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# Quantifying CaCO<sub>3</sub> Microprecipitates Within Developing Surface Mats of Marine Stromatolites Using GIS and Digital Image Analysis

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The unique geochemical coupling of organic molecules and mineral CaCO<sub>3</sub> provides a fluorescence signature detectable using conventional confocal scanning laser microscopy (CSLM). The surface microbial mats of open-water marine stromatolites (Bahamas) exist in a continuum of states ranging from a Type 1 (i.e., nonlithifying) to Type 2 (i.e., lithified micritic laminae present) to Type 3 (i.e., fused grain layer). An approach was developed here, that utilizes geographical information systems (GIS) and digital image analysis, coupled with CSLM to estimate concentrations of calcium carbonate precipitates in developing marine stromatolites. We propose that the area occupied by particles within each image can be used to estimate concentrations of precipitates. Fluorescent polymeric microbeads and bacteria were used to calibrate the approach. We used this approach to demonstrate that CaCO<sub>3</sub> precipitates in lithifying layers were quantifiable and significantly different ( $p < 0.0001$ ) from those in nonlithifying layers. The approach provided a useful tool for the unambiguous assessment of relative changes in microbial precipitates occurring over small ( $\mu\text{m}$  to mm) spatial scales, and that characterize the formation of lithified layers (micritic laminae) in open-water marine stromatolites.

30 **Keywords** biofilm, CaCO<sub>3</sub>, GIS, stromatolite, precipitate, carbonate, bacteria

## INTRODUCTION

35 Marine stromatolites in subtidal regions of the Bahamas are microbially produced laminated (i.e. layered) macrostructures of sediment grains called “ooids,” and microscopic CaCO<sub>3</sub> precipitates. The precipitates are produced through the interactions of specific microbial communities and the local geochemical en-

vironment (Reid et al. 2000), and are embedded within a mucous matrix of extracellular polymers (EPS) produced by bacteria.

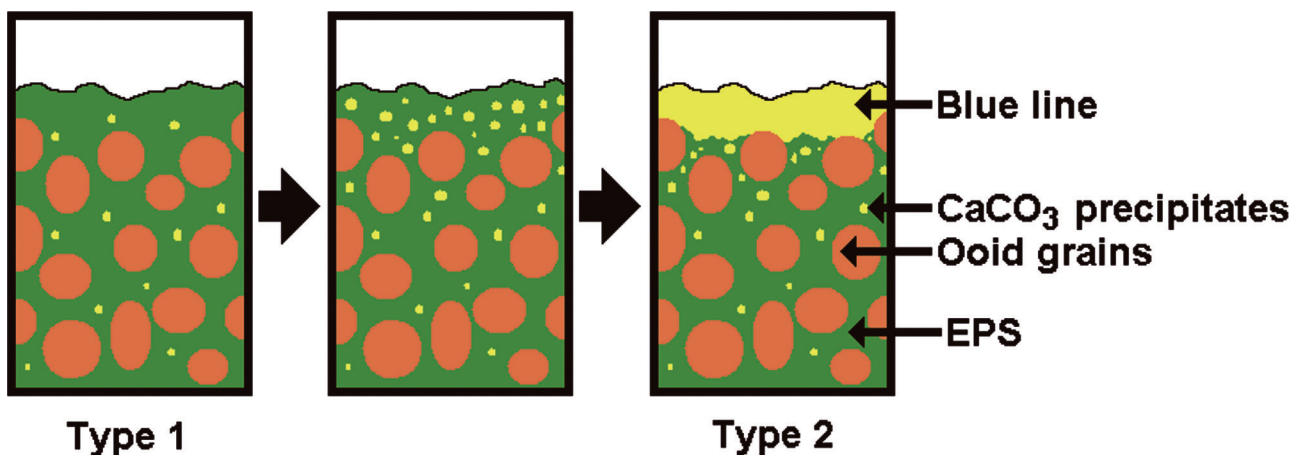
An important attribute of stromatolites is the microbially mediated precipitation of CaCO<sub>3</sub> into horizontal micritic laminae within the microbial surface mat layer. The underlying laminations of stromatolites, therefore, represent a chronology of former surface mats (Reid et al. 2000). This represents a characteristic feature that defines stromatolites, and contributes to their structural integrity and longer-term preservation through the geological (fossil) record (Grotzinger and Knoll 1999).

Recent work on open-water marine stromatolites in the Bahamas has shown that the microbial mats of marine stromatolites typically cycle among three different microbial communities, termed Types 1, 2, and 3, which exhibit different physical structural integrities. Type 1 mats consist of microbial cells embedded within a viscous matrix of secreted extracellular polymers (EPS). The abundant and sticky EPS of *Schizotrix* sp. trap and accrete sediment grains (ooids), but also inhibit the precipitation process (Decho and Kawaguchi 2003). Thus, precipitation is limited to small, isolated precipitates randomly scattered within the EPS matrix (Figure 1). The formation of micritic laminae is a characteristic of a Type 2 mat (Reid et al. 2000), and begins with the organized precipitation of many small precipitates within a localized area of the mat (Figure 1). This is facilitated by the concentrated activities of heterotrophic sulfate-reducing bacteria (Visscher et al. 2000). Type 3 mats consist of an abundant population of the coccoid cyanobacterium *Solentia* sp. and randomly-orientated *Schizotrix* filaments below a calcified biofilm (Reid et al. 2000). As the transition from a Type 1 to Type 2 mat occurs, the small precipitates increase in abundance over time, within a localized horizontal area of the mat eventually forming a continuous layer of precipitate (termed a “blue line” in Reid et al. 2000) that is typically 20–60  $\mu\text{m}$  thick. Therefore, the transition from a Type 1 to Type 2 mat is represented (in nature) as a continuum of states that may be characterized by changes in the relative densities of precipitates within specific regions of a mat sample. A newly formed Type 1

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**Figure 1.** Diagram showing the developing precipitation at the surface of a Type 1 mat. As the relative density of precipitates increases within a localized horizontal area, consolidation occurs into a micritic lamina (i.e., blue line). The formation of a blue line characterizes the transformation to a Type 2 mat.

mat will have relatively few precipitates. At the other extreme, a Type 2 mat will contain very densely packed precipitates organized into a thin horizontal layer (blue line) (sensu Reid et al. 2000).

Recent evidence suggests that microbially mediated precipitation of  $\text{CaCO}_3$  in stromatolites occurs within the biofilms of the surface mat layers (Reid et al. 2000). The EPS matrix, secreted by the dominant mat cyanobacteria, *Schizothrix* sp. (Kawaguchi et al. 2003), inhibit precipitation through the chelation of  $\text{Ca}^{++}$  ions. (Kawaguchi and Decho 2002b) This is most prominent in the newly developing surface biofilm of an un lithifying Type 1 mat. Here, precipitates begin as small islands of  $\text{CaCO}_3$  relatively dispersed throughout the EPS matrix, also containing cells and ooids (Kawaguchi and Decho 2002a). As a Type 1 mat develops into a Type 2 mat, there are changes in the microspatial organization (and activities) of the resident microbial communities (Visscher et al. 2000), the partial degradation of EPS (Decho et al. submitted) by sulfate reducers and other bacteria (Visscher et al. 2000) and a concomitant release of  $\text{Ca}^{++}$  ions. The abundance of precipitates increases in concentration until forming a dense interlocking horizontal layer within a Type 2 mat. (Visscher et al. 2000) This blue line may be important in physical stabilization against wave action of the surface microbial mat, and appears to facilitate subsequent colonization of ooid grains by endolithic cyanobacteria that results in a Type 3 mat (MacIntyre et al. 2000).

When examining marine stromatolites, the surface mat layers exist in a continuum of states ranging from Type 1 to Type 2 and/or Type 3. Therefore, an important parameter in the developing stromatolite mat layers is the accumulation of  $\text{CaCO}_3$  precipitates. Thus, it is important to develop an approach to quantitatively assess the progressive accumulation of  $\text{CaCO}_3$  precipitates within a developing stromatolite mat layer. The information will be used later as an objective approach to classification of stromatolite mats as either Type 1, 2, or 3.

Image analysis techniques have been used to examine the structure of biofilms (see citations within Yang et al. 2000). Our previous work has focused on acquiring digital images with a confocal scanning laser microscope (CSLM), classification of images using image analysis software (i.e., Erdas Imagine), conversion of images into maps. Specific functions of the Geographical Information Systems (GIS) software, ArcView GIS, were used to compute areas occupied by specific elements in relationship with others (i.e., microbored canals within ooid grains) within each map corresponding to an image (Petrisor and Decho 2004). In the present study, the method was applied to images of natural stromatolites obtained using CSLM, and was used to quantify differences in  $\text{CaCO}_3$  precipitate concentration between Type 1 and Type 2 mat layers.

**MATERIALS AND METHODS**

All stromatolites, from which samples were collected, came from a subtidal marine environment at Highborne Cay in the Exuma Chain of islands in the Bahamas (76° 49' W; 24° 43' N) (Decho and Kawaguchi 1999). This site is under current investigation through the Research Initiative on Bahamian Stromatolites (RIBS) project (<http://www.home.duq.edu/~stolz/RIBS/index.html>). Freshly collected, intact stromatolites were carefully cut into approx. 4 cm<sup>3</sup> blocks using a rock saw. Immediately after collection, the stromatolite blocks were preserved in 3% buffered formaldehyde in seawater. Sections were initially trimmed using a sterile razor, then placed in BEEM embedding molds. Nanoplast resin (Ted Pella Co., Redding, CA, USA) and catalyst were thoroughly mixed on site and then added to moulds containing the stromatolites sample. The molds were placed in a temperature-controlled heat block at 25°C for 48–60 h to allow slow penetration and complete mixing of the Nanoplast resin with the hydrated sample. After penetration of the medium, the temperature was raised to 40°C for 48 h to dry and then 60°C for 48 h to harden the medium into blocks. The resulting blocks

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were thick sectioned, mounted on glass microscope slides using Epon 812 and then observed using CSLM (488/520 nm; excitation/emission).

Images were obtained using an MRC 1024MP(7) single and multiphoton system (BioRad Laboratories, Hercules, CA.) equipped with a Nikon Eclipse TE 300 compound microscope (Nikon, Tokyo, JAPAN). Image resolution was 512 × 512 pixels. For CSLM imaging, three internal detectors were used, each with a 6-position emission filter wheel and a variable confocal aperture. Sample slides were viewed using a Nikon Plan Apo 20× objective. Colored composite images were exported in a Bitmap format. Different parameters related to image acquisition (i.e., iris, gain, power of the lasers, etc.) were set up to provide images with the best visual differences between the features of interest (CaCO<sub>3</sub> precipitates and ooids). As a result, the values of these parameters are specific to each image.

Images were sampled randomly from within mat layers, identified prior to analysis using an optical microscope as belonging to either Type 1 or Type 2 mats. A total of 25 Type 1 and 35 Type 2 images were collected. Each image was classified using supervised classification in Erdas Imagine 8.5 (Leica Geosystems GIS & Mapping, LLC, Atlanta, Georgia, USA). The method allows the user to select sample pixels corresponding to each feature (exopolymer, ooids, or CaCO<sub>3</sub> precipitates) to define specific signatures, and later on the computer performs the classification

based on the collection of signatures (Decho and Kawaguchi 1999). In this particular case, it was not necessary to differentiate between the EPS and interstitial spaces. Both were assigned to a general class labeled exopolymer. We used 10–25 areas within a given sample to define each feature, depending on the quality of the image. Following the classification, images were exported into an Arc View GIS 3.X format (Environmental Systems Research Institute, Inc., Redlands, California, USA). The area occupied by CaCO<sub>3</sub> precipitates within each image was computed using Equation 1, and was expressed as a percentage of the total area.

$$\text{Percentage area (\% area)} = \frac{AP}{262,144} \quad [1]$$

where:

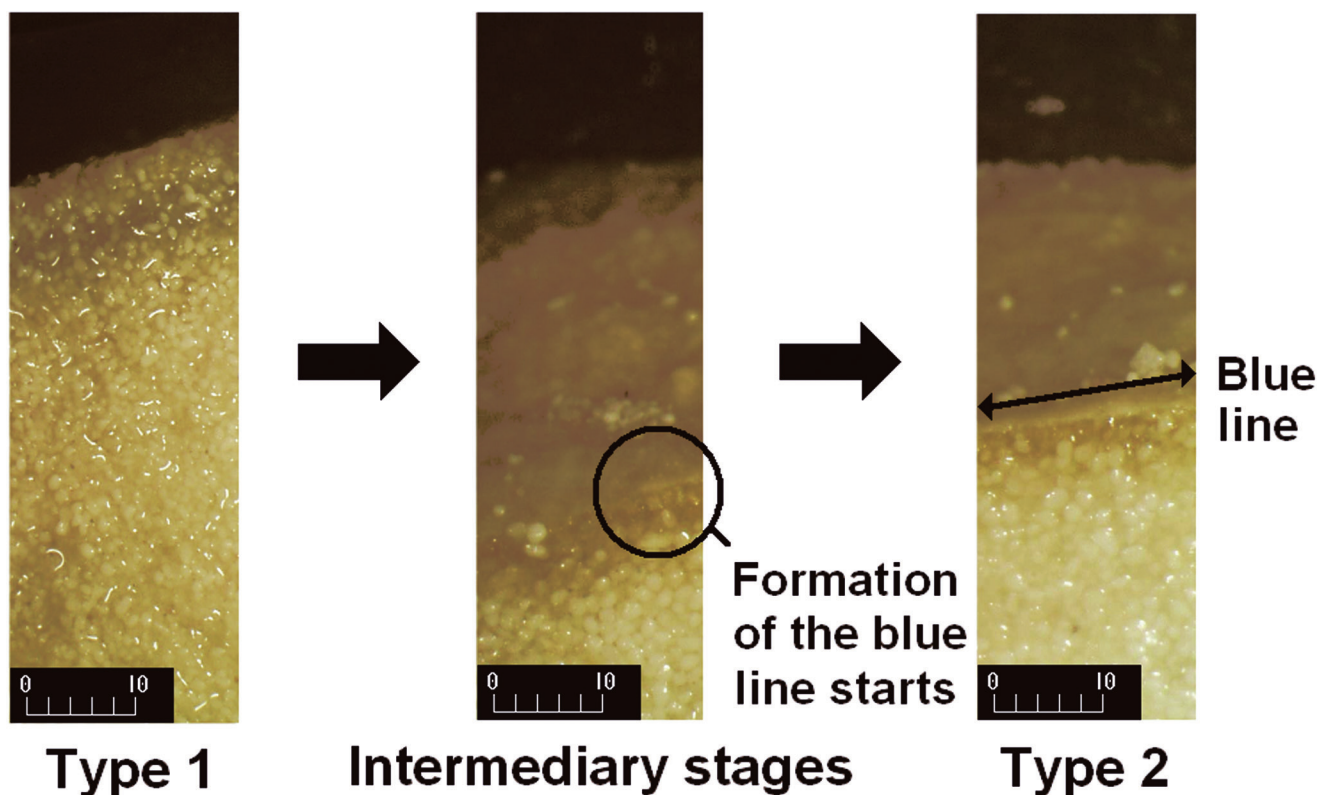
AP is the area occupied by precipitates within the image, in pixels, and

262,144 = 512 × 512 is the area of each image, in pixels.

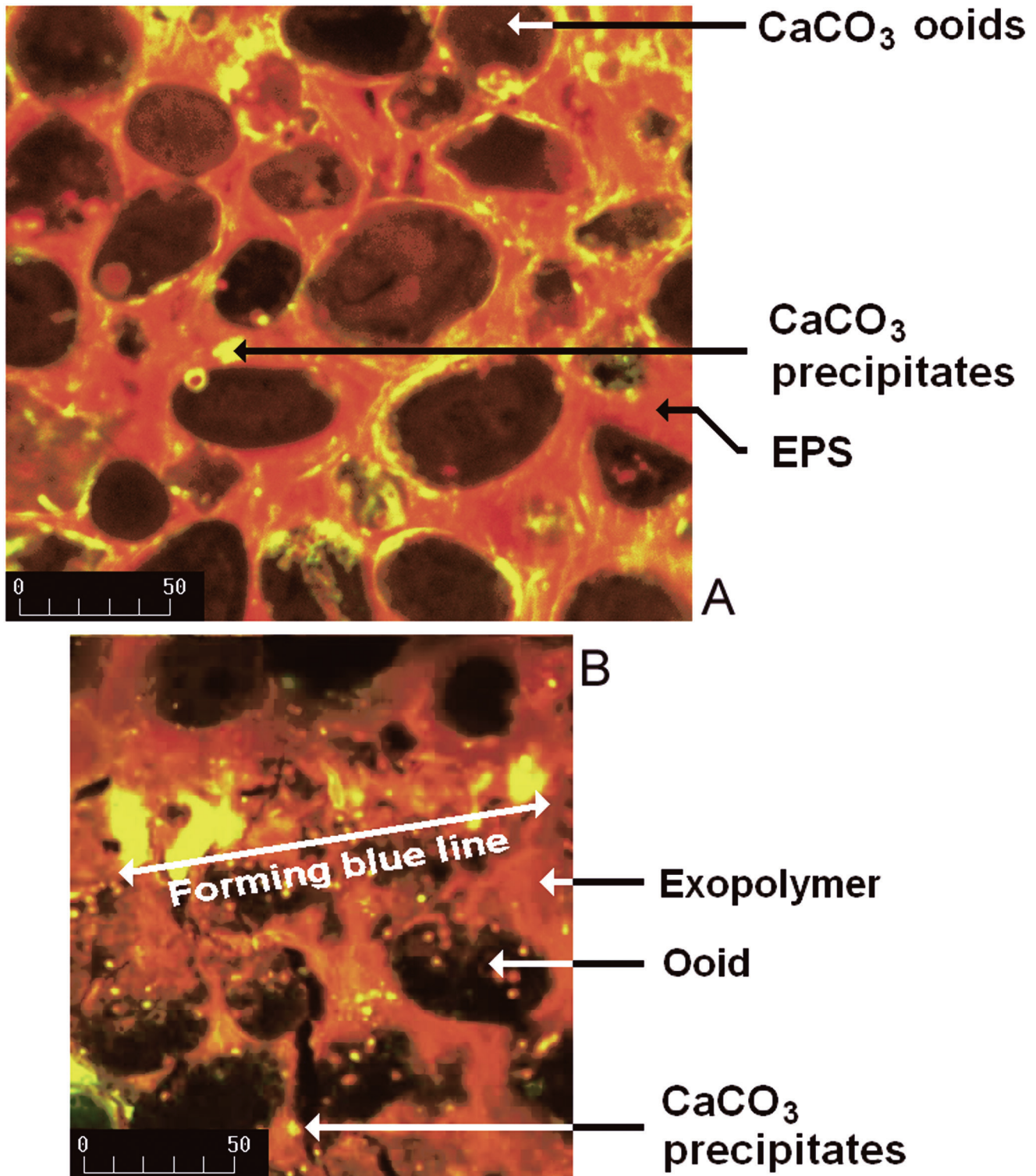
**RESULTS**

The process of transformation of Type 1 mats in Type 2 mats and the formation of the micritic lamina is illustrated in Figure 2. This figure displays actual images of the mechanism described in Figure 1.

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**Figure 2.** Light microscopy images of different surface mat stages illustrating the transformation of Type 1 mats into Type 2 mats and the formation of a blue line. Approximate scale: 0.1 cm.



**Figure 3.** (a) Typical image of a Type 1 stromatolite mat. Note that CaCO<sub>3</sub> precipitates are dispersed throughout the EPS matrix and interstitial space rather than concentrated in a certain region. (b) Typical image of a developing CaCO<sub>3</sub> precipitates of a “blue line” in a Type 2 mat. Note accumulating area of dense precipitates (yellow).

**Table 1**  
Statistical properties of the area occupied by CaCO<sub>3</sub> precipitates

	Percentage	
	Type 1 (N = 25)	Type 2 (N = 35)
Mean	0.039	0.091
Confidence interval of the mean	[0.031, 0.047]	[0.078, 0.105]
Standard deviation	0.020	0.040
Confidence interval of the standard deviation	[0.015, 0.027]	[0.032, 0.052]
<i>t</i> -test (equality of means)	6.71	
<i>p</i> -value of the <i>t</i> -test	<0.0001	
F-test (equality of variances)	4.10	
<i>p</i> -value of the F-test	0.0006	

**Q2** Precipitates within each image in pixels (column labeled “Area”), and the percentage represented by this area from the total area of the image (column labeled “Percentage”) for each type of mat.

**190** Typical images of Type 1 and Type 2 mats were displayed in Figure 3a and, respectively, Figure 3b. It was readily apparent (i.e., by visual examination) be noticed that CaCO<sub>3</sub> precipitates are typically more abundant in Type 2 mats, than in Type 1 mats.

**195** The results are presented in Table 1. For each type of mat, the table displays the areas as a percentage of the total area of the image (column labeled “Percentage”). The test for the equality of variances indicated a highly significant difference (*p* = 0.0006) between the two mat types (Type 1 and Type 2 mats). Therefore, the Satterthwaite *t*-test for samples with unequal variances was used. The test indicated that the area occupied by CaCO<sub>3</sub> precipitates was significantly lower in Type 1 than in Type 2 mat layers (*p* < 0.0001).

**DISCUSSION**

**205** The surface mats of open-water, marine stromatolites typically cycle among three distinct communities (Reid et al. 2000). Most observations (i.e., samples) involve communities in transition, along a continuum between two or three distinct endpoints. A difficult parameter in assessing changes in microbial communities is the relative time of sampling during community transition. Therefore, characterizing where these communities lie (along the continuum) becomes difficult. In the microbial communities of stromatolite mats, the transition between Type 1 (nonlithifying) and Type 2 (lithifying) may be characterized by the relative concentrations of calcium carbonate (CaCO<sub>3</sub>) precipitates that occur within the surface biofilm layer. As the mat community reaches the Type 2 stage, there will exist a dense thin (i.e., approx. 20–60 μm) layer of precipitate, resulting from the activities of the microbial communities.

All images used in the study exhibited a high variability (Type 2 mats being more variable than Type 1 mats). Due to our methodology, stromatolite sample sections were mounted on slides and tentatively assigned as Type 1 or Type 2. It is important to note that these designations do not indicate that a given section is typical for the mat type. Samples exhibiting the transition of Type 1 mats to Type 2 mats were assigned to the closest end stage of the process. The net results of our studies show that Type 2 mats clearly exhibit statistically higher concentrations of CaCO<sub>3</sub> precipitates.

Precipitation of CaCO<sub>3</sub> appears to be a tightly controlled process within Bahamian stromatolite mats. The EPS, abundantly secreted by cyanobacteria, inhibit precipitation. Precipitation in the EPS-laden matrix of Type 1 communities is largely confined to small, isolated clusters. CaCO<sub>3</sub> crystals are often amorphous in shape, and is likely due to EPS inhibiting crystal growth. It is only through the (partial) heterotrophic degradation of EPS in localized regions of the surface biofilm matrix of the stromatolites, that precipitation abundantly occurs. In physical terms, these microbially mediated changes alter the mat surface from a sticky surface (Type 1) that fosters sediment accretion and upward growth of the mat, to a non sticky, lithifying surface (Type 2) that is more physically resilient to wave action and disruption.

The autofluorescence (488/520 nm; excit./emiss) of CaCO<sub>3</sub> provided a convenient signature that was used to quantify the concentration and relative dispersion of precipitates within a mat layer. It is not yet fully understood, what biogeochemical interactions mediate precipitation of CaCO<sub>3</sub> within stromatolites mats. Unique mineral/organic associations ultimately form the amorphous and crystalline matrix of CaCO<sub>3</sub> precipitates, and likely contribute to the autofluorescence signal of the CaCO<sub>3</sub> matrix. The smaller molecular weight (i.e., <30 kDa) protein fraction of EPS that is specifically enriched in aspartic acid has been shown to serve as nucleation sites to form small crystals (Reitner et al. 2000; Kawaguchi and Decho 2002a). This is consistent with the immobilized-matrix/crystal nucleation (IC) hypothesis for CaCO<sub>3</sub> and other biominerals (Wheeler and Sikes 1984). As the precipitation continues to grow in frequency and size, other organic molecules likely become incorporated and the matrix becomes largely amorphous, rather than crystalline as determined by X-ray diffraction studies (Decho, personal observation). The abundant precipitates eventually merge into a single layer. The specific organic/mineral source of the autofluorescent signal is currently under investigation.

The present study has addressed an important parameter in developing stromatolite mat layers: the accumulation of CaCO<sub>3</sub> precipitates into a structured layer. Micritic laminae are a defining feature of stromatolites (Grotzinger and Knoll 1999). Here, we demonstrate that the GIS/ Digital Image Analysis approach can be used, objectively and quantitatively, to analyze the autofluorescence signature of microscopic CaCO<sub>3</sub> precipitates that occur within the surface microbial mats of stromatolites, and contribute to the formation of micritic laminae. When one continues to examine marine stromatolite samples at any point

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in time, the surface mat layers of samples will exist in a continuum of states ranging from Type 1 to Type 2 and/or Type 3. It will be critical to develop an unambiguous approach, using the accumulation of CaCO<sub>3</sub> precipitates, for the classification of stromatolite mats as either Type 1, 2, or 3.

## REFERENCES

- 275 Decho AW, Kawaguchi T. 1999. Confocal imaging of in-situ natural microbial communities and their extracellular polymeric secretions using Nanoplast resin. *Biotechniques* 27:1246–1252.
- 280 Decho AW, Kawaguchi T. 2003. Extracellular Polymers (EPS) and Calcification within modern marine stromatolites. In: Krumbein WE, Paterson D, Zavarzin GA, editors. *Fossil and Recent Biofilms: A Natural History of Life on Earth*. Dordrecht, The Netherlands. Kluwer Academic Publishers. p. 227–240.
- 285 Grotzinger JP, Knoll AH. 1999. Stromatolites in PreCambrian carbonates: evolutionary mileposts or environmental dipsticks? *Annu Rev Earth Planet Sci* 27:313–358.
- 290 Kawaguchi T, Al Sayegh H, Decho AW. 2003. Development of an indirect competitive enzyme-linked immunosorbent assay to detect extracellular polymeric substances (EPS) secreted by the marine stromatolite-forming cyanobacteria, *Schizothrix sp.* *J Immunoassay Immunochem* 24:29–39.
- Kawaguchi T, Decho AW. 2002a. A laboratory investigation of cyanobacterial extracellular polymeric secretions (EPS) in influencing CaCO<sub>3</sub> polymorphism. *J Crystal Growth* 240:230–235.
- Kawaguchi T, Decho AW. 2002b. Isolation and biochemical characterization of extracellular polymeric secretions (EPS) from modern soft marine stromatolites (Bahamas) and its inhibitory effect on CaCO<sub>3</sub> precipitation. *Prep Biochem BioTechnol* 32:51–63. 295
- MacIntyre IG, Prufert-Bebout L, Reid RP. 2000. The role of endolithic cyanobacteria in the formation of lithified laminae in Bahamian stromatolites. *Sedimentology* 47:915–921. 300
- Petrisor AI, Decho AW. 2004. Using geographical information techniques to quantify the spatial structure of endolithic boring processes within sediment grains of marine stromatolites. *J Microbiol Methods* 56:173–180. 305
- Reid RP, Visscher P, Decho AW, Stolz J, Bebout BM, Macintyre IG, Paerl HW, Pinckney JL, Prufert-Bebout L, Stepe TF, DesMarais DJ. 2000. The role of microbes in accretion, lamination, and early lithification of modern marine stromatolites. *Nature* 406:989–992. 310
- Reitner J, Theil V, Zankl H, Michaelis W, Worheide G, Gautret P. 2000. Organic and biogeochemical patterns in cryptic microbialites. In: Ridding RE, Avramik SM, editors. *Microbial Sediments*. Berlin: Springer-Verlag. p. 149–160. 315
- Visscher PT, Reid RP, Bebout BM. 2000. Microscale observations of sulfate reduction: evidence of microbial activity forming lithified micritic laminae in modern marine stromatolites. *Geology* 28:919–922. 320
- Wheeler AP, Sikes CS. 1984. Regulation of carbonate calcification by organic matrix. *Amer Zool* 24:933–944.
- Yang X, Beyenal H, Harkin G, Lewandowski Z. 2000. Quantifying biofilm structure using image analysis. *J Microbiol Meth* 39:109–119.