Metazoa* (insects, fish and nematodes), *Viridiplantae* (green algae and land plants), *Rhodophyta* (red algae), *Straemophiles* (including diatoms) and *Fungi*. Additionally, a small number of genes similar to viruses and plasmids (mobile) were recovered from the metagenomes. Overall, the data suggests that the microbialites in Cuatro Ciénegas are composed of diverse, closely interacting, autotrophs and heterotrophs, including *Bacteria*, *Archaea*, eukaryotes and viruses.

A

	Archaea	Cyano-bacteria	Proteo-bacteria	Other Bacteria	Eukarya	Mobile
PAII	1.8%	18.0%	37.4%	31.9%	10.6%	0.3%
RM	0.7%	74.4%	11.2%	9.0%	4.4%	0.3%

B

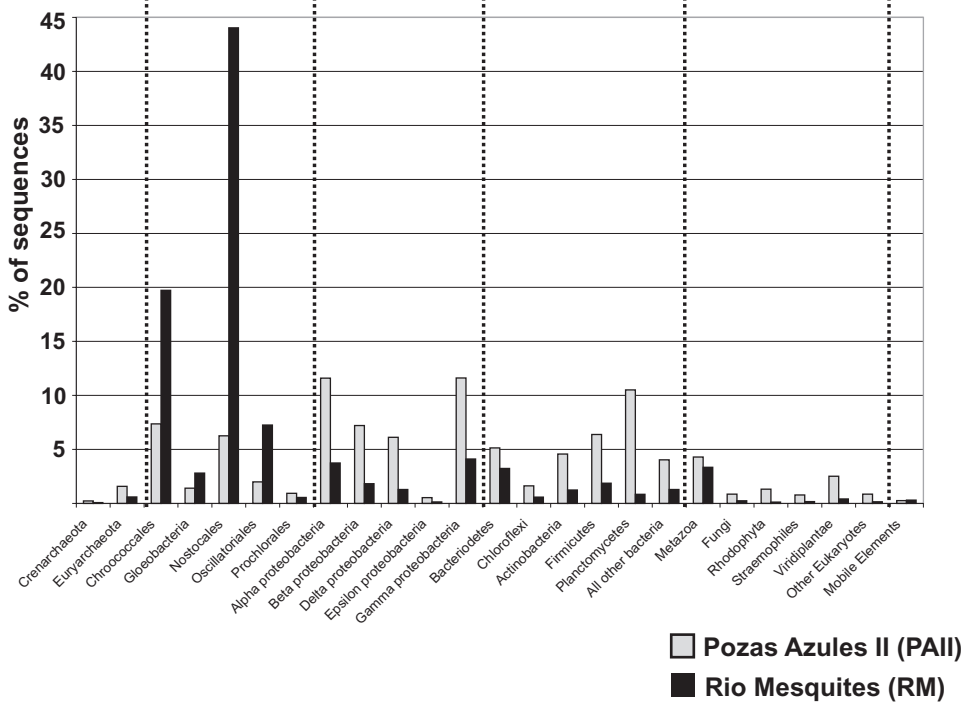


Fig. 2. Taxonomic composition of the microbialite metagenomes based on sequence similarities to the SEED database. A total of 58 593 sequences in the Pozas Azules II metagenome and 30 030 sequences in the Rio Mesquites metagenome had significant similarities to the SEED database.
A. Percentage of identifiable sequences from each metagenome with best BLAST similarity to *Archaea*, cyanobacteria, proteobacteria, other *Bacteria*, *Eukarya* and mobile elements. The percentage in each category is based on the sum of the subdivisions listed in (B). PAII is Pozas Azules II and RM is Rio Mesquites.
B. More detailed breakdown of taxonomic composition of each microbialite metagenome.

Subsystem overview

To compare the major metabolic functions of the Cuatro Ciénegas microbialite metagenomes with those of other freshwater and marine ecosystems, the abundance of genes belonging to each major subsystem category in the SEED platform was determined (Dinsdale *et al*., 2008). Non-parametric ANOVA was used to determine which subsystems were statistically over- or under-represented in the microbialite metagenomes compared with previously sequenced metagenomes of free-living microbes from marine and freshwater environments. Figure 3A shows the percentage of sequences belonging to each major subsystem category in each metagenome type and demonstrates that the gene content of these microbial communities are similar except for a few key areas. Genes involved in membrane transport ($P = 0.002$), phosphorus metabolism ($P = 0.001$) and regulation/cell signalling ($P = 0.007$) were significantly more abundant in the microbialite metagenomes than the free-living marine and freshwater microbial metagenomes. Over-representation of the phosphorus subsystem is logical considering the extremely low phosphorus levels in Cuatro Ciénegas. The over-representation of cell signalling genes is consistent with the high density of microbes found within microbialite communities, and the fact that the microbial communities are secreting and embedded in biofilms.

Nutrient cycling: phosphorus

The pools of Cuatro Ciénegas exhibit the lowest phosphorus content reported in continental waters, which affects the structure and function of the local biological commu-

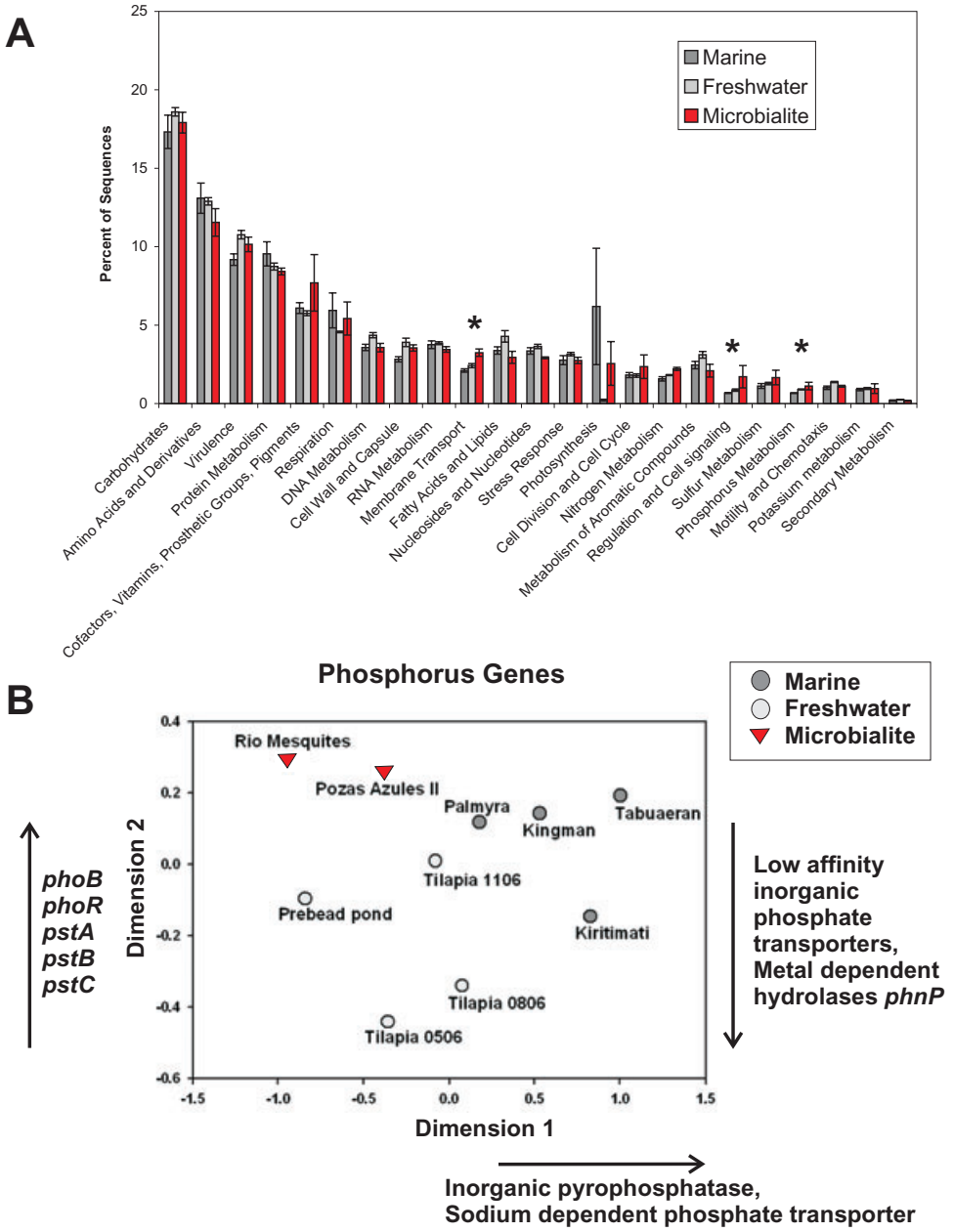


Fig. 3. A. Percentage of sequences belonging to each major subsystem category in marine, freshwater and microbialite metagenomes. Asterisk indicates subsystems that are significantly over-represented in the microbialite metagenomes compared with marine and freshwater microbial metagenomes.
B. Comparison of the distribution of phosphorus genes associated with microbial communities from microbialites, freshwater and marine environments, identified with multidimensional scaling. Each community had a distinctive phosphorus gene signature. Genes that were important in positioning of the metagenomes in the two-dimensional space are provided on the axis, the direction of the arrow indicates where higher abundances of these genes were found.

nities (Elser *et al*., 2005a,b). In fact, it has been suggested that one of the main reasons why there are living microbialites in this region is because stoichiometric nutrient constraints prevent snails and potentially other eukaryotes from effectively grazing the microbialites (Elser *et al*., 2005a). Oncolites similar to those found in Cuatro Ciénegas are also actively forming in the German river Alz, where phosphorus levels are extremely low (Hagele *et al*., 2006). In Rio Mesquites, phosphorus enrichment experiments have demonstrated significant increases in primary production and biogenic calcification, along with major decreases in biomass C:P and N:P ratios in oncolites after P enrichment (Elser *et al*., 2005b). Furthermore, these changes were accompanied by massive alterations in

the microbial community structure in which diverse cyanobacteria-dominated communities shifted to a lower-diversity diatom-dominated community (Elser *et al*., 2005b), signifying that the microbial communities in CCB are especially adapted to life in low phosphorus conditions. As phosphorus is required for many critical cell functions, it is expected that microbes in Cuatro Ciénegas will be especially adapted to scavenge phosphorus, and utilize alternative phosphorus sources. Consistent with this hypothesis, both microbialite metagenomes were distinctive from the marine and freshwater metagenomes (Fig. 3) and contain numerous genes involved in phosphate metabolism, cyanobacterial phosphorus uptake, polyphosphate metabolism and phosphonate and alkylphosphonate utilization (Fig. 4A).

The phosphate regulon consists of several genes such as transporters and hydrolytic enzymes that are inducible under phosphorus starvation, acting to control cellular responses to low extracellular phosphate concentrations and assimilate phosphorus from the environment. Genes for the two-component regulatory system of inorganic phosphate (P_i) signal transduction (*phoB* and *phoR* in Gram-negative bacteria) were found in both the Pozas Azules II and Rio Mesquites microbialite metagenomes. Both microbialite metagenomes also contain the cytoplasmic membrane proteins involved in the transport of orthophosphate (*PstA, PstB, PstC*), the periplasmic P_i-binding protein *PstS*, and phosphate-inducible predicted ATPase *PhoH*. These genes were more abundant in the microbialite metagenomes compared with previously sequenced metagenomes from free-living freshwater and marine microbial communities, and caused the separation between the microbialites and other metagenomes (Fig. 3B). The Pozas Azules II metagenome also contains *PhoQ*, a transmembrane histidine kinase, and *PhoU*, which mediates signal transduction in the Pho regulon. Other genes induced by phosphate starvation, such as alkaline phosphatase, which is used to remove phosphate groups from compounds such as nucleotides and proteins, were abundant in both microbialite metagenomes.

Phosphonates are organophosphorus compounds containing a carbon-to-phosphorus bond (Kononova and Nesmeyanova, 2002). Bacteria have been documented to utilize phosphonates through two main mechanisms: the hydrolysis of specific substrates using a phosphonatase enzyme, or the hydrolysis of a broad range of substrates using the C-P lyase pathway (Kononova and Nesmeyanova, 2002). Genes involved in both of these strategies were present in the microbialite metagenomes. These included phosphonatase genes (phosphonoacetaldehyde hydrolase, 2-aminoethylphosphonate transporters and aminotransferase), as well as several C-P lyase pathway genes (*phnB, phnK*, *phnL*). The C-P lyase pathway for phosphonate utilization has recently been described in the marine diazotroph *Trichodesmium*, where it has been suggested to help explain the prevalence of *Trichodesmium* in oligotrophic environments (Dyhrman *et al*., 2006). In addition, it has been suggested that phosphonates emerged early in the evolution of the planet and may be prebiotic carriers of phosphorus (DeGraaf *et al*., 1997). Thus, it is of particular interest that microbialites, similar to the stromatolites known on early Earth, can utilize this phosphorus source.

Low environmental phosphate concentrations often cause cells to accumulate polyphosphate, which is a polymer containing up to several hundred orthophosphate residues (Kornberg, 1995). Polyphosphate can serve as a phosphorus reservoir, providing a stable and sufficient level of phosphorus (Kornberg, 1995). Polyphosphate metabolism genes (polyphosphate kinase and exopolyphosphatase) were abundant in both microbialite metagenomes. Polyphosphate is a strong chelator of metals, including calcium (Kornberg, 1995; Lin and Singer, 2005), and genes involved in the metabolism of polyphosphate may therefore play an important role in controlling carbonate precipitation in the Cuatro Ciénegas microbialites.

One important known microbial adaptation to low phosphate concentrations is the use of sulfolipids instead of phospholipids as membrane lipids (Van Mooy *et al*., 2006). Sulfolipids are utilized in some marine *Alphaproteobacteria* (Abraham *et al*., 2004), and marine cyanobacteria are known to use this strategy in oligotrophic waters (Van Mooy *et al*., 2006). This ability has also recently been demonstrated for a *Bacillus* strain isolated from Cuatro Ciénegas (Alcaraz *et al*. 2008). Consistent with this known adaptation, genes for sulfolipid biosynthesis were identified in both Cuatro Ciénegas microbialites.

Nutrient cycling: nitrogen

Both microbialite metagenomes contained genes involved in assimilatory nitrate reduction, ammonia assimilation and metabolism, nitrogen fixation and allantoin degradation (Fig. 4B). However, genes for nitrification were not observed among the sequences from either metagenome. The most abundant nitrogen cycling genes were involved in ammonia metabolism, and it appears that this ammonia is being produced through a combination of nitrate/nitrite ammonification, nitrogen fixation and allantoin degradation.

The concentration and the $\delta^{15}N$ values of dissolved inorganic nitrogen (DIN) in the system are critical parameters in identifying the availability of specific nitrogen species and in determining the processes associated with nitrogen assimilation and cycling. The concentration of DIN (in the form of nitrate) is very high in Pozas Azules II and Rio Mesquites, with values of 60 and 150 μM

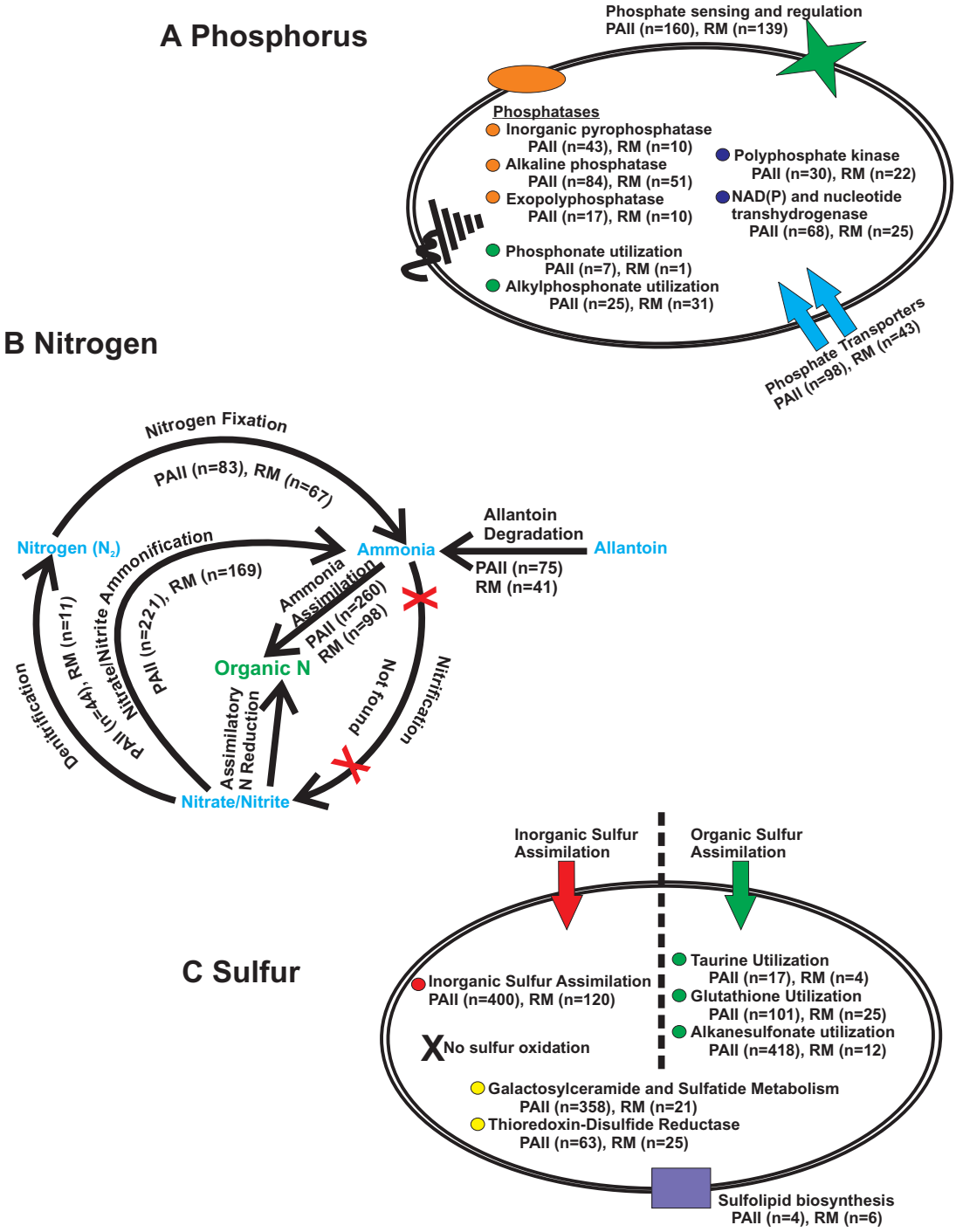


Fig. 4. Overview of microbialite metabolism based on similarities to the SEED database. RM = Rio Mesquites, PAII = Pozas Azules II, n = the number of sequences from each metagenome with BLAST similarities to the given gene or process. (A) phosphorus cycling, (B) nitrogen cycling and (C) sulfur cycling.

respectively. The $\delta^{15}N$ composition of the DIN is extremely enriched with values of +13‰ for Pozas Azules II and +16.5‰ for Rio Mesquites. The enriched $\delta^{15}N$-DIN values coupled with high nitrate concentrations in all the spring-fed pools and rivers is suggestive of the dominance of denitrification in an oxygen-depleted aquifer leading to the ^{15}N-enriched nutrient pool throughout the CCB (Cline and Kaplin, 1975).

The $\delta^{15}N$ of organic nitrogen isolated from within the microbialite can help identify the dominant nitrogen

cycling processes that are associated with the biomineralization process. The $\delta^{15}N$ values of intracrystalline organic matter from the microbialites have a narrow range and vary from an average of +9.5‰ in the Pozas Azules II thrombolite to +11.5‰ in the Rio Mesquites oncolite. Algal photoautotrophic biomass, measured as the $\delta^{15}N$ values of filtered water column particulate organic nitrogen, has $\delta^{15}N$ values of +10–+11‰, similar to values observed in the intracrystalline organic matter isolated from the microbialite. These $\delta^{15}N$ values of organic matter are consistent with the typical +3–+5‰ isotopic fractionation associated with nitrate assimilation by aquatic photoautotrophs in an environment where nitrate availability is not limiting (Fogel and Cifeuntes, 1993). Metagenomic results confirm the presence of the genes for nitrate and nitrite reductase, which are required for the breakdown of assimilated intracellular nitrate to nitrite and eventually to ammonia for amino acid synthesis.

Despite the visual presence of heterocystous cyanobacteria, measured nitrogenase activity in microbial mats from this region (Falcon *et al*., 2007), and the presence of nitrogen fixation genes in both microbialite metagenomes, there is a complete lack of a nitrogen isotopic signature resulting from nitrogen fixation (which would produce $\delta^{15}N$ values of 0–+2‰). As nitrogen fixation is a metabolically energy intensive process requiring abundant iron, the dominance of this process in this nitrate-rich and iron-poor karst terrain is not predicted (Karl *et al*., 2002). However, given the extremely high concentrations of isotopically-enriched nitrate in the system, it is possible that the biomass generated through nitrogen fixation is not enough to significantly influence the $\delta^{15}N$ value of the total biomass. Future studies will need to reconcile the genomic and isotopic data through determination of nitrogen fixation rates, isotopic signatures and gene expression on small spatial scales.

Nutrient cycling: sulfur

The sulfur cycle also likely plays an important role in the lithification of microbialites. Visscher and colleagues (2000) suggested that cyanobacterial photosynthesis, sulfate reduction and anaerobic sulfide oxidation in stromatolitic microbial mats at Highborne Cay, Bahamas lead to the precipitation of calcium carbonate, whereas aerobic respiration and aerobic sulfide oxidation lead to calcium carbonate dissolution (Visscher *et al*., 1998; Dupraz and Visscher, 2005). Using the silver foil technique, it was shown that sulfate reduction occurred at the site of carbonate precipitation in Bahamian stromatolites and lithifying hypersaline microbial mats (Visscher *et al*., 2000; Dupraz *et al*., 2004). Sulfate reduction leads to an increase in pH, which favours carbonate precipitation (Hammes and Verstraete, 2002; Braissant *et al*., 2007). The Cuatro Ciénegas microbialite metagenomes contain genes for sulfate reduction, but genes for sulfur oxidation were not observed among the sequences (Fig. 4C).

Sulfate-reducing bacteria were traditionally thought to exist only in anoxic environments, however, some sulfate-reducing bacteria can tolerate, and even utilize, oxygen (Baumgartner *et al*., 2006). However, sulfate-reducing bacteria living in oxic environments need protection from reactive oxygen species, such as free radicals and peroxidases. Both metagenomes contain a number of genes involved in protection from oxidative stress and reactive oxygen species, including superoxide dismutase, catalase, peroxidase and glutathione reductase. Although the microbialites are growing in environments that are supersaturated in oxygen, the microbes produce exopolymeric substances and create biofilms on the surface of the microbialite, which can lead to the development of steep chemical/redox gradients and locations suitable for sulfate reduction. Along with other heterotrophic and autotrophic bacteria, sulfate-reducing bacteria are known to produce large amounts of exopolymeric substances, which interact with calcium, creating elevated local calcium concentrations that favour the precipitation of calcium carbonate (Bosak and Newman, 2005; Braissant *et al*., 2007).

The $\delta^{34}S$ of dissolved sulfate in the rivers and pools is an important indicator of the sources and cycling of sulfur within the CCB. The $\delta^{34}S$ of dissolved sulfate ranges from +8‰ to +30‰ (Aldama Rodriguez *et al*., 2005), consistent with a source derived from the weathering of ancient marine evaporates which surround the CCB. Interestingly, at the bottom of Pozas Azules II near the inflow of subsurface waters, filamentous sulfur-oxidizing microbial mats were observed. This suggests that some reduced sulfur is generated in the aquifer and oxidized upon exposure to oxidizing surface waters. However, based on the enriched $\delta^{34}S$ of dissolved sulfate in the pools and river, reduced sulfur cycling is not strongly influencing the overall sulfur reservoir.

The sulfur isotopic composition of organic matter isolated from within the microbialites can reveal important information on the sources and redox-dependent cycling of sulfur at the site of carbonate precipitation. Isotopic analyses demonstrate that the intracrystalline organic sulfur from the Pozas Azules II microbialite has a $\delta^{34}S$ value of –25‰ and the Rio Mesquites microbialite has a $\delta^{34}S$ value of –19.8‰. This extremely negative value of $\delta^{34}S$ is indicative of the process of dissimilatory sulfate reduction. The heterotrophic process of dissimilatory sulfate reduction leads to the formation of ^{34}S-depleted HS^- with a fractionation of approximately –40‰ (Canfield, 2001). Thus, the ^{34}S-depleted values observed in the intracrystalline organic matter of the Pozas Azules II

microbialite are associated with the incorporation of sulfur that has undergone dissimilatory sulfate reduction.

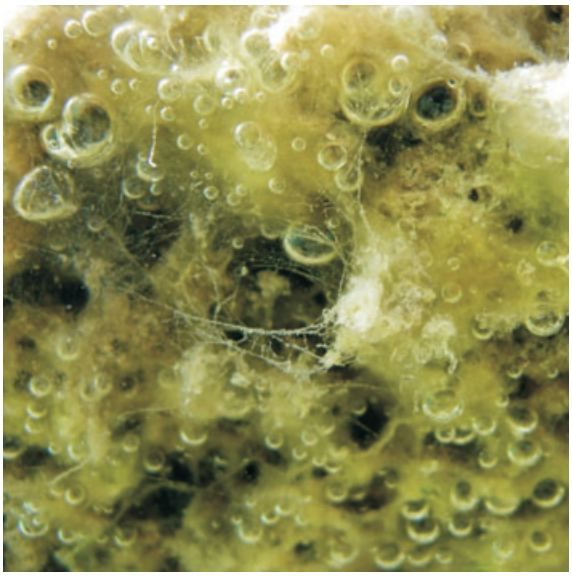
Genes for utilization of taurine, alkanesulfonate and glutathione as sulfur sources were recovered from both metagenomes. Taurine is thought to be a major osmolyte produced by snails, worms and diatoms (Visscher *et al*., 2000; Yancey, 2005), and glutathione and alkanesulfonates can serve as the sole source of sulfur or nitrogen for some microbes (Visscher *et al*., 2000). The sulfonates are a group of organosulfur compounds that are known to be present in aquatic environments (Visscher *et al*., 1999). Through experimental manipulation, Visscher and colleagues (1999) demonstrated that sulfate-reducing bacteria in stromatolite mats could utilize low-molecular-weight sulfonates (Visscher *et al*., 1999). In *Escherichia coli*, the genes for utilizing alternative sulfur sources such as sulfonates are expressed under sulfate starvation (Eichhorn *et al*., 2000). However, in the microbialites, the issue may not be that the cells are starved for sulfate, rather that there are many alternative sulfur sources available for use. For example, the genes for sulfonate utilization may be used to access sulfonated residues present in exopolymeric substances (Visscher *et al*., 1999; Braissant *et al*., 2007). The metagenomic data suggests the importance of examining alternate sulfur utilization pathways in microbialites.

The most abundant sulfur metabolism gene in the Pozas Azules II microbialite metagenomes was arylsulfatase ($n = 316$). Arylsulfatase is greater than five times more abundant than any other sulfur metabolism gene in the Pozas Azules II metagenome. Additionally, this gene was found in the Rio Mesquites metagenome ($n = 10$), and a microbialite metagenome from Highborne Cay, Bahamas ($n = 3$; F. Rohwer, unpublished), where it was also one of the most abundant sulfur genes. Arylsulfatase (EC 3.1.6.1) is a glycosulfohydrolase involved with desulfation of sulfated polysaccharides. Arylsulfatase activity has been found in numerous genera of bacteria, and in the digestive glands of a variety of marine animals that consume algae. This enzyme is thought to facilitate digestion and absorption of algal polysaccharides by cleaving the sulfate ester bonds in dietary polysaccharides (Hoshi and Moriya, 1980; Akagawa-Matsushita *et al*., 1992; Kim *et al*., 2005). Here we propose an additional function for arylsulfatase in the degradation and utilization of sulfur-rich exopolysaccharides, such as those produced by sulfate-reducing bacteria (Braissant *et al*., 2007). Another sulfatase present in both metagenomes that may serve a similar function is *N*-acetylgalactosamine 6-sulfate sulfatase (GALNS). In humans, deficiencies of some sulfatases (e.g. arylsulfatase B, GALNS), as well as other glycosidases identified in the metagenome (e.g. alpha-glucosidase, beta-glucosidase, beta-galactosidase) lead to lysosomal storage disorders such as mucopolysaccharoidosis (Diez-Roux and Ballabio, 2005). This suggests that these genes may be involved in the degradation of exopolysaccharides, which are known to play a key role in carbonate precipitation (see below).

Biofilms and extracellular polymeric substances

The biofilms on the surface of the Cuatro Ciénegas microbialites are readily observable with the naked eye (Fig. 5). Both microbialites also contained a number of genes associated with the 'Widespread Colonization Island' (WCI) (Tomich *et al*., 2007). The WCI contains the tight adherence (Tad) macromolecular transport system, which encodes the machinery required for the assembly of adhesive fimbrial low-molecular-weight protein (Flp) pili involved in biofilm formation (Tomich *et al*., 2007). Tad loci have been identified in approximately 30% of completely sequenced bacterial genomes, and all archaeal species examined (Tomich *et al*., 2007). Several studies have linked quorum sensing pathways to Tad gene expression and biofilm formation (Davies *et al*., 1998; Decho, 1999; Ghannoum and O'Toole, 2004; Tomich *et al*., 2007). The S-adenosylmethionine synthetase gene, which is involved in synthesis of autoinducer-2, was identified in both microbialite metagenomes, further supporting this interaction. In addition, a large number of genes involved in chemotaxis, and both gliding and flagellar motility were identified in both microbialite metagenomes. Gliding motility genes such as those for pilus assembly are known to play an important role in adhesion and biofilm formation, while flagellar motility is critical for approaching surfaces and counteracting repulsive forces (O'Toole and Kolter, 1998; Telford *et al*., 2006).

The microbial communities that comprise biofilms are embedded in a matrix of EPS, including exopolysaccharides, DNA, proteins and lipids (Flemming *et al*., 2007). EPS is known to affect biofilm development in some microorganisms (Ghannoum and O'Toole, 2004). EPS is produced by a variety of microorganisms, including cyanobacteria, aerobic heterotrophs and sulfate-reducing bacteria (Decho, 1990; Sutherland, 2004; Braissant *et al*., 2007). A number of genes involved in EPS biosynthesis were recovered from both metagenomes. The dominant pathways identified were those for alginate metabolism, colonic acid biosynthesis, sialic acid metabolism and rhamnose-containing glycans (Fig. 5). Alginate is an acidic polysaccharide produced by *Pseudomonas aeruginosa*, *Pseudomonas syringae* and *Azotobacter vinelandii*, which has been implicated in biofilm formation (Davies, 1999). Colanic acid is an exopolysaccharide produced by *E. coli* that impairs the initial attachment of cells, but is critical for the formation of complex three-dimensional structure and depth of biofilms (Danese *et al*., 2000). In



Gene	Abundance	
	Pozas Azules II	Rio Mesquites
EPS Production		
Alginate metabolism	131	67
CMP-N-acetylneuraminate biosynthesis	57	40
Colanic acid biosynthesis	50	44
Exopolysaccharide biosynthesis	20	24
Rhamnose-containing glycans	298	148
dTDP-rhamnose synthesis	188	81
Sialic acid metabolism	213	97
EPS Degradation		
Arylsulfatase	310	10
N-acetylgalactosamine 6-sulfate sulfatase (GALNS)	53	2
Alpha-galactosidase	8	0
Beta-galactosidase	27	7
Alpha-glucosidase	9	6
Beta-glucosidase	15	11
Maltodextrin glucosidase	34	19
Chitinase	12	1

Fig. 5. Close-up view of the surface of a Rios Mesquites microbialite and table with abundance of extracellular polymeric substance (EPS) synthesis pathways and genes potentially involved in the degradation of EPS.

P. aeruginosa, rhamnolipids play a role in maintaining heterogenous biofilm architectures, and are believed to maintain cell-free channels to allow transport through the biofilm (Ghannoum and O'Toole, 2004). Sialic acid is known to be part of the capsular material produced by pathogens such as *Neisseria meningitidis* and *E. coli* K1 (Sutherland, 2004). It has been demonstrated that some bacteria (e.g. *Klebsiella* spp.) produce maximum amounts of exopolysaccharide in the absence of phosphate, which might favour exopolysaccharide production by certain bacteria in the low phosphate conditions of Cuatro Ciénegas (Farres *et al*., 1997).

The EPS matrix can sequester dissolved and particulate substances from the environment, providing nutrients for microorganisms in the biofilm (Decho, 1990). EPS compounds aid in the development of sharp geochemical gradients and stable microenvironments by binding and concentrating organic molecules and ions close to cells (Decho, 2000). This allows for localization of microbial biogeochemical processes, and can contribute to biomineralization (Decho, 2000; Dupraz and Visscher, 2005). In particular, EPS binds calcium ions in an organic matrix, which inhibits the precipitation of calcium carbonate. Subsequent microbial degradation of this EPS (by heterotrophic bacteria, including sulfate-reducing bacteria) results in release of the EPS-bound calcium, favouring the localized precipitation of calcium carbonate (Decho *et al*., 2005; Dupraz and Visscher, 2005). Both microbialite metagenomes contain numerous hydrolases, lyases, glycosidases, chitinases and sulfatses (Fig. 5), which are likely to function in the degradation of EPS. Through the production and decay of EPS, the microbial biofilms are critical to the development of localized conditions suitable for carbonate precipitation.

Carbon photoautotrophy

Photosynthesis is the main process on earth that converts light into chemical energy (Bryant and Frigaard, 2006). Cyanobacteria have been previously described as important within the Cuatro Ciénegas microbialites (Falcon *et al*., 2007), and bubbling oxygen is observable on the surface of the microbialites (Fig. 5). Cyanobacteria are known to be a major component of other microbialite systems, such as marine stromatolites in the Bahamas (Reid *et al*., 2000) and freshwater oncoids in Germany (Hagele *et al*., 2006). In fact, the cyanobacteria in stromatolites may have been critical to the production of an oxygenated atmosphere (Des Marais, 1991).

Cyanobacteria have a circadian clock, an endogenous time-keeping mechanism which generates and maintains a 24-h periodicity to gene expression patterns (Golden and Canales, 2003; Lakin-Thomas, 2006). The circadian clock allows diazotrophic cyanobacteria to alternate photosynthesis and nitrogen fixation, and is responsive to environmental cues such as changes in light and temperature. Cyanobacterial circadian clock genes were identified in both metagenomes, including *KaiA*, *KaiB*, *KaiC*, which are the dominant circadian oscillator genes, the circadian input kinase (*CikA*), which relays environmental information to the central oscillator, light-dependent period (*ldpA*), which senses the redox state of the cell, and the *Synechococcus* adaptive sensor (*SasA*)

a clock-associated histidine kinase. These genes allow for temporal regulation of gene expression, which will lead to alternating activity of different microbial groups involved in carbonate precipitation (Garcia-Pichel *et al.*, 2004; Dupraz and Visscher, 2005).

Light-dependent and light-independent reactions of photosynthesis were abundant in both of the microbialite metagenomes. Genes for both Type I photosynthetic reaction centres, which have Fe-S clusters as terminal electron acceptors, and Type 2 reaction centres, which use quinones (Bryant and Frigaard, 2006), were identified in both metagenomes. Genes for Photosystem I and II, which perform the light-induced charge separation across the photosynthetic membrane, were present in both metagenomes, including numerous *Psa* genes (*PsaA, PsaB, PsaE, PsaF, PsaL*) and *Psb* genes (*PsbA, PsbB, PsbC, PsbD, PsbE, PsbV, PsbW*). A number of genes for tetrapyrroles (including chlorophyll), phycobilisome genes (allophycocyanin, phycocyanin, phycoerythrin and phycoerythrocyanin) and the photoreceptor phytochromes were also identified in the metagenomes, consistent with photosynthesis by cyanobacteria. Phycoerythrin absorbs green light (540–570 nm), phycoerythrocyanin absorbs yellow light (~570 nm), phycocyanin absorbs yellow-orange light (620–655 nm), allophycocyanin absorbs red light and phytochromes absorb in the red to near infrared (650–740 nm) (Ke, 2001; Quail, 2002), which suggests that the microbialite cyanobacteria can access a variety of wavelengths of light. In addition, proteorhodopsin was observed in both metagenomes.

Examination of the $\delta^{13}C$ in the carbonate organic matter system of Cuatro Ciénegas also strongly supports the presence of aerobic photoautotrophy. The $\delta^{13}C$ values of DIC ranged from +4.1‰ in Rio Mesquites to +4.5‰ in Pozas Azules II, consistent with a DIC source derived from chemical weathering of ancient marine limestone. The $\delta^{13}C$ of intracrystalline organic matter in the microbialites is –27‰ for the Rio Mesquites oncolite and –25‰ for the Pozas Azules II thrombolite. Recognizing that most aerobic photoautotrophs preferentially assimilate dissolved CO_2 as a carbon source, the $\delta^{13}C$ values of the intracrystalline organic matter are consistent with the enzymatic fractionation [$\varepsilon p_{(CO_2\text{-biomass})}$] associated with oxygenic photosynthesis (Fogel and Cifeuntes, 1993) (Fig. 6A).

Uptake of carbon dioxide can raise the pH locally, which promotes the precipitation of calcium carbonate (Riding, 2006). Both microbialite metagenomes contain numerous genes for carbon dioxide fixation through the Calvin–Benson cycle, which is indicative of photosynthetic organisms (e.g. cyanobacteria). In addition, both metagenomes contain carboxysome genes, such as the catalyst carbonic anhydrase, and the Pozas Azules II metagenome contains several cyanobacterial carbon dioxide concentrating mechanism and high-affinity carbon uptake proteins (Badger and Price, 2003).

Carbonate precipitation: coupling between autotrophy and heterotrophy

Cyanobacteria, sulfate-reducing bacteria and aerobic heterotrophs have all been implicated in controlling calcium carbonate precipitation (Dupraz and Visscher, 2005; Visscher and Stolz, 2005). To address the relative contribution of autotrophic and heterotrophic processes to calcium carbonate precipitation, the $\delta^{13}C$ in the carbonate system was accurately determined. The $\delta^{13}C$ values of DIC ranged from +4.1‰ in Rio Mesquites to +4.5‰ in Pozas Azules II. Utilizing thermodynamic-isotope equilibrium considerations for the precipitation of carbonates from ambient waters (Emrich *et al.*, 1970; Mook *et al.*, 1974), the predicted $\delta^{13}C$ value for the Rio Mesquites oncolites is +6.2‰, and for the Pozas Azules II thrombolites is +6.7‰ (Fig. 6B). However, the measured $\delta^{13}C$ values of the Rio Mesquites oncolites (+1.1‰) and the Pozas Azules II thrombolites (+2.2‰) are both significantly depleted relative to equilibrium considerations (Fig. 6C). These results suggest that either the microbialites are precipitating in disequilibrium or that, more likely, a ^{13}C-depleted source of carbon is available and strongly influencing the ^{13}C-DIC at the site of carbonate precipitation (Andres *et al.*, 2006). The visible oxygen bubbles on the surfaces of the microbialites, microscopy-based occurrence of cyanobacteria and metagenomic confirmation of genes attributed to a RuBisCO-based photosynthetic pathway confirm that the intracrystalline organic matter contains photoautotrophic biomass with $\delta^{13}C$ values between –25‰ and –27‰. Respiration of this ^{13}C-depleted photoautotrophic biomass by heterotrophic organisms (aerobic or anaerobic) would release ^{13}C-depleted CO_2 (~–26‰) to the DIC reservoir, resulting in a localized negative shift in the ^{13}C-DIC. The depleted $\delta^{13}C$ values of the microbialite carbonate can only be explained through the addition of a ^{13}C-depleted source of carbon to the local DIC reservoir. Using a simple mass balance calculation with ambient water (+4‰) and remineralized organic matter (–26‰) as end-members and sole contributors to the local DIC reservoir, it is predicted that at least 20% of the carbon in the carbonate mineral matrix is derived from heterotrophic respiration (Fig. 6D).

These results clearly suggest that the microbialites are precipitating carbonate minerals at sites adjacent to or in direct association with heterotrophic respiration where the remineralized autotrophic carbon is being added to the local reservoir of DIC. This finding is consistent with previous studies which document the importance of biologically mediated isotopic fractionation associated with the

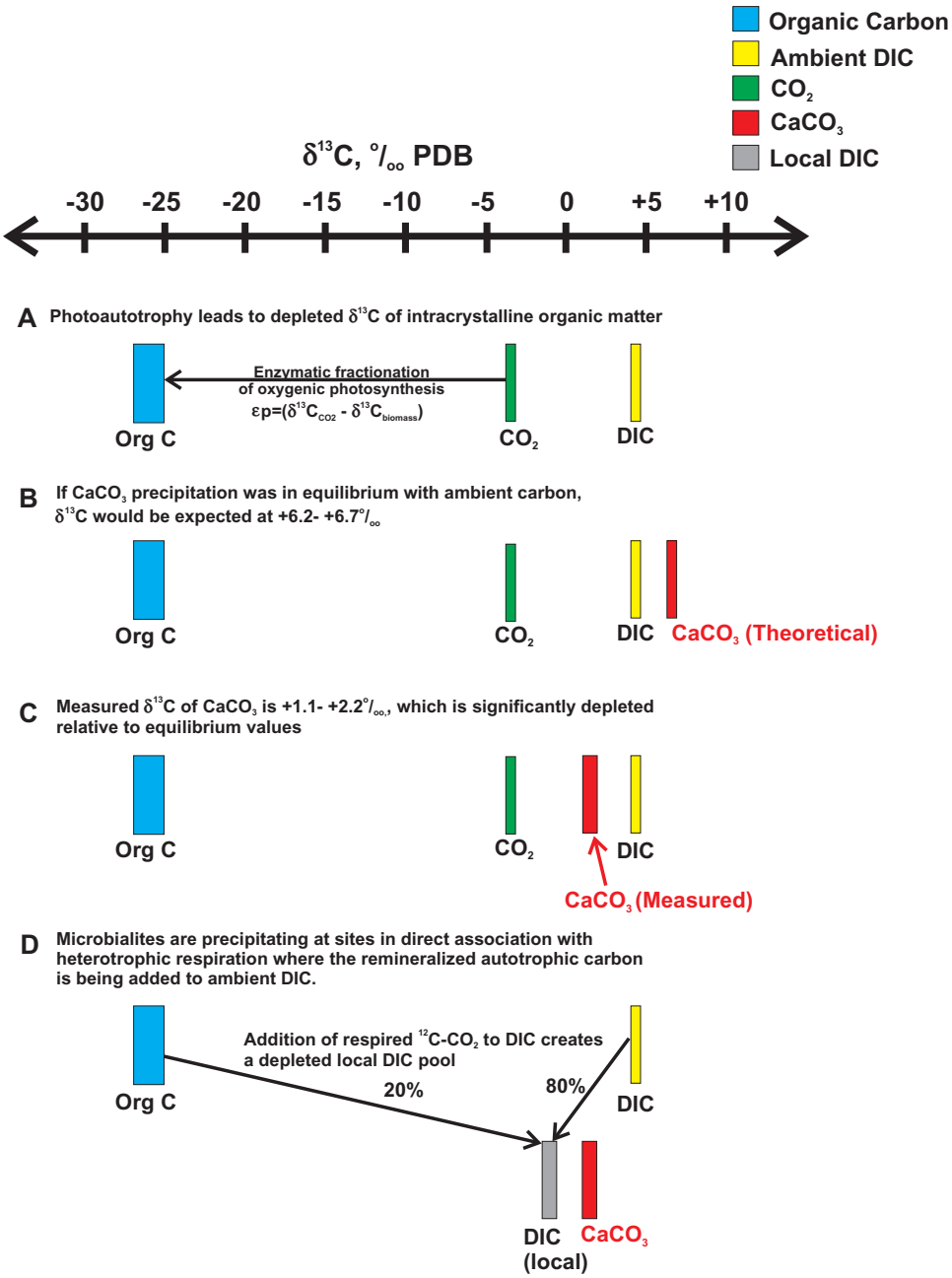


Fig. 6. Calcium carbonate precipitation is the result of close coupling of autotrophy and heterotrophy in the microbialites. PBD = Pee Dee Belemnite.
A. Photoautotrophy leads to a depleted $\delta^{13}C$ of intracrystalline organic matter (–25‰ in Pozas Azules II and –27‰ in Rio Mesquites).
B. If calcium carbonate precipitation occurred in equilibrium with ambient carbon (measured DIC is +4.5‰ in Pozas Azules II and +4.1‰ in Rio Mesquites), the calcium carbonate $\delta^{13}C$ should be +6.7‰ for Pozas Azules II and +6.2‰ for Rio Mesquites.
C. Measured $\delta^{13}C$ of the calcium carbonate are significantly depleted relative to equilibrium calculations (+2.2‰ for Pozas Azules II and +1.1‰ for Rio Mesquites).
D. To produce the observed values, there must be a local DIC pool at the site of carbonate precipitation that is depleted relative to the ambient DIC. This could be created through addition of respired ^{12}C-CO_2 to DIC where heterotrophs are respiring photoautotrophic biomass. To obtain the observed calcium carbonate $\delta^{13}C$ values, it is predicted that at least 20% of the carbon in the carbonate mineral matrix is derived from heterotrophic respiration.

Table 1. Auxiliary metabolic genes identified in the viral fraction from Pozas Azules II (SEED 4440320.2; NCBI 28335) and Rio Mesquites (SEED 4440321.3; NCBI 28357).

Subsystem	Gene	Pozas Azules II phage (*n*)	Rio Mesquites phage (*n*)
Carbohydrates	Enolase	7	2
Carbohydrates	Transketolase	15	3
Carbohydrates	Phosphoglycolate phosphatase	8	1
Cell wall and capsule	GDP-mannose 4,6-dehydratase	54	0
Cell wall and capsule	dTDP-glucose 4,6-dehydratase (*rfbB*)	74	1
Cell wall and capsule	Glucose 1-phosphate thymidylyltransferase	16	0
Cell wall and capsule	*N*-acetylneuraminate synthase	22	2
Cofactors, vitamins, Prosthetic groups, pigments	Nicotinamide phosphoribosyltransferase	26	19
Cofactors, vitamins, Prosthetic groups, pigments	Thymidylate synthase *thyX*	83	19
Cofactors, vitamins, Prosthetic groups, pigments	Cysteine desulfurase	10	3
Cofactors, vitamins, Prosthetic groups, pigments	GTP cyclohydrolase I type 1	24	3
Lipid metabolism	Sulfolipid sulfoquinovosyldiacylglycerol biosynthesis protein	1	0
Lipid metabolism	Sulfolipid (UDP-sulfoquinovose) biosynthesis protein	1	0
Nucleotide metabolism	Thymidylate synthase	45	3
Nucleotide metabolism	Deoxycytidine triphosphate deaminase	20	0
Nucleotide metabolism	Inosine-5′-monophosphate dehydrogenase	24	2
Nucleotide metabolism	Deoxyuridine 5′-triphosphate nucleotidohydrolase	27	36
Nucleotide metabolism	Ribonucleotide reductase	*Total = 241*	*Total = 170*
	Class Ia *(*aerobic)	76	61
	Class Ib (aerobic)	0	68
	Class II (B12-dependent)	163	6
	Class III (anaerobic)	2	35
Phosphate metabolism	Phosphate starvation-inducible protein *phoH*	21	6
Phosphate metabolism	Inorganic pyrophosphatase	5	0
Phosphate metabolism	Exopolyphosphatase	0	3
Sulfur	Arylsulfatase	5	0

photosynthetic uptake and respiratory release of carbon to the locally available DIC at the site of carbonate precipitation (Des Marais *et al*., 1989; Ferris *et al*., 1997; Andres *et al*., 2006). Extracellular polymeric substances and the structural complexity of the microbial biofilm are likely critical to the tight spatial coupling of autotrophic and heterotrophic processes and the maintenance of a depleted local DIC pool. Due to the abundance of sulfate in the system, as well as the genomic and isotopic evidence for sulfate reduction, we speculate that sulfate reduction may be the dominant heterotrophic process influencing microbialite precipitation in Cuatro Ciénegas. These findings are consistent with observations from other microbialite systems (Dupraz and Visscher, 2005), but need further experimental validation.

Horizontal gene transfer of metabolic genes

Concurrent analysis of viral and microbial metagenomes from the same sample has the advantage of identifying candidate genes that are being horizontally transferred via transduction (Dinsdale *et al*., 2008). The metagenomes of the coexisting viruses from each of these microbialite samples have recently been described (Desnues *et al*., 2008). The majority of the identifiable sequences were most similar to phages, which are viruses that infect bacteria. Phages are known to carry auxiliary metabolic genes, which can function in the host cell during infection, allow for adaptation to different environments, and serve as a genetic reservoir for transfer between bacterial hosts (Breitbart *et al*., 2007). Horizontal gene transfer may be facilitated in the microbialite biofilm due to high cell densities (Wolfaardt *et al*., 1999). Although the role of viruses in horizontal gene transfer in the microbialites of Cuatro Ciénegas has not been quantified experimentally, it is notable that a number of auxiliary metabolic genes involved in nucleotide recycling, phosphate metabolism, sulfolipid biosynthesis, folate biosynthesis and capsular and extracellular polysaccharides, were identified in purified viral fractions from the Cuatro Ciénegas microbialites (Table 1). The genes involved in nucleotide metabolism, phosphate metabolism and lipid metabolism likely reflect adaptations to the extremely low phosphorus levels in Cuatro Ciénegas. Future studies need to experimentally determine the role of viruses in transfer of these metabolic genes.

Notably, several genes listed in Table 1 may be involved in the biosynthesis and degradation of EPS. For example, dTDP-glucose 4,6-dehydratase (*rfbB*) was particularly abundant in the Pozas Azules II viral metagenome. This

gene is responsible for the production of rhamnose precursors that are required for the exopolysaccharide synthesis, and increasing *rfbB* activity correlates with the amount of exopolysaccharide produced (Degeest *et al*., 2001; Barreto *et al*., 2005; Peant *et al*., 2005). Although this gene has not previously been found in a phage genome, bioinformatic analyses suggest that this gene has likely undergone multiple horizontal gene transfer events (Omelchenko *et al*., 2003). In addition, GDP-mannose 4,6-dehydratase gene, which is involved in colonic acid biosynthesis, was abundant in the Pozas Azules II viral metagenome. This gene was recently shown to be present in Mimivirus and is suspected to be horizontally transferred (Moreira and Brochier-Armanet, 2008). These examples suggest that viruses may play an important role in the horizontal transfer of genes for exopolysaccharide synthesis, thus possibly contributing to the role of diverse microbes in carbonate precipitation.

Conclusions

This study demonstrates the presence of a complex, redox-dependent, highly adapted community of microbes in the living microbialites of Cuatro Ciénegas, Mexico. The Cuatro Ciénegas microbialite metagenomes have a metabolic composition that is distinct from other marine and freshwater microbial communities, and were enriched in genes for phosphorus metabolism. The microbialite metagenomes contain a large number of genes involved in the establishment and development of biofilms (including the WCI, motility, production and utilization of EPS and quorum sensing), which allow for creation of a complex, spatially structured environment. In addition to the spatial complexity of the biofilm, microbial activity is tightly controlled by sensory and regulatory systems (such as the Pho regulon), cell–cell communication and timing mechanisms (e.g. circadian clock in cyanobacteria). The coordinated metabolic activities of these microbes create chemical microenvironments that favour carbonate precipitation. Isotopic measurements of the intracrystalline organic matter demonstrate the importance of heterotrophic respiration of photoautotrophic biomass in the precipitation of calcium carbonate.

Isotopic signatures preserved within ancient microbialites are often used as proxies to interpret past chemical conditions and microbiological processes on early Earth. The primary limitation to this strategy is our lack of understanding regarding the complex interactions between microbial diversity, chemical processes and the preservation of geochemical signatures in carbonate minerals and intracrystalline organic matter. In order to interpret the environmental and evolutionary significance of microbialites throughout the geologic record, it is vital to develop an integrated view of how chemical processes and microorganisms lead to the precipitation of modern microbialites. This study demonstrates how coupling metagenomic and isotopic approaches can elucidate the identity and metabolic capabilities of microbialite communities and lead to an understanding of the processes associated with carbonate precipitation.

Experimental procedures

Study site and sample collection

The study site of Cuatro Ciénegas is located in the Chihuahuan desert of north central Mexico at 26°59′02.09″N, 102°03′16.96″W (Fig. 1A). Two distinct microbialite morphologies were sampled: spherical oncolites from Rio Mesquites, and domal thrombolites from Pozas Azules II (Fig. 1). Rio Mesquites is located at 26°54′45.17″N, 102°06′54.00″W and Pozas Azules II is located at 26°52′20.83″N, 102°04′38.56″W (Fig. 1B). The two sites are located approximately 3.7 miles from each other, and based on distinct carbon and nitrogen isotopic signatures are not believed to have direct subsurface connections (D. Hollander, unpublished). Both microbialites are pure calcite based on X-ray diffraction analysis (A. Nitti and D. Hollander, unpublished).

Samples for metagenomic sequencing were collected in July 2005, while those for isotopic analyses were collected in July 2006. Water column temperatures in both Rio Mesquites and Pozas Azules II ranged between 27°C and 30°C at the time of sampling, depending on depth. Dissolved species concentrations reflected the chemical weathering of an ancient carbonate platform deposit (dominated by bicarbonate and sulfate). Consistent with previous work of Elser and colleagues (2005a), nutrient measurements indicated abundant nitrate (60 μM in Pozas Azules II and 150 μM in Rio Mesquites) and non-detectable quantities of dissolved phosphorus (< 0.1 μM). Insignificant amounts of ammonia were present at both sites. Both Rio Mesquites and Pozas Azules II were supersaturated in oxygen and had near-neutral water column pH ranging between 7.5 and 8.1.

Metagenomic sequencing

To determine the composition and metabolism of the microbial communities associated with the living microbialites in Cuatro Ciénegas, Mexico, metagenomic sequencing was performed on homogenized samples from the surface layers of a small round oncolite from Rio Mesquites (Fig. 6C) and a large shelf-like thrombolite from Pozas Azules II (Fig. 6D). For the Rio Mesquites metagenome, DNA was extracted from approximately 5 g of the oncolite using the Mo Bio SOIL DNA extraction kit (Mo Bio; Solano Beach, CA) according to the manufacturer's instructions. This method of DNA extraction was unsuccessful on the Pozas Azules II microbialites, possibly due to the abundance of exopolymeric substances. In order to extract DNA of sufficient quality from this microbialite, it was necessary to use a freeze/thaw, CTAB, phenol : chloroform extraction. For the Pozas Azules II sample, the apparent 'microbial mat' fraction of the thrombolite was removed from the remaining material with a 1 ml pipette, and

250 μl aliquots of this material were placed in microfuge tubes and pelleted. This pellet was then re-suspended into 200 μl of TESC [100 mM Tris, pH 8, 100 mM EDTA, 1.5 M NaCl, 1% CTAB (w/v)] and subjected to three cycles of freezing in liquid nitrogen, then thawing at 37°C. Next, the sample was incubated for 30 min at 50°C with 44 μl of 10% SDS and 12 μl proteinase K, while being vortexed every 5 min. Finally, the sample was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1), then once with chloroform, and precipitated overnight at –20°C in isopropanol. DNA was pelleted, washed with 70% ethanol, air dried and re-suspended in 50 μl of sterile water. The DNA was further purified with a silica column from a Qiagen DNeasy kit (Qiagen; Valencia, CA), then ethanol precipitated and re-suspended in H_2O.

In order to obtain sufficient DNA for pyrosequencing, the DNA from both microbialites was amplified using Genomiphi (GE Healthcare; Piscataway, NJ). For the Pozas Azules II microbialite, it was necessary to dilute the DNA 1:100 to get effective Genomiphi amplification, which was likely due to the presence of inhibitors. Six separate reactions were amplified and later pooled before sequencing to minimize amplification biases. After amplification, the DNA was purified with a Qiagen DNeasy silica column and ethanol precipitated. This Genomiphi-amplified DNA was then sequenced using the 454 Pyrosequencing technology (Margulies *et al*., 2005; Edwards *et al*., 2006), yielding a total of 13.3 Mb of sequence data from Rio Mesquites and 33.8 Mb of sequence data from Pozas Azules II. The average sequence lengths were 107 and 104 bp for the Rio Mesquites and Pozas Azules II microbialites respectively.

Bioinformatic and statistical analyses

All metagenomic sequences were compared by BLASTX against the SEED non-redundant database on 27 October 2007, using the MetaGenome Rapid Annotation using Subsystem Technology (MG-RAST) server (http://metagenomics.nmpdr.org). Taxonomic composition of the microbial community was also determined by BLASTN against the Ribosomal Database Project (Cole *et al*., 2007), with significant alignments producing an *E*-value $< 1 \times 10^{-5}$ over at least 50 nucleotides. To explore the metabolic potential of the microbialites, the distribution of sequences that showed similarity to the SEED platform were compared with the metabolic potential of previously sequenced, free-living freshwater and marine microbial communities. The metabolic comparisons were conducted based on the percentage of sequences showing similarities to each major metabolic process within the SEED platform using a non-parametric analysis of variance. The datasets used for comparison were water column (free-living) microbes from four coral reef sites in the Central Pacific and inshore marine samples from the east coast of America (marine), and from four aquaculture ponds in Eastern California (freshwater) (Dinsdale *et al*., 2008). The accession numbers for the datasets used for comparison are as follows: Kingman (SEED 4440037.3, NCBI 28343), Kiritimati (SEED 4440041.3, NCBI 28347), Palmyra (SEED 4440039.3, NCBI 28363), Tabuaeran (SEED 4440279.3, NCBI 28367), Marine DMSP1 SEED 4440364.3, NCBI 19145), DMSP2 (SEED 4440360.3, NCBI 19145), Van (SEED 4440365.3, NCBI 19145), Van2 (SEED 4440363.3, NCBI 19145), Tilapia 11/05 (SEED 4440440.3, NCBI 28387), Prebead (SEED 4440416.3, NCBI 28407), Tilapia 08/06/06 (SEED 4440422.3, NCBI 28603), Tilapia 04/06 (SEED 4440413.3, NCBI 28405).

The phosphorus subsystem was compared in more detail using a multidimensional scaling (MDS) approach on the percentage of sequences that showed similarities to each protein. For this analysis the four coral reef (Kingman, Kiritimati, Palmyra, Tabuaeran) and four freshwater samples (Tilapia 11/05, Prebead, Tilapia 08/06/06, Tilapia 04/06) were used. The MDS was conducted on a dissimilarity matrix constructed using a hierarchical cluster analysis calculated on squared Euclidian distances. The MDS was conducted on a single random start with stress levels set at 0.0001 and 100 iterations.

Chemical and isotopic analyses

Water samples were collected from 10 cm below the surface of the water in 20 l carboys, and were subsequently processed on site for nutrient and isotopic analyses. Immediately after collection, water samples were passed through pre-combusted 0.7 μm GF/F glass fibre filters using a mild pressure of 10 psi. Both the filtrate and the residues on the GF/F filters were stored frozen for subsequent analyses.

A 250 ml aliquot of the filtrate was collected for δ^{13}C-DIC (dissolved inorganic carbon) analysis in a fired glass bottle until overflowing. The sample was then poisoned with approximately 200 μl of saturated $HgCl_2$ solution and the glass bottle quickly sealed with an Apiezon greased ground glass stopper. Samples were stored in the cold and dark until they could be frozen (within 3 days) and were analysed within 2 weeks of collection.

A 50 ml aliquot of the filtrate was analysed for NO_3^-, NO_2^-, NH_4^+, PO_4^{3-} on an autoanalyser (Atlas *et al*., 1971; Grasshoff, 1976; Gordon *et al*., 1993). Aliquots of the filtrate were thawed and prepared for isotopic analysis of nitrate according to the ammonia diffusion method (Sigman *et al*., 1997). Samples were initially conditioned by adding MgO to reduce the DON blank, and incubating for 5 days at 65°C. The samples were then boiled to reduce the volume to 25% of the initial volume. Devarda's alloy was added to the samples to catalyse the reduction of nitrate to ammonium and acidified filter packets were then added to trap the evolved ammonium. The sample mixtures were incubated for an additional 4 days at 65°C, after which the samples were placed on a shaker for an additional 3 days. After shaking, the filter packets were removed and dried in a desiccator. Filter packets remained stored until the day of analysis, when the filters were packed in silver capsules just prior to combustion.

Particulate organic matter retained on pre-combusted GF/F filters were thawed completely, placed in clean Petri dishes and dried overnight in an oven at 60°C. Dried filters were then scraped to remove all organic matter and the top layer of the GF/F filter. The scraped samples were homogenized, packed in aluminum boats and stored in a desiccator until the day of isotopic analysis when δ^{15}N and δ^{13}C were measured simultaneously.

Samples of both types of microbialites were prepared for isolation of intracrystalline organic matter according to

previously described methods (Ingalls *et al.*, 2003). Briefly, samples were ground finely with a mortar and pestle and immersed in a 5% HClO solution for 5 days to remove intercrystalline organic matter. Samples were then thoroughly rinsed with deionized water to remove HClO and subsequently placed in a mild acid solution (0.5 N HCl) inside dialysis bags (3500 Da) until all carbonate was neutralized by the acid and only the insoluble organic matter trapped within the carbonate crystalline matrix remained. The bags were then placed in a tank of deionized water to remove salts. To ensure equilibriation of pH inside and outside the bags, water in the tank was changed daily for a period of 4 days. On day 5, the bags were removed from the tanks and the samples were individually transferred to centrifuge tubes and then frozen. Once frozen, samples were lyophilized, homogenized and subsequently packed into tin capsules for $\delta^{13}C$ and $\delta^{15}N$ analysis. Solid phase carbonate minerals sampled from both microbialites were prepared for isotopic analysis by treatment of the finely ground samples with 5% HClO solution, which were then rinsed with deionized water and dried at low temperature. In preparation for $\delta^{34}S$ analysis, a subsample was decarbonated and dried.

All isotopic analyses, except sulfur and $\delta^{13}C$-DIC, 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 $\delta^{13}C$ and $\delta^{18}O$ 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 analyser (EA). Samples were introduced via an autosampler into the combustion furnace of the EA set at 1050°C. Flash combustion converts nitrogen and carbon in the sample to pure N_2 and CO_2, which are eluted off a gas chromatograph column and carried by a stream of helium gas to the mass spectrometer, where the ^{15}N and ^{13}C abundances are measured based on their mass to charge ratios. Natural abundances of each stable isotope are expressed as per mil (‰) units using delta notation, e.g. $\delta^{15}N = [(R_{sample}/R_{standard}) - 1] \times 1000$, where $R = {}^{15}N/{}^{14}N$. Sulfur isotopic analyses were conducted at Indiana University using a Finnigan MAT252 mass spectrometer coupled to an EA. Water samples were analysed for $\delta^{13}C$-DIC using a Gas Bench coupled to ThermoFinnigan Delta Plus XL dual-inlet mass spectrometer. Dissolved inorganic carbon was analysed on the gas bench by placing the sample (600 μl) in a sealed exetainer. The exetainer was flushed with helium to replace air in the headspace and to purge CO_2 dissolved in the water. Phosphoric acid was added, the sample was shaken then left to equilibrate for 1 h. The CO_2 produced was introduced into the mass spectrometer in continuous flow mode.

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