PROCEEDINGS OF THE 12TH SYMPOSIUM ON THE GEOLOGY OF THE BAHAMAS AND OTHER CARBONATE REGIONS

Edited by
R. Laurence Davis and Douglas W. Gamble

Production Editor:
Douglas W. Gamble

Gerace Research Center
San Salvador, Bahamas
2006

Front Cover: Crinoids in waters of San Salvador, Bahamas. Photograph by Sandy Voegeli, 2003.

Back Cover: Dr. H. Leonard Vacher, University of South Florida, Keynote Speaker for the 12th Symposium and author of "Keynote Address – Plato, Archimedes, Ghyben Herzberg, and Mylroie", this volume , p. ix. Photograph by Don Seale.

Wallace Press, Concord, NH.

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ISBN 0-935909-77-X

MICROBIAL ECOLOGY IN MODERN STROMATOLITES FROM SAN SALVADOR, BAHAMAS

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ABSTRACT

Storr's Lake, a hypersaline lake on San Salvador Island, Bahamas, that contains magnesium enriched calcium carbonate lithified mats consisting of filamentous microorganisms, diatoms, cyanobacteria, associated bacteria, and trapped sediment. We used microbiological techniques including 16S rRNA analyses, activity assays, and microscopy techniques to evaluate the microbial ecology of the Storr's Lake mats. Because of the high sulfate content (76-150 g/L), we looked for sulfate-reducing bacteria (SRB). A laser scanning confocal electron microscope (LSM) and a JEOL 6430F field scanning electron microscope (FE-SEM) equipped with light element electron dispersive X-ray spectrometry (EDS) were used to examine the stromatolites for the elemental composition and presence of biogenic features. The 16S rRNA analysis indicates the presence of five sulfur-reducing genera of bacteria including *Desulfovibrio, Desulfobulbus, Desulfococcus, Desulfobacter,* and *Desulfobacterium.* The *Desulfobulbus* 16S rRNA biofilm concentrations in are significantly higher (140.3 $ng/g_{sediment}$) than those reported previously. The PLFA analysis reveals a diverse microbial population with a high turnover rate. The FE-SEM analyses indicate rod-like, spheroid-shaped features, dumbbells, filaments, biofilms and diatoms as components of the fractured stromatolites. The biofilm is composed of thick filaments and web-like, vesiculated film. Small spheres were observed (<0.1μm) in the biofilms as well as various crystals tightly associated with filamentous microorganisms that may be abiotic precipitates.

INTRODUCTION

Stromatolites are organosedimentary structures formed by microbial mats. Microbial mats contribute to the formation of stromatolites by trapping inorganic particles and by precipitating calcium carbonate. Some fossil stromatolites are 3.5 billion years old (Aitken, 1967). Modern stromatolites are described from a number of environments, including fresh (Fritsch, 1950; Osborne et al., 1982), brackish (Monty, 1967), marine (Logan, 1961; Dill et al., 1986) and hypersaline waters (Walter, 1976). Modern stromatolites often differ from ancient stromatolites in their patterns of lamination and internal fabric development (Neumann et al., 1988). In addition, the earliest stromatolites lacked metazoan grazers. The appearance of metazoan grazers, including gastropods, limited the size of modern stromatolites (Garrett, 1970).

Bahamian stromatolites have been found in less than 10 m of water in subtidal current channels and sand shoals near Lee Stocking Island on the eastern margin of the Bahamas platform (Dravis, 1983; Dill et al., 1986). They are thought to be protected from bacteriovors by periodic burial beneath the sand (Aalto and Shapiro, 1991). Stromatolites are also found in Storr's Lake, an enclosed hypersaline body of

water on San Salvador Island (Hattin, 1982). Storr's Lake stromatolites are approximately 2000 years old, approximately half the age of the marine Bahamian stromatolites. The Storr's Lake hypersaline stromatolites are potential modern day analogs to some ancient stromatolitic structures (Hoffmann et al., 1999).

The microbial composition of mats that form stromatolites varies depending on the environment, but the basic mat structure does not. The components of these mats are cyanobacteria, phototrophic and aphotic bacteria, sulfur oxidizing bacteria and sulfur reducing bacteria (SRB), eukaryotic algae, and diatoms (Reid et al., 2000; Visscher et al., 1998). Some of these components have been described in mats from Storr's Lake stromatolites (Neumann et al., 1977), which are poorly laminated and are primarily formed by the mat-forming cyanobacterium *Phormidium* (Mann and Hoffman, 1984). Among the Storr's Lake cyanobacteria, *Phormidium, Microcoleus, Calothrix, Lyngbya, Cyanostylon, Rivularia, Spirulina* and *Scytonema* have been described. *Beggiatoa* and *Chromatium* were reported among the sulfur oxidizing bacteria (Neumann et al., 1988). Various algae (Elliott, 1994) and diatoms were also found in these mats, but the composition of the SRB has not, to our knowledge, been described. The SRB, along with other heterotrophic bacteria play a major role in the precipitation of aragonite in stromatolites.

MATERIALS AND METHODS

Field Sampling

Stromatolites were sampled from six locations in Storr's Lake, which is on the east side of San Salvador, Bahamas (Figure 1). This lake is rimmed with dwarf *Rhizophora mangle* and salinities vary from 70 to 100 ppt, depending on rainfall. The stromatolites collected were of the bulbous crust type described by Neumann et al. (1988). The six sampling sites shown in Figure 1 spanned from the south, at Dim Bay (Site 1), to the north, near Polly Hill Plantation (Site 6). Samples (water, stromatolites, and associated biofilm) were obtained at depths from 0.1 - 0.5 m and kept cold (~ 3.0°C) until laboratory processing. Water temperature was measured in the field, dissolved oxygen concentration determined with a test kit (Chemetrics, Calverton, VA), and salinity with a refractometer (Fisher Scientific, Fairlawn, NJ).

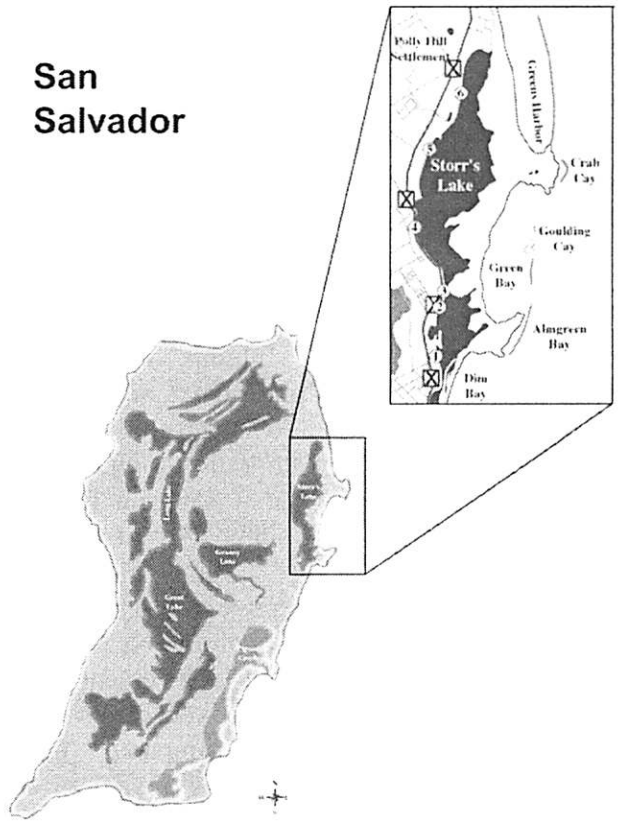

Figure 1. San Salvador, Bahamas and location of Storr's Lake with sampling sites1-6. Modified from a map published in Gerace et al., 1998.

Microbial Activity

Triplicate samples were collected for sites 1, 2, and 3. The samples included scrapings form the lithified top and underneath areas of the stromatolites. All samples were placed in sterile 25 ml tubes. Storr's Lake water samples (10 ml) were added directly to the reaction tubes and one gram of stromatolite scrapings were added to 9.0ml of sterile 3.2% Instant Ocean in reaction

tubes. Tetrazolium-linked dehydrogenase activity was assayed by adding 0.5 ml of a 1.5mg/ml solution of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl-2H-tetrazolium Cl (INT). All samples were incubated at ambient temperature for three hours. Vials were then frozen and returned to the laboratory where the concentration of the reduced product (Formazan) was determined spectrophotometricaly at an absorbance of 490nm. Absorbance was converted to concentration using standard formazan solutions. Dry weights were determined for solid samples and results presented on a dry weight basis (Smith and Hayasaka, 1986).

Ion Chromatography

Chloride, sodium, lithium, manganese, calcium, nitrite, nitrate, phosphate, and sulfate biofilm concentrations were measured with a Dionex DX500 ion chromatograph equipped with a conductivity detector, and a 250-mm Dionex IonPac AS14 Analytical column (4-mm ID, 16-µm bead; Dionex Corp., Sunnyvale, CA), operated at ambient temperatures. A 3.5 mM sodium carbonate/1 mM sodium bicarbonate buffer solution was used as the eluent (1.2 mL/min). Samples were taken from the supernatant of a solution prepared from stromatolite biofilm diluted 200X in deionized water vortexed for 1 minute then centrifuged for 5 minutes at 2500 rpm

Microscopy

Samples for electron microscopy analysis were preserved in 10% formaldehyde on the day they were collected. These samples were obtained from the same sites and collected under the same conditions as the samples that were used for the 16S rRNA analysis. Samples were either air dried, chemical or critical point dried, and subsequently coated with platinum. A JEOL 6340F field emission scanning electron microscope (FE-SEM) equipped with a light element electron dispersive X-ray spectrometry (EDS) system and a Philips XL30 environmental electron microscope (ESEM) was used for imaging and elemental analysis.

Molecular Analysis

The methods for extraction and purification of microbial 16S rRNA from stromatolites were based on those reported by Moran et al. (1993). Stromatolites samples (2.84 cm^2 X 10 cm) were collected in the field. Samples were partitioned in 2-cm length segments and weighed into a 50 mL polypropylene centrifuge tube (Nalgene 3110-0500). Stromatolite samples were then stored at –78 °C at least 2 d prior to extraction.

Ogligonucleotide probes were utilized for determining quantities of SRB 16sRNA present in stromatolites. Oligonucleotide probes were synthesized at the Molecular Genetics Facility of the University of Georgia using an ABI DNA/RNA synthesizer (Model 394). The oligonucleotide sequences which hybridize to specific 16S rRNA of SRB phylogenetic groups are reported in Devereux et al. (1992).

Oligonucleotides received from the University of Georgia were quantitated based on optical densities reported by manufacturer (Sambrook et al., 1989). The oligonucleotides were constituted in sterilized dH2O to a concentration of 100 ng/µL. The labeling of oligonucleotides was similar to the method of Frischer et al. (1996) and Stahl and Amann (1991). After extraction and purification concentrations of 16S rRNA in samples were then quantitated based on standard curves of 16S rRNA isolated from pure cultures of *Desulfovibrio desulfuricans*, *Desulfobulbus propionicus*, *Desulfococcus multivorans*, *Desulfobacter* sp. (BG-8), and *Desulfobacterium* (BG-33). Standard curves consisted of 16S rRNA that ranged from 100 to 12.5 ng. The r^2 values for plots of 16S rRNA concentration vs. band intensity were greater than 0.950 for all 5 cultures.

RESULTS

Microbial Activity

Results on INT-Linked Dehydrogenase activity of Storrs Lake stromatolite biofilm and water samples are shown in Figure 2. The microbial activity in samples from the top of the stromatolites, Samples 2, 5, and 8, is 2-3 orders of magnitude greater than biofilm samples beneath the top layers in sample 3, 6, and 9 (Figure 2). The activity in Storr's Lake water samples 1, 4 and 7 taken next to the stromatolites were in all cases higher than the biofilm. This activity correlated with microscopic examination of the stromatolite biofilms where the top of the structures appeared very robust and active while the bottom or deeper layers appeared to be a "microbial graveyard" (Byrne et al., 2000) dominated by diatom frustules and mineral deposits. The deeper layers had more fossilized diatom and filamentous structures with fewer intact microorganisms present.

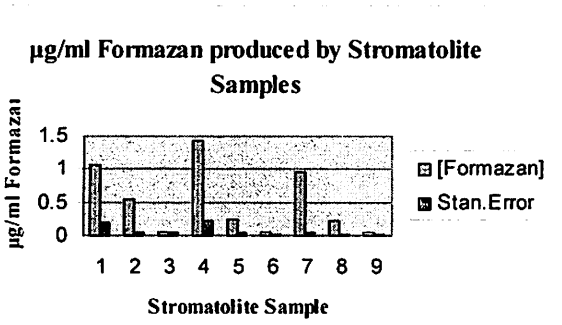

Figure 2. INT-Linked Dehydrogenase Activity of Stromatolite Samples from San Salvador, Bahamas. #1,4,and 7 are from the water mass, in ug/ml Formazan/10ml/hr. #2, 5 and 8 are from top scrapings of stromatolites; #3, 6 and 9 are scrapings from beneath the top layers of stromatolites and are in µg/ml Formazan/g/hr.

Microscopy

Examination of the stromatolite and biofilm with LSM revealed a diverse microbial community. The LSM allows observation of samples in three dimensions. The samples stained with AO reveals a diverse microbial population that is intimately associated with biofilm (Figure 3). These include a variety of filamentous microorganisms as well as rods and chains of rods. On the surface and edges of the mats numerous types of filamentous microbial species were observed. Bacteria and cyanobacteria species were seen intermixed in various configurations. This included what appeared as rosettes characteristic of *Thiothrix* spp. as seen in Figure 3A. The presence of *Thiothrix* spp., a sulfide oxidizing filamentous species, was subsequent confirmed by immunofluorescence as previously described (Brigmon et al., 1996). There appeared to be an organization to the microbial mats where the filamentous organisms formed a layer with the larger spherical structures on the surface as noted in Figure 3B. Note that Figure 3B has a 30 µm depth of field with the spherical structures positioned on top of the filaments. Figure 3C demonstrates several large coccoid structures or dumbell structures producing what appears to be polysaccharide material with smaller associated bacteria. Figure 3D is from a stromatolite surface. Note the attachment of the filamentous organisms to detrital particles in 3D.

All stromatolite samples contain both filamentous and nonfilamentous cyanobacteria, one-celled algae, and fungi. For the most part, the cyanobacteria are tightly adhered to the detrital particulates and are easily identified with an epifluorescence microscope using both autofluorescence and acridine orange for observation.

The microbial mats of the shore and mud flats of Storr's Lake are similar in appearance with a light brown exterior. The mats on the surface and shores have a gray crusty exterior appearance. Just under the surface is a light brown viscous layer comprised mostly of cyanobacteria that ranges from 1 to 100 mm in thickness depending on the site. The aquatic mats had the thicker layers. Microscopic examination demonstrated bacteria associated with the cyanobacteria. Beneath the brown layer was a green layer that appeared to contain predominantly *Phormidium* species that seemed to vary from 3-6 mm depending on location.

Beneath the green layer was generally a purple layer around 6-10 mm in thickness. The purple layer contained what appeared to be numerous bacterial species predominated by purple sulfur bacteria including *Chromatium* spp. The LSM allows visualization of these layers including refractive sulfur globules in sulfur bacteria without disturbing the components (Wiggli et al., 1999).

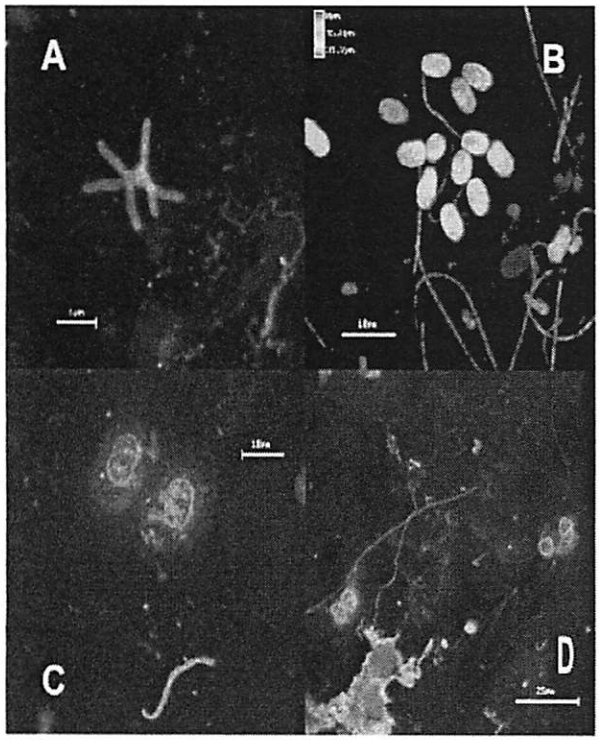

Figure 3. Laser Confocal Microscope images A. Filamentous sulfur oxidizing as evidenced by this likely Thiothrix spp. rosette. B. There is an organization to the microbial mats where the filamentous organisms formed a layer with the larger spherical structures on the surface. Note that B. has a 30 μm depth of field. C. Several large cocoid structures with polysaccharide material and smaller associated bacteria. D. Attachment of filamentous bacteria and cyanobacteria to detrital particles.

Filamentous components of the mats included cyanobacteria, fungi, and colonial aggregations of intertwined species with particulate matter including diatom shells. The filaments varied in diameter from 4 mm to 12 mm. Many filaments were tightly wound with detrital material. The larger sized filaments are not so tightly bound to the solid material and include 12 μm diameter *Oscillatoria* and 3-8 μm diameter *Lyngbya* and *Spirulina* filaments. Numerous motile microorganisms including *Oscillatoria* were observed in fresh unfixed biofilm samples by Laser Scanning Microscopy. The cyanobacteria are different from those that normally observed as there is a higher proportion of phycobillin pigments. The chlorophyll in these cells fluoresced bright red when excited by green light, but there was almost no fluorescence emission when the cells were excited by blue light indicating a high proportion of phycobillin pigments. Diatoms are much less abundant than cyanobacteria and include *Pinnularia*, *Navicula* and *Achnanthes*. Other algal groups (e.g. green algae or red algae) are not observed.

Electron Microscopy Examination

Four distinct populations or sizes of coccoid or spheroidal forms are commonly seen in the stromatolite biofilms with the FE-SEM. The large forms have a mean size of 5.5 μm, medium forms are 2.0 μm, the small are 0.55 μm, and the smallest are 0.13 μm. The larger two are within the acceptable size range for bacteria, while the small are at the lower size limit (Byrne et al., 2001). Figure 4 is an SEM of the large coccoid forms (mean size 5.5 μm) generally observed in the biofilm samples. The size is well within the acceptable size range of bacteria. Smaller coccoid forms (mean size .13 μm) were also observed thickly embedded in biofilm. These are below the acceptable size for bacteria and are presumably abiotically precipitated. With the exception of the diatoms, the fossilized organic remains are composed of calcium carbonate enriched with magnesium.

A variety of shapes and sizes of crystals were also observed in the biofilm (Figure 5). These crystals were seen both in the unfixed fresh biofilms as well as the fixed and dried samples so they were not artifacts of sample preparation. The crystals were most evident on the marginal

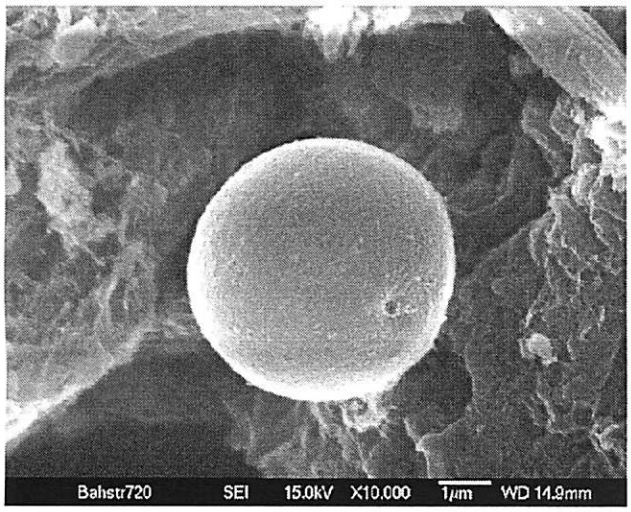

Figure 4. SEM of the large coccoid forms (mean size 5.5 μm) generally observed in the biofilm samples. The size is well within the acceptable size range of bacteria.

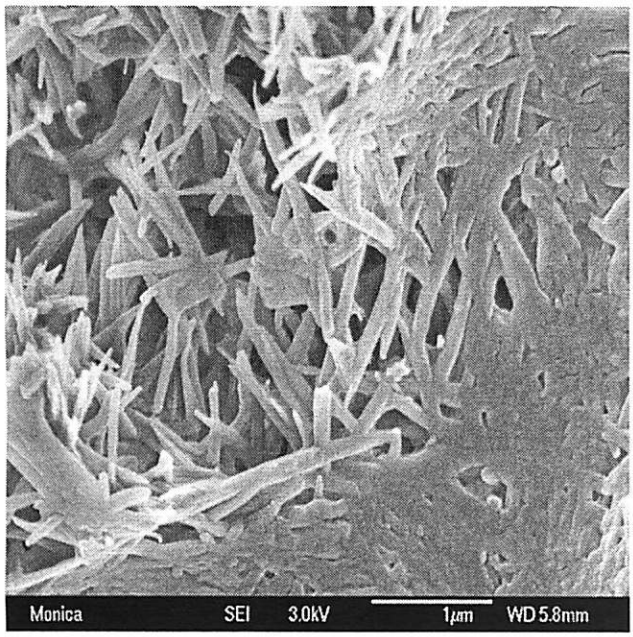

Figure 5. Various forms of crystals are thickly embedded in biofilm including these smaller filaments. These may be abiotic precipitants and were often seen intertwined with bacteria filaments.

areas between the harder mineralized surface of the growing stromatolite and the softer biofilm covering. The areas between crystals also served as a surface for microbial attachment within the biofilm.

Various types of fossilized cyanobacteria were observed within the biofilm/stromatolite matrix by microscopy. A majority of these fossilized structures appeared to be filamentous. An example of fossilized filamentous cyanobacteria observed microscopically in the biofilm is illustrated in Figure 6. Both live and fossilized filamentous cyanobacteria filaments were observed to be embedded in the film by attachment to living microorganisms, diatom shells, detritus, and other biomaterial buildup. In some cases large mineral deposits were also seen associated with cellular structures as demonstrated in Figure 6. The fossilized material also appeared to form conduits in the biofilms so water and nutrients could circulate.

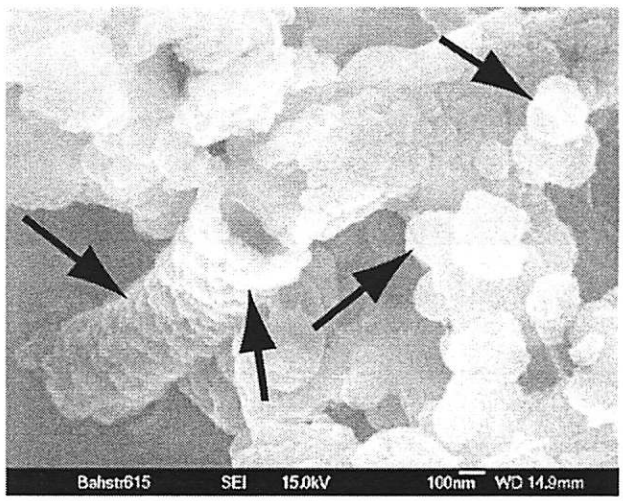

Figure 6. An example of fossilized filamentous cyanobacteria from the biofilm. Note mineral encrustations on and next to filament.

It is evident that Mg enriched calcium carbonate deposits have accumulated on the cell wall (Figure 6). A collapsed septated fungal filament is prominent in Figure 7. Also in Figure 7 are ovoid to rod-shaped structures that may be representative of sulfate-reducing bacteria. Figure 8 illustrates what appears to be a

cyanobacteria filament protruding from a crystal. This figure demonstrates the complex biogeochemical processes occurring within this evaporite system. Whether the crystal formed as a result of water evaporation and associated mineral precipitation or the filament grew in spite of the crystal is not clear. However, it does clearly show that the biological activity occurs along with the geochemical reactions. This particular filament appeared intact and healthy. In many instances microorganisms were seen attached to various types of crystalline as well as mineral formations.

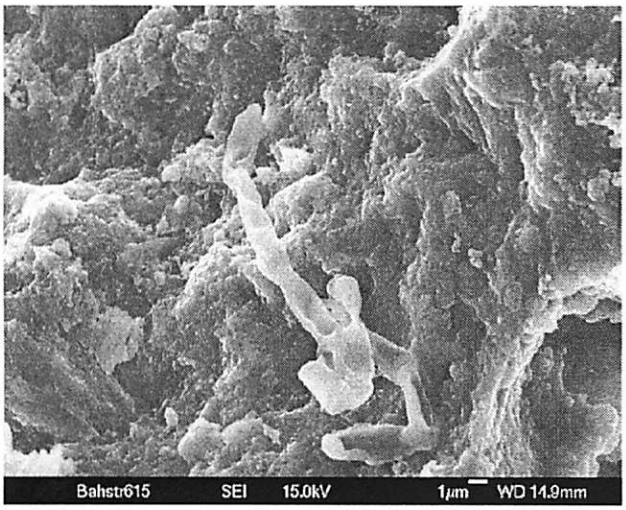

Figure 7. A collapsed septated fungal filament is prominent as are ovoid to rod-shaped structures that may be representative of sulfate-reducing bacteria.

The different sphere-like structures are also observed by SEM were also observed with LSM. There are also dumb bell-shaped precipitates that are similar in morphology and size to those described in laboratory experiments by Chafetz (1992) and Warren et al. (2001). Both Chafetz (1992) and Warren et al. (2001) attributed these shapes to bacterially induced or passive biomineralization processes. Spherical–shaped deposits occur on dead cyanobacteria filaments. These deposits are similar to those described in lithified microbial mats by Chafetz (1992).

Molecular Analysis

Results outlined in Table 1 demonstrate that the 16S rRNA from the five groups of SRB tested in this study were present. The 16S rRNA from the *Desulfobulbus* group was the most abundant with 140ng/$g_{sediment}$ detected while the *Desulfobacterium* group has the least amount of 16S rRNA present (Table 1).

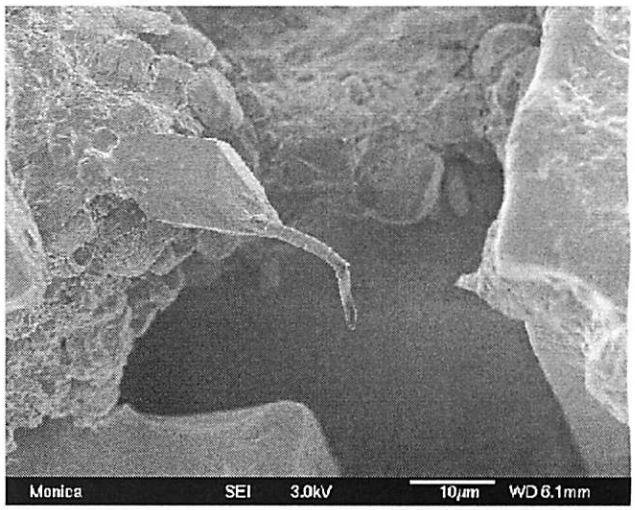

Figure 8. A cyanobacteria filament protruding from a crystal. Typical of this evaporite system.

Probe	Group Targeted	Concentration (ng/$g_{sediment}$)
687-704	*Desulfovibrio*	24.2
660	*Desulfobulbus*	140.3
814	*Desulfococcus*	73.5
129	*Desulfobacter*	63
221	*Desulfobacterium*	14.3

Table 1. Concentrations of 16S rRNA that hybridized with sulfate-reducing bacterial probes

Collectively, these data indicate that SRB are prevalent in the stromatolite sediments of Storr's Lake. This result is not surprising given the concentrations of sulfate (Table 2) that are available for use as a terminal electron acceptor. Overall, the stromatolite 16S rRNA concentrations are on the same order of magnitude as those observed in other marine systems (Devereux et al., 1996; King 1999).

However, the *Desulfobulbus* 16S rRNA concentrations in the stromatolite biofilm were significantly higher than those reported by other investigators in environmental samples (Devereux et al. 1996; King 1999). The reason for the higher *Desulfobulbus* 16S rRNA concentrations relative to other SRB groups identified in the stromatolite is unclear.

Water Chemistry

The chemical analysis is of great interest so far as the microbiology is concerned (Table 2). Results from the ion chromatography demonstrate the hypersaline nature of Storr's Lake. In addition the water at the sampling sites was extremely viscous with biofilm at the time of sampling. Even after clarifying through filtration and centrifugation the samples had to be diluted with deionized water (DI) 200-fold for ion analysis. Not shown in Table 2 is oxygen measurements taken below 1 cm depth that were all below detection so the conditions appeared extremely anoxic with the field tests. The hydrogen sulfide (H_2S) was evident by it's odor at the time of testing as well as the results in Table 2.

Site	Cl^- g/l	Tot P g/l	SO_4^{-2} g/l	Na^+ g/l	K^+ g/l	Mn^+ g/l	Ca g/l	DO mg/l 1 cm	H_2S mg/l	Temp C°
Site 1	672	ND	116	1294	292	66	0	0	2	37.5
Site 2	726	ND	116	914	215	<1	33	3	3	42
Site 3	834	<2	150	998	229	<1	35	5	2	35
Site 4	566	ND	76	615	153	<1	36	2	1	34
Site 5	675	ND	108	679	172	<1	33	3	1	32.5
Site 6	632	ND	96	564	143	<1	27	2	1	33

Table 2. Anion & Cation Concentrations, and Dissolved Oxygen, sulfide, and temperature in Storr's Lake at the surface of stromatolites.

Chloride (Cl^-), sulfate (SO_4^{-2}), sodium (Na^+), potassium (K^+), and calcium (Ca) are all in the grams per liter (g/l) range. Site 1 had no detectable calcium and the only site with detectable manganese (Mn^+). No nitrate, ammonia, or nitrite was detected. Phosphate was only detected in trace quantities at Site 3. Water temperatures at the six sites ranged from 32°C to 42°C at the time of sampling. Ambient air temperature was around 35°C. Most interesting was the drop off in water oxygen concentrations from the 0-5 ppm range at the 1 cm below surface depth to non-detectable at 10 cm at all six sites. In all these areas tested the biofilm comprised the full water column in depth. These oxygen concentration gradients reflect the high oxygen demand of the microbial mat/hypersaline system at Storr's Lake.

DISCUSSION

The distribution pattern of the layers of microorganisms in the biofilms observed associated with the stromatolites of Storr's Lake are similar to those reported for other marine environments (Paerl et al., 2001) and hypersaline ponds (Caumette et al., 1994). Terrestrial microbial crusts also show similarities in that they are dominated by filamentous cyanobacteria, particularly sheath-forming and polysaccharide secreting species (Mazor et al., 1996). The aquatic mats are similar in that they contain a matrix of layers containing predominately cyanobacteria forming a structural matrix with precipitated material at the surface (Wiggli, 1999). Recent work has shown that while cyanobacteria appear to make up a large amount of the biomass in these systems, they may not be the main agents of bacterially mediated precipitation (Paerl et al., 2001).

Extensive extraction techniques were developed for isolation of bacterial RNA for molecular characterization from the Storr's Lake stromatolite biofilm. The high salinity, mineral content, and polysaccharide material in samples appeared to cause initial interference. The results however, do give a precise answer as to the key constituents of the diverse microbial population, the sulfate reducers. Future work could involve the use of these molecular probes for *in situ* hybridization studies to determine their position

and potential function in the microbial mats. It would also be of interest to determine the spatial distribution in the stromatolite and if they metabolize in the fossilized material where sulfate and other nutrients may still be available.

These Storr's Lake stromatolites are made up of a combination of old fossil material primarily composed of cyanobacteria and trapped detrital sediments. It appears that the stromatolites in Storr's Lake are still undergoing sediment accumulation facilitated by the layered biofilm of highly active bacteria and cyanobacteria that are observable with epifluoresence. Storr's Lake is intimately connected to the San Salvador aquifer and the carbonate rocks and the conduits they contain. The pattern of stromatolite formation in Storrs Lake including some small islands in the middle may be directly linked to these biogeochemical processes. Knowledge of the ecology of these karst aquifers on this and other Bahamian Islands is limited as to the groundwater flow and energy flux (Davis and Johnson, 1989). The interaction of the microbial mats, stromatolites, and this unique ecosystem need to be better understood. By increasing our understanding of the geomicrobial ecology of these rare environments, valuable information will be gained on these unique natural resources.

CONCLUSIONS

In this study direct sampling and examination of microbial activities in ecological niches are assessed in situ in a unique aquatic system. The microbial activity found here reveals a dynamic system where the stromatolites are still forming. The diverse microbial communities of Storr's Lake appear to correlate with the distribution of geochemical patterns in other mineralized systems (Brigmon et al., 1994). During sampling, the biofilm layers were well defined along the crusty shoreline where the stromatolites predominate. The biofilm shows well-defined stratification with depth. The active biological community appears dependent on both chemical and solar energy.

As commercial development on this island continues, impacts on the aquatic resources will likely increase. Sites such as Storr's Lake provide a unique opportunity for microbial ecological testing. Conclusions concerning the impact of future stresses (i.e. island development) on the microbial community can be made from future monitoring.

ACKNOWLEDGMENTS

We would like to thank Dr. Donald T. Gerace, Chief Executive Officer, and Vincent Voegeli, Executive Director of the Gerace Research Center, San Salvador, Bahamas, for their support. We would also like to thank Earthwatch for their collaboration on sampling and Louis Hulse at the NASA Johnson Space Center for use of the scanning electron microscope. The investigations of P. Morris and M. Byrne were supported by NASA Astrobiology Grant NAG9-1113, NAG9-1114.

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