Quantifying CaCO₃ 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 CaCO₃ 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 CaCO₃ 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 (µm to mm) spatial scales, and that characterize the formation of lithified layers (micritic laminae) in open-water marine stromatolites.

Keywords biofilm, CaCO₃, GIS, stromatolite, precipitate, carbonate, bacteria

INTRODUCTION

Marine stromatolites in subtidal regions of the Bahamas are microbiologically produced laminated (i.e. layered) macrostructures of sediment grains called “ooids,” and microscopical CaCO₃ precipitates. The precipitates are produced through the interactions of specific microbial communities and the local geochemical environment (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 microbiologically mediated precipitation of CaCO₃ 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 horizontal 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 µm 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.

Recent evidence suggests that microbially mediated precipitation of CaCO₃ 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 Ca⁺⁺ ions. (Kawaguchi and Decho 2002b) This is most prominent in the newly developing surface biofilm of an unlithifying Type 1 mat. Here, precipitates begin as small islands of CaCO₃ 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. in press) by sulfate reducers and other bacteria (Visscher et al. 2000) and a concomitant release of 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 CaCO₃ precipitates. Thus, it is important to develop an approach to quantitatively assess the progressive accumulation of CaCO₃ 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 CaCO₃ 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 cm³ 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
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 (CaCO3 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 CaCO3 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 CaCO3 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.

**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$_3$ precipitates are dispersed throughout the EPS matrix and interstitial space rather than concentrated in a certain region. (b) Typical image of a developing CaCO$_3$ precipitates of a “blue line” in a Type 2 mat. Note accumulating area of dense precipitates (yellow).
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 CaCO3 precipitates are typically more abundant in Type 2 mats, than in Type 1 mats.

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”) for each type of mat.

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

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.

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 CaCO3 precipitates.

Precipitation of CaCO3 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. CaCO3 crystals are often amorphous is 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 CaCO3 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 CaCO3 within stromatolites mats. Unique mineral/organic associations ultimately form the amorphous and crystalline matrix of CaCO3 precipitates, and likely contribute to the autofluorescence signal of the CaCO3 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 CaCO3 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 CaCO3 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 CaCO3 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
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 CaCO₃ precipitates, for the classification of stromatolite mats as either Type 1, 2, or 3.

REFERENCES