Biogeochemical cycling and microbial diversity in the thrombolitic microbialites of Highborne Cay, Bahamas

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ABSTRACT

Thrombolites are unlaminated carbonate build-ups that are formed via the metabolic activities of complex microbial mat communities. The thrombolitic mats of Highborne Cay, Bahamas develop in close proximity (1–2 m) to accreting laminated stromatolites, providing an ideal opportunity for biogeochemical and molecular comparisons of these two distinctive microbialite ecosystems. In this study, we provide the first comprehensive characterization of the biogeochemical activities and microbial diversity of the Highborne Cay thrombolitic mats. Morphological and molecular analyses reveal two dominant mat types associated with the thrombolite deposits, both of which are dominated by bacteria from the taxa Cyanobacteria and Alphaproteobacteria. Diel cycling of dissolved oxygen (DO) and dissolved inorganic carbon (DIC) were measured in all thrombolitic mat types. DO production varied between thrombolitic types and one morphotype, referred to in this study as ‘button mats’, produced the highest levels among all mat types, including the adjacent stromatolites. Characterization of thrombolite bacterial communities revealed a high bacterial diversity, roughly equivalent to that of the nearby stromatolites, and a low eukaryotic diversity. Extensive phylogenetic overlap between thrombolitic and stromatolitic microbial communities was observed, although thrombolite-specific cyanobacterial populations were detected. In particular, the button mats were dominated by a calcified, filamentous cyanobacterium identified via morphology and 16S rRNA gene sequencing as Dichothrix sp. The distinctive microbial communities and chemical cycling patterns within the thrombolitic mats provide novel insight into the biogeochemical processes related to the lithifying mats in this system, and provide data relevant to understanding microbially induced carbonate biomineralization.

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INTRODUCTION

Thrombolitic microbialites are unlaminated carbonate build-ups that are the by-products of microbes and their environment (Burne & Moore, 1987). Although the external features of thrombolites are comparable to other microbialites, they are, however, differentiated by their internal meso- and microstructures. Thrombolites are characterized by clotted internal fabrics, which contrast with the laminated fabrics of stromatolites (Aiken, 1967; Kennard & James, 1986). Much like their laminated stromatolitic counter parts, thrombolites represent an important milieu for the evolution of life (Kennard & James, 1986). Thrombolites have a long geological record with definitive fossils dating back to 1.92 Ga (Kah & Grotzinger, 1992) and represent one of the earliest organosedimentary depositing ecosystems. Although once prevalent on the ancient Earth, modern accreting thrombolites are rare. One of the few modern sites of active thrombolitic development is the island of Highborne Cay, located along the west margin of Exuma Sound, Bahamas (Fig. 1a; Reid et al., 1999). This site is ideal for studying lithifying mats, as intertidal thrombolitic microbialites are juxtaposed to the subtidal stromatolitic communities (Fig. 1b) with both ecosystems experiencing similar environmental conditions with respect to salinity, temperature, and wave action.
Although the processes of microbialite development have been well-studied in modern stromatolites (e.g. Visscher et al., 1998; Reid et al., 2000; Dupraz & Visscher, 2005; Papineau et al., 2005), the mechanisms of thrombolitic microbialite formation have not (Thompson et al., 1990; Moore & Burne, 1994). In the open marine stromatolites of Highborne Cay, Bahamas, the primary mechanism of accretion is the trapping, binding, and precipitation of calcium carbonates by a surficial microbial mat community (Reid et al., 2000). Those stromatolitic microbial mats conducive to lithification comprised several key microbial functional groups including cyanobacteria, aerobic heterotrophs, sulfide-oxidizing bacteria, sulfate-reducing bacteria, and fermentative bacteria (Visscher & Stolz, 2005; Stolz et al., 2009). Together, the delicate balance of physiochemical processes and metabolic activities, including alteration of the saturation index and properties of microbially produced exopolymeric matrices, are necessary for lithification to occur (for review Dupraz et al., 2008). The metabolic activities of various microbial functional groups, conducting photosynthesis, respiration and fermentation, influence the precipitation and/or dissolution of carbonate, thereby controlling whether these microbial induced structures are preserved in the fossil record (Dupraz & Visscher, 2005). The emerging lithified laminae characteristic of these communities are formed from combined geochemical conditions that promote net precipitation (e.g. the depth horizon where cyanobacterial and sulfate-reducing activities peak in close proximity) and where a nucleation template is available (Visscher et al., 1998; Dupraz et al., 2008).

The process of thrombolite formation has been the subject of a long-standing debate. Previously, it has been suggested that thrombolites are merely ‘bioturbated prokaryotic stromatolites’ disrupted by the secondary colonization of eukaryotes (Walter & Heys, 1985). Other theories have suggested that thrombolites are predominately eukaryotic reefs formed by the activities of eukaryotic green algae from the genus 

![Fig. 1 Thrombolites of Highborne Cay, Bahamas. (a) Map of the Bahamas with arrow point to the location of Highborne Cay. Bar, 150 km. (b) Image of the thrombolitic build-ups (t) along the intertidal zone. Thrombolites are in close proximity to the subtidal stromatolites (s) and coralline algal reef platform (r). Bar, 100 cm. (c) Cross-section of thrombolite depicting the clotted carbonate fabric. Bar, 25 mm.](image-url)

algae (Feldmann & McKenzie, 1998). Elsewhere, the formation of freshwater thrombolites growing in Green Lake, Fayetteville, NY has been attributed to the activity of the coccolid cyanobacteria of the genus 

![Acetabularia spp.](image-url)

*Synechococcus* sp. (Thompson et al., 1990). In addition, the formation of the marine thrombolites in Lake Clifton, Western Australia has been associated with the enrichments of the filamentous cyanobacterium 

![Scytonema sp](image-url)

*Scytonema* sp. (Moore & Burne, 1994). Despite these previous studies on specific ecotypes associated with thrombolite formation the comprehensive microbial diversity and elemental cycling of carbon and oxygen, both critical factors in lithification, are poorly understood in the thrombolitic microbialites.

In this study, we present results from microbial diversity and biogeochemical analyses of the thrombolitic microbialites at Highborne Cay, Bahamas. The close proximity of these thrombolitic communities to the well-studied stromatolites facilitates a comparative study of the biogeochemical and microbial diversities within each community. Specifically, we compare the microbial diversity, and biogeochemical activities [i.e. dissolved oxygen (DO) and inorganic carbon dynamics] of the dominant thrombolitic mat types to the adjacent stromatolitic microbialites. By characterizing the similarities and differences between these two microbialitic communities we can refine models for thrombolite formation and accretion, thus potentially improving our understanding of thrombolite preservation in the fossil record.

**MATERIALS AND METHODS**

**Field site description and sample collection**

Microbialite samples used in this study were collected from intertidal thrombolitic build-ups and subtidal Type 2 stromatolites located on the windward side of Highborne Cay, an island in the northern Exumas, Bahamas (76°49′ W, 24°8′ N). All samples were collected from these microbialite communities in July 2007. Sections of each mat type measur-
ing approximately 2.5 cm × 2.5 cm × 1.5 cm were collected in triplicate and used for subsequent productivity assays. Three additional core samples (similar dimensions) were collected, saturated with RNAlater (Ambion, Austin, TX, USA) to stabilize the nucleic acids, and then frozen at −20 °C. Frozen samples were returned to the laboratory where they were stored at −80 °C until DNA extraction. Finally, two subsets of samples were collected from corresponding thrombolitic mats for microscopic examination. One set was examined immediately in the field and the other set was stored in filtered seawater containing 4% formaldehyde, and returned to the laboratory for microscopic and petrographic analyses. All chemicals used in this study were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted.

**Light-level and thin section microscopy**

Freshly collected thrombolitic mats were sampled and immediately sectioned (0.5 cm) using a rock saw. Cross-sections of the thrombolitic mats were immersed in filtered seawater and examined with bright field microscopy using an Olympus SX12 microscope (Olympus, Center Valley, PA, USA). To characterize the thrombolitic microstructure, formaldehyde-fixed thrombolitic mat samples were prepared for petrographic thin sectioning. Fixed samples were embedded in a custom built vacuum chamber and infiltrated with the cold setting embedding resin Epofix (EMS, Hatfield, PA, USA). The embedded samples were fixed to glass slides using low temperature Hillquist epoxy (Hillquist, Denver, CO, USA), polished by hand with a 5-μm grit to a final thickness of between 40 and 50 μm. The sections were then examined using an Olympus BH-2 polarizing petrographic microscope and imaged using an Olympus PM-10AM photomicrographic system.

**Biogeochemical cycling: dynamics of oxygen and inorganic carbon**

Thrombolitic and stromatolitic mat sections collected for analyses of dissolved oxygen (DO) and dissolved inorganic carbon (DIC) exchange were placed in individual flux chambers. The flux chambers are sealed transparent plexiglass containers that are filled with 160 mL of filter-sterilized seawater and carbon (DIC) exchange were placed in individual flux chambers (Olympus, Center Valley, PA, USA). To characterize the thrombolitic microstructure, formaldehyde-fixed thrombolitic mat samples were prepared for petrographic thin sectioning. Fixed samples were embedded in a custom built vacuum chamber and infiltrated with the cold setting embedding resin Epofix (EMS, Hatfield, PA, USA). The embedded samples were fixed to glass slides using low temperature Hillquist epoxy (Hillquist, Denver, CO, USA), polished by hand with a 5-μm grit to a final thickness of between 40 and 50 μm. The sections were then examined using an Olympus BH-2 polarizing petrographic microscope and imaged using an Olympus PM-10AM photomicrographic system.

Dissolved oxygen measurements

To measure the levels of DO within the thrombolitic mats, a 0.5 mL water sample was removed from each chamber every 3 h over a 36-h period. The DO in each sample was measured in triplicate using a Clark-type oxygen microelectrode with guard cathode (Revsbech et al., 1983) connected to a PA-2000 picoameter (Unisense, Aarhus, Denmark). After each sampling, an equal volume of sterile water was replaced to avoid the creation of a headspace. The measured DO values represent a summation of photosynthetic O2 production and O2 consumption. The latter comprises biological processes such as aerobic respiration and sulfide oxidation, as well as abiotic chemical consumption processes. In microbial mats, aerobic respiration is considered to be the dominant O2 sink (Revsbech & Jørgensen, 1986), but in stromatolites of Highborne Cay, approximately one third of O2 consumption is attributed to sulfide reoxidation (Visscher et al., 1998). In this study, we used the DO measurements as an indicator of the overall microbial activity. DO dynamics correlate with diel processes, as photosynthetic production, aerobic heterotrophy and chemolithotrophic sulfide oxidation fluctuate as a function of shifting biogeochemical conditions. The increase of DO during the daytime combined with the decrease of DO during the early night provided an estimate for the community O2 production (Des Marais & Canfield, 1994).

Dissolved inorganic carbon measurements

In addition to those samples collected for DO, supplementary water samples (3 mL) were also extracted from each chamber and analyzed for DIC. These DIC water samples were filtered with a 0.45-μm pore size syringe filter and injected into a nitrogen purged sealed 10-mL serum bottle, and stabilized by the addition of 0.3 mL of 1 m NaOH. The samples were stored at 4 °C until further analysis. Subsequently, 0.3 mL of 0.1 m HCl was added to convert all HCO3− and CO32− to CO2 and 100 μL of headspace were extracted from the serum bottles and injected into a gas chromatograph thermal conductivity detector (GC-TCD; Shimadzu GC14A; Shimadzu Instruments, Columbia, MD, USA) to quantify the CO2.
Standards prepared from pure CO₂ were run and plotted to generate a standard curve that was used to calculate the CO₂ concentration (in ppm) for each sample. DIC is consumed (i.e. net loss from the overlying water) during peak photosynthesis, which occurs during the late morning/early afternoon. DIC is produced (i.e. net increase in the overlying water) during nighttime when aerobic respiration processes and less importantly sulfide oxidation are producing this metabolite (Des Marais & Canfield, 1994).

**Microelectrode depth profiling of oxygen**

To examine the in situ activity of photosynthesis within the button thrombolitic mats, depth profiles for oxygen were generated using a polarometric Clark needle electrodes with an outer diameter of 0.4 mm and a sensing tip diameter of 10–20 μm (Visscher et al., 1991). The oxygen signal was registered on a Keithley model 385 picoameter with customized variable polarization. Microelectrodes were deployed under natural light and temperature. Profiles were measured in 0.2 mm increments during peak photosynthetic activity (between noon and 2:30 PM) with the aid of a National Aperture motorized ministage MM-3M-EX-2.0 and a National Aperture programmable Servo 3000 MC4B controller box. Due to the hardness of the thrombolitic samples, electrode breakage occurred frequently, however, in total, nine O₂ depth profiles were obtained in the button microbialitic mats.

**Biomass analyses**

As outlined above, great care was taken to use similar sample sizes of each mat type. Samples were weighed and photographed. The discernable surface areas of each sample were calculated by analyzing the overhead photos with Canvas 11 (ACD Systems of America, Miami, FL, USA).

The chlorophyll in each sample was determined by standard acetone extraction (Inskeep & Bloom, 1985) and chlorophyll a (Chlα) was used as a biomass proxy. The formaldehyde was removed from the sample, rinsed in distilled water, and ground with a mortar and pestle. Weight-equivalent portions of each homogenized sample were placed in a test tube to which 4°C acetone was added. The resulting slurry was covered with foil to prevent pigment dispersion by exposure to light and placed at 4°C overnight. Samples were then centrifuged at a low speed to pellet the sediment and 1 mL of supernatant was transferred to a quartz cuvette and scanned using a spectrophotometer (300–800 nm). The absorption peak of 665 nm was used to calculate the Chlα concentration in each sample.

**DNA extraction**

Total genomic DNA was isolated from the thrombolitic mats using a xanthogenate DNA extraction method as previously described (Green et al., 2008; Foster et al., 2009). Briefly, thrombolitic mat samples were vertically sliced and six slices weighing between 50 and 80 mg were added to a bead cocktail containing 0.2 g of three sterile zirconia beads sizes (2.4, 0.7 and 0.1 mm; BioSpec Products, Inc., Bartlesville, OK, USA). To this cocktail an extraction buffer was added containing 100 mM Tris–HCl pH 8.0, 100 mM potassium phosphate buffer pH 8.0, 1% (w/v) cetethyltrimethyl ammonium bromide, and 2% (w/v) sodium dodecyl sulfate. The samples were vortexed for 2 min and incubated for 2 h at 65°C with a xanthogenate solution containing 2.5 μM ammonium acetate and 8.2% (w/v) potassium ethyl xanthogenate to remove excess extracellular polymeric substances. After treatment the samples were processed as previously described using components (C4–C6) of the MoBio PowerSoil DNA kit (MoBio, Carlsbad, CA, USA; Green et al., 2008). DNA extracted from the thrombolitic mat samples was quantified spectrophotometrically from six replicate samples, normalized and pooled for downstream applications.

**Small subunit rRNA gene library synthesis and screening**

Three distinct clone libraries were generated for both the button and pink thrombolitic mats using DNA extracted from thrombolitic mats. The first clone library targeted the 16S rRNA genes of each thrombolitic mat type and was generated by PCR amplification of genomic DNA with the universal bacterial primers 27f (AGAGTTGTATCTGGCTCAG) and 1525r (TAAGGAGGTATCCAGCC; Lane, 1991). The second clone library was generated using the phylum-specific Cyanobacteria primers Cya359 (GGGGATYTTCGGCAATGGG) and an equimolar mixture of Cya781a (GACTACTGTTGGATCTGCCGTCAG) and Cya781b (GACTAGCGGTATCTAATCCATT; Nübel et al., 1997). The third library targeted the eukaryotic diversity in the button and pink thrombolitic mats and was generated using the universal Eukarya 18S rRNA gene primers 18SF (ACCTGGTATATCTAATCCACT; Lane, 1991) and 18SR (TGATCTCTTCYGCAGGTTCAC; Moon-van der Staay et al., 2001). Other than the primer sets, the construction of the three libraries was identical. Genomic DNA from the button and pink thrombolitic mats was PCR amplified using the GoTaq PCR core kit (Promega, Madison, WI, USA) and 1.5 mM MgCl₂. The PCR conditions included an initial denaturation at 94°C for 2 min, 30 cycles of amplification at 94°C for 1 min, 58°C for 2 min, elongation at 72°C for 2 min and a final extension at 72°C for 7 min. The amplified PCR products were purified using the Ultra-Clean PCR kit (MoBio), and cloned using the Original TA cloning kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer’s instructions. Clones were sequenced by the University of Florida Interdisciplinary Center for Biotechnology Research DNA sequencing core facility (UF-ICBR) using traditional Sanger sequencing with an ABI 3130 DNA sequencer (Applied Biosystems, Foster
City, CA, USA). Sequences were screened for plasmid contamination and low-quality sequence data using the software package SEQUENCHER (Gene Codes, Ann Arbor, MI, USA).

Small subunit rRNA gene sequence analysis

The partial 16S rRNA gene sequences recovered from the button and pink thrombolitic mats were aligned to known 16S rRNA gene sequences using the Greengenes 16S rRNA NAST alignment tool (DeSantis et al., 2006). The aligned sequences were then screened for putative chimeric sequences using the Bellerophon tool on the Greengenes website (Huber et al., 2004). A total of 28 suspect chimeric sequences were identified and removed from the thrombolitic mat clone libraries. The remaining aligned sequences were characterized with the Greengenes Automatic Taxonomic Classification and DNA maximum likelihood (DNAML) tools and then imported into the ARB software package (Ludwig et al., 2004). Sequences were inserted into a tree composed of near-full length sequences, employing the ARB parsimony option employing a 50% maximum frequency bacterial filter while maintaining the overall topology of the tree. Inserted sequences and closely related sequences were retained while removing other sequences from the tree. Trees were exported from ARB and opened using the software package WinFig (http://www.schmidt-web-berlin.de/winfog/).

For calculation of diversity indices, operational taxonomic units (OTUs) defined by 97% sequence similarity were determined using the Complete Linkage Clustering tool at the Ribosomal Database Project’s Pyrosequencing pipeline. These OTUs were further refined by visual inspection of the trees generated within ARB. All diversity calculations were performed according to formulae described by Schloss & Handelsman (2005) for the software package DOTUR, implemented within Excel (Microsoft Corporation, Redmond, WA, USA).

Sequences recovered from the eukaryotic 18S rRNA gene libraries were compared to the GenBank database using the nucleotide basic local alignment and search tool (BLASTN; Altschul et al., 1997). These gene sequences were aligned using the online software package CLUSTALW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html; Larkin et al., 2007), and a guide tree generated within ARB. OTUs were identified by visual inspection, and diversity calculations performed as described above.

Automated rRNA intergenic spacer analysis

The automated rRNA intergenic spacer analysis (ARISA) method was used as an independent and rapid assessment of bacterial diversity in the button-type thrombolitic mats. In prokaryotes, the heterogeneity of the intergenic-transcribed spacer (ITS) region between the 16S and 23S rRNA gene facilitates a rapid screening of community diversity (Fisher & Triplett, 1999; Yannarell & Triplett, 2004; Havemann & Foster, 2008). ARISA was performed on 10 independent DNA extractions from button thrombolitic mats using the universal bacterial ITS primer set S-D-Bact-1522-b-S-20 and L-D-Bact-132-a-A-18, which amplifies between position 1452 of the 16S rRNA gene and position 115 of the 23S rRNA gene (Ranjard et al., 2001; Cardinale et al., 2004). The PCR conditions for ITS amplification and ARISA were performed as previously described in Havemann & Foster (2008).

To examine the dominant peaks, eight PCR amplifications of the ITS region were performed as described in Havemann & Foster (2008), pooled, and ethanol precipitated. Products were suspended in a solution containing 1 mM Tris–HCl (pH 8.5) and DNA loading dye (10 mM Tris–HCl pH 7.5, 50 mM EDTA pH 8.0, 0.03% bromophenol blue, 15% Ficoll 400) and separated on a 2.0% agarose gel. DNA bands that corresponded to peaks of interest, based on base pair size, were excised and purified using a QiaGen gel extraction kit according to manufacturer’s protocol (QiaGen, Valencia, CA, USA). Samples were then cloned as described above. Twenty-four positive clones for each target band were selected and sequenced at the UF-ICBR core facility. The sequences of the 16S–23S rRNA ITS regions were analyzed to delineate their closest relative using the BLAST alignment tool in GenBank.

Nucleotide sequence accession numbers

All nucleotide sequences recovered in this study have been deposited in the GenBank database. The bacterial 16S rRNA genes have been assigned accession numbers GQ483823–GQ484263, eukaryotic 18S rRNA genes assigned GQ483646–GQ483822, and the ITS sequences have been assigned GQ484264–GQ484354.

RESULTS

Morphology of thrombolitic mat types

Within the fringing reef complex along the eastern margin of Highborne Cay (Fig. 1a,b), thrombolitic microbialites occupied the intertidal zone at four (Sites 4, 5, 6, and 8) of the 10 research sites designated by Andres & Reid, 2006. Although not as widespread as their subtidal stromatolitic counterparts, the thrombolitic structures were pronounced (Fig. 1b) ranging in size from a few cm to over 1 m in length and 0.5 m in height. The thrombolites were juxtaposed (1–2 m) to the subtidal stromatolites and both structures were subjected to periodic burial events by oolitic sand grains like their stromatolitic counterparts. Vertical cross-sections of the thrombolitic structures showed a distinctive clotted mesostructure (Fig. 1c) primarily composed of the calcium carbonate polymorph aragonite (98%; data not shown) present as sand grains and microcrystalline (micritic) precipitates.
Analyses of the thrombolite surfaces revealed distinctive microbial mat communities, up to 3 cm thick. Visual observation of the thrombolitic mats showed two dominant types, an irregular nodous community referred to in this study as ‘button mats’, and a smooth, flat mat referred to as ‘pink mats’. The button mats (Fig. 2a) were located on the seaward thrombolites and were littered with small (1–2 cm) round domal structures that when examined in cross-section, showed a pronounced community of vertically orientated filamentous cyanobacteria. The filamentous cyanobacteria were heavily calcified and exhibited morphological characteristics such as basal heterocysts, tapered apical ends and a lateral branching pattern that runs parallel to the trichome. Together these morphological characteristics suggested that the dominant cyanobacteria are affiliated with the genus Dichothrix of the order Nostocales. Filaments of Dichothrix sp. that were dissected out of the thrombolites were subject to 16S rRNA gene sequencing, and the recovered sequences were highly similar (>97%) to the previously described Calothrix sp. (97%). This result supports the morphological identification of the dominant cyanobacteria; however, there are no published 16S rRNA gene sequences currently available for any cultivated Dichothrix species. Petrographic thin sections of these button mats also revealed an abundance of filamentous Dichothrix sp. within the upper layers of the thrombolitic communities (Fig. 2c; see also Planavsky et al., 2009). These Dichothrix sp. filaments exhibited calcification occurring around the exopolymeric sheath in the upper few mm of the mat and appeared to degrade as they progressed downward into the thrombolite (Fig. 2c). In the lower levels of the mat, the surrounding sediment grains became more cemented (data not shown).

The pink mats were so called due to the patchy areas of pigmented Chlorophyta cells colonizing the community surface of near shore thrombolites (Fig. 2d). Overlaying the pink encrusted thrombolitic mat was a field of exopolymeric substances (EPS) that when submerged extended approximately 1 cm above the mat surface (Fig. 2e). Cross-sections under a petrographic microscope revealed the presence of a thin layer of micrite associated with the EPS (Fig. 2f) suggesting that this community is capable of lithification, perhaps in a manner more similar to the nearby stromatolites as there was no calcification occurring around individual, or even groups, of cells. The sediment grains appear to be loose in the upper surface of pink mats and more compact in the lower levels of this mat, although once again, calcification or cementation is not as pronounced as in the button-type mats. In addition to the two dominant button and pink mat types, another mat type was observed less frequently and comprised mostly of Cladophora spp. algae resembling turf. These turf-like communities appeared to colonize sporadically the surfaces of the button mats, and were only found on those thrombolite structures that were not subjected to daily or weekly burial events.

**Thrombolitic mat productivity**

To characterize the metabolic potential of the various thrombolitic mats, samples of each mat type were incubated in individual flux chambers for 36 h and measured in triplicate for DO and DIC under natural and filtered sunlight (Fig. 3). These experiments were conducted in August and November 2006 and in July 2007. Due to the high reproducibility of the data only those results from July 2007 are reported as they were coordinated with molecular diversity analyses. In addition to the aforementioned thrombolitic mat types (button, pink, turf) a fourth sample, in which the button mats were partially colonized with the algal Cladophora spp. turf (i.e. button-turf), was also examined. Together these four thrombolitic mat samples were compared to the adjacent stromatolitic mats for diel DO and DIC dynamics. As mentioned above, the biomass of the each mat type was comparable. The average surface area was 7.20 ± 0.73 cm² and
sample weight was 8.04 ± 1.22 g (Table 1). The Chl\textsubscript{a} content, however, varied among mat types, reflecting differences in phototroph biomass. Button (25.08 μg) and button-turf (20.89 μg) mats contained the most chlorophyll-rich biomass, whereas pink thrombolitic mats contained the least (9.54 μg; Table 1).

**Dissolved oxygen**

The measured levels of DO, which represent photosynthetic production in excess of consumption were light- and mat type-dependent. Of the five samples types examined, the button thrombolitic mats exhibited the most rapid rates of increase as well as highest levels of DO under natural light (Fig. 3a). The DO in these button thrombolitic mats peaked at 12:00PM (0.36 mM) whereas the remaining samples were significantly lower than the buttons at midday (P > 0.002), indicating that the button mats supported much higher microbial activities than the other mat types. The pink thrombolitic mats had the second highest levels of DO at midday (0.127 mM) and was significantly higher than the button-turf, turf, and stromatolite samples (P > 0.002). The dimensions of the different mat types were very similar and we decided not to normalize the patterns to Chl\textsubscript{a} as the variation in this biomass indicator may reflect differences in the community composition that are characteristic of the various mat types (Table 1). However, even when normalized to Chl\textsubscript{a}, the button mat types still displayed significantly greater DO production rates than any other mat type.

It should be noted that the rates of photosynthesis were much higher than the rate of DO increase suggested. Element cycling is tight in microbial mats and a large part of the oxygen that is produced is often immediately consumed by respiration and sulfide oxidation. By midnight, however, the levels of DO dropped precipitously in the button mat types (0.11 mM), and a near linear decrease in the DO level suggested a fairly constant rate of consumption during the diel cycle. The remaining mat types showed only a small decrease in DO throughout the night, again indicating much less microbial activity than the button mat type. By noon the next day the DO rates again began to rise in all samples clearly demonstrating a diel rhythm to the oxygenic photosynthesis in the thrombolitic communities under natural light (Fig. 3a). It should also be noted that the DO dynamics in the button mat type were much more pronounced than those in stromatolitic mats, which have been shown to have high rates of photosynthesis and aerobic respiration (Visscher et al., 1998). Under neutral density filtration in which 50% of the PAR was removed, the DO levels were relatively unchanged in all mat types and did not show a pronounced change in primary productivity throughout the diel cycle (Fig. 3b). With the exception of the button mat types, the DO levels in all other mat samples were undersaturated, which may have been caused by a rapid initial consumption of oxygen.

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**Table 1 Biomass and Chl\textsubscript{a} measurements of thrombolitic mats**

<table>
<thead>
<tr>
<th>Mat type</th>
<th>Light conditions</th>
<th>Weight (g)</th>
<th>Surface area (cm(^2))</th>
<th>Chl\textsubscript{a} (μg per sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Button</td>
<td>Full light</td>
<td>8.76</td>
<td>6.80</td>
<td>27.75</td>
</tr>
<tr>
<td>Button-turf</td>
<td>Full light</td>
<td>9.17</td>
<td>6.90</td>
<td>31.10</td>
</tr>
<tr>
<td>Turf</td>
<td>Full light</td>
<td>9.93</td>
<td>6.50</td>
<td>13.65</td>
</tr>
<tr>
<td>Pink</td>
<td>Full light</td>
<td>8.27</td>
<td>8.30</td>
<td>13.89</td>
</tr>
<tr>
<td>Stromatolite</td>
<td>Full light</td>
<td>8.65</td>
<td>6.60</td>
<td>18.03</td>
</tr>
<tr>
<td>Button</td>
<td>Filtered light</td>
<td>7.14</td>
<td>8.00</td>
<td>25.08</td>
</tr>
<tr>
<td>Button-turf</td>
<td>Filtered light</td>
<td>7.03</td>
<td>8.10</td>
<td>20.89</td>
</tr>
<tr>
<td>Turf</td>
<td>Filtered light</td>
<td>6.31</td>
<td>6.40</td>
<td>21.52</td>
</tr>
<tr>
<td>Pink</td>
<td>Filtered light</td>
<td>6.47</td>
<td>7.30</td>
<td>9.54</td>
</tr>
<tr>
<td>Stromatolite(^a)</td>
<td>Filtered light</td>
<td>8.65</td>
<td>6.60</td>
<td>18.03</td>
</tr>
</tbody>
</table>

\(^a\)Type 2 stromatolites were used as they represent a transitional stromatolitic mat type.
Dissolved inorganic carbon (DIC, or CO₂, HCO₃⁻, CO₃²⁻)
Under natural light, the measured levels of DIC, which represent heterotrophic respiration in excess of CO₂ consumption (e.g. carbon fixation, and a much lesser extent acetogenesis, methanogenesis) showed very little variation between the five mat samples (Fig. 3c). Of the various mat types the button-turf sample had the highest levels of DIC peaking at $2 \times 10^4$ ppm at 9:00 PM. The DIC level in the experimental chambers with the pink thrombolitic mats remained relatively low until midnight, at which point, it increased fourfold from $2.9 \times 10^3$ to $1.2 \times 10^4$ ppm. However, this increase was transient, and the CO₂ level dropped to $5 \times 10^3$ ppm by 3:00 AM. Under 50% filtered light the rates of DIC concentration was higher at all time points with the peaks of CO₂ occurring at 3:00 AM (5 × 10⁴ ppm; Fig. 3d). These high levels of DIC remained throughout the 36-h cycle only dropping midday to 1.5 to $2.5 \times 10^3$ ppm even in the presence of the elevated DO. During the second evening, the maximum CO₂ levels were lower in all mat types, most noticeably in the pink thrombolitic mats. The high dynamic DO pattern was repeatedly observed in button mat samples and was not apparent in the DIC results.

Oxygen depth distribution in button thrombolitic mats
To generate a spatial profile of oxygenic activity in the button thrombolitic mats, microelectrodes were replicated nine times generating vertical profiles of O₂ distribution between 12:30 and 2:30 PM. All replicate profiles showed a similar oxygen distribution, with multiple oxygen peaks that mostly likely resulted from the patchy distribution of the cyanobacteria in these mats. The maximum depth of O₂ penetration was between 8 and 14 mm. One of the nine replicates O₂ profiles is depicted in Fig. 4 revealing three O₂ peaks with clear minima (but not anoxia) in between. In this profile, three distinct peaks of O₂ were observed at 1.6 mm (0.85 mM), 3.4 mm (0.79 mM), and 4.8 mm (0.70 mM) below the surface suggesting a complex, patchy microbial community of highly active photosynthetic organisms. Although the specific location of each peak varied slightly between each profile, all replicates contained multiple peaks of O₂. Oxygen profiling of the pink mats was not possible due to the extensive carbonate precipitation in these pink thrombolitic mats, which prohibited microelectrode penetration.

Microbial diversity and composition in thrombolitic mats
Cultivation-independent microbial community characterization was performed on the two dominant thrombolite mat types, button and pink, by generating clone libraries small subunit rRNA genes (SSU rRNA) of Bacteria, Cyanobacteria, and Eukarya. Additionally, the ITS region between the small and large subunit RNAs was amplified using bacterial domain primers for ARISA analysis, and additional cloning and sequencing was performed on these amplicons. These analyses were not conducted on the turf mats as they exhibited lower productivity rates and were not as abundant as the button and pink mats.

These sequence data were initially analyzed to generate diversity indices for each primer set and thrombolite type (Table 2). These analyses indicate that the level of bacterial sequence diversity in thrombolitic mats is similar to that of stromatolites from Highborne Cay and Shark Bay, Australia (Foster & Green, 2010). Analysis of the sequence data from the domains Bacteria and Eukarya, and phylum Cyanobacteria indicates lower sequence diversity for the buttons mat type. Similar diversity levels for the class Alphaproteobacteria were measured for the two mat types. Due to the high diversity of Bacteria in these systems and the size of the clone libraries, the coverage of the domain Bacteria was moderate (49–59%; Table 2). Much more robust coverage was observed for the domain Eukarya and for the Cyanobacteria (80–90% and 81–84%, respectively).

The composition of the microbial community was examined in more detail by generating high-level classification of sequences (domains Bacteria and Eukarya, Figs 5 and 8, respectively) and phylogenetic trees (class Alphaproteobacteria and phylum Cyanobacteria; Figs 6 and 7, respectively). At the phylum level, the button thrombolitic mats (Fig. 5a) were dominated by Proteobacteria, specifically the class Alphaproteobacteria (65%) and the Cyanobacteria (36%). The other
predominant phyla (and classes) detected in the button mats included the Planctomycetes (19%), Deltaproteobacteria (13%), and the Spirochaetes (10%). In the pink thrombolitic mats, the phylum-level diversity was higher (Fig. 5b). The Alphaproteobacteria were again the dominant taxa in the pink mats (58%), while the Cyanobacteria represented only 19% of the recovered sequences. The pink mats were also rich in Deltaproteobacteria (38%), Planctomycetes (23%), and Firmicutes (17%). A significant portion of the Alphaproteobacterial sequences had low DNA ML values (0.8–0.89) indicating relatively low similarity to previously described isolates or environmental sequences, whereas the cyanobacterial values were higher (0.9–1). Although it is likely that archaea are present within the community, it was not included in this study as in other microbialite systems archaea account for <5% of the total microbial populations (Burns et al., 2004; Goh et al., 2009; Foster & Green, 2010).

Alphaproteobacteria community composition

The Alphaproteobacteria were the largest taxon represented in the cultivation-independent sequences analyses of the thrombolitic microbial mats. The histograms depict the number of OTUs recovered from each phylum and the associated environmental sequences. Actinobacteria (38%), Planctomycetes (23%), and Deltaproteobacteria (19%) were predominant phyla (and classes) detected in the button mats (Fig. 5a). In the pink thrombolitic mats (Fig. 5b), the phylum-level diversity was higher. The Alphaproteobacteria (13%), Planctomycetes (19%), and Deltaproteobacteria (13%) made up the dominant taxa in the pink mats. The pink mats were also rich in Gammaproteobacteria (19%), and the Cyanobacteria (10%). In the pink thrombolitic mats, the Gammaproteobacteria (19%) and the Cyanobacteria (10%) were the dominant taxa. The pink thrombolitic mats were also rich in Actinobacteria (38%), Planctomycetes (23%), and Deltaproteobacteria (19%).

Table 2 Small subunit ribosomal RNA gene diversity analyses of clone libraries

<table>
<thead>
<tr>
<th>Domain Bacteria</th>
<th>Domain Eukarya</th>
<th>Phylum Cyanobacteria</th>
<th>Class Alphaproteobacteria</th>
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</thead>
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<tr>
<td>Sequences</td>
<td>OTUs</td>
<td>Total</td>
<td>Button Pink</td>
</tr>
<tr>
<td>328</td>
<td>185</td>
<td>157</td>
<td>110</td>
</tr>
<tr>
<td>OTUs</td>
<td>Singlets</td>
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</tr>
<tr>
<td>114</td>
<td>11</td>
<td>94</td>
<td>11</td>
</tr>
<tr>
<td>OTUs</td>
<td>Doublets</td>
<td></td>
<td>Button Pink</td>
</tr>
<tr>
<td>49</td>
<td>5</td>
<td>18</td>
<td>2</td>
</tr>
<tr>
<td>OTUs</td>
<td>&gt;5% (Sum)</td>
<td></td>
<td>Button Pink</td>
</tr>
<tr>
<td>0</td>
<td>6%</td>
<td>0%</td>
<td>63%</td>
</tr>
<tr>
<td>Shannon</td>
<td></td>
<td></td>
<td>Button Pink</td>
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<tr>
<td>4.10</td>
<td>1.10</td>
<td>4.29</td>
<td>1.72</td>
</tr>
<tr>
<td>Evenness</td>
<td></td>
<td></td>
<td>Button Pink</td>
</tr>
<tr>
<td>0.95</td>
<td>0.94</td>
<td>0.97</td>
<td>0.86</td>
</tr>
<tr>
<td>Chao1</td>
<td>(confidence)</td>
<td></td>
<td>Button Pink</td>
</tr>
<tr>
<td>314</td>
<td>(264/394)</td>
<td>203</td>
<td>(150/307)</td>
</tr>
<tr>
<td>OTUs</td>
<td>% Coverage</td>
<td></td>
<td>Button Pink</td>
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<tr>
<td>65%</td>
<td>59%</td>
<td>49%</td>
<td>86%</td>
</tr>
</tbody>
</table>

*Clone library was generated with general bacterial primers.
*Clone library was generated with general eukaryal primers.
*Clone library was generated with cyanobacterial primers.
Total represents the pooled sequences of the button and pink thrombolitic mats.
The values were calculated based on a 97% similarity threshold.
The values were calculated based on a 97% similarity threshold.
*Number of OTUs at 97% similarity threshold or higher that represent >5% of the population. The percentage of the total community these OTUs represent are in parentheses.
Values in parentheses represent the lower and upper 95% confidence intervals associated with Chao1 nonparametric estimator.
*Percent coverage of the clone library using the formula (1 – [N-singlets]/N).

Fig. 5 Phylum-level diversity of thrombolitic microbial mats. The histograms depict the number of OTUs recovered from each phylum and the associated environmental sequences. Actinobacteria (38%), Planctomycetes (23%), and Deltaproteobacteria (19%) were the dominant phyla detected in the button mats (a), and the Cyanobacteria (10%), Planctomycetes (19%), and Deltaproteobacteria (13%) made up the pink thrombolitic mats (b). The pink thrombolitic mats were also rich in Gammaproteobacteria (19%), and the Cyanobacteria (10%). In the pink thrombolitic mats, the Cyanobacteria represented only 19% of the total community, whereas the cyanobacterial values were higher (0.9–1). Although it is likely that archaea are present within the community, it was not included in this study as in other microbialite systems archaea account for <5% of the total microbial populations (Burns et al., 2004; Goh et al., 2009; Foster & Green, 2010).
thrombolic mats. Within the Alphaproteobacteria the most dominant order in both thrombolic types at was the Rhodobacterales (~70% of the Alphaproteobacterial sequences), a highly diverse assemblage of phototrophic and heterotrophic marine organisms (Fig. 6). Roughly, 10% of the Rhodobacterales sequences belong to the genera the Rhodobacter and Roseobacter, which are extremely common in coastal marine waters (DeLong & Karl, 2005). Apart from these genera, the Rhodobacterales sequences from the thrombolites were clustered into 20 groups, consisting of one or more OTUs. None of these clusters was exclusive to thrombolites, and contained sequences detected in both natural and artificial stromatolites from Highborne Cay. Some clusters (i.e. Clusters 2, 3, 4, 6–8, 11, 13, and 17–20) appear to be unique to the Highborne Cay microbialite environment. The absence of close-isolated representatives, and the high metabolic diversity within the order, makes it difficult to assign a putative metabolic function to the micro-organisms from which these sequences were derived. For example, the single most abundant OTU within the thrombolites was found within both the button and pink mat types and belongs to Cluster 3. This OTU was most closely related to a Sargasso Sea clone and to sequences derived from the stromatolites of Highborne Cay.

**Cyanobacterial community composition**

The thrombolite cyanobacterial community, while less diverse than the Alphaproteobacteria (Table 2) was nonetheless a complex consortium. The community contained species from the orders Nostocales (heterocystous), Pleurocapsales (coccoid, non-heterocystous), Oscillatoriales (filamentous, non-heterocystous) and Chroococcales (coccoid, non-heterocystous). The cyanobacterial sequences generated with the Bacterial and Cyanobacterial primers were grouped into 17 distinct clusters of one or more OTUs (Fig. 7). Unlike the Alphaproteobacteria, a number of recovered cyanobacterial lineages unique to the thrombolites were identified (Fig. 7 black clusters; 1–3, 7 and 12). Sequences from the unique clusters were most similar to gene sequences from organisms derived from the heterocystous genera Rivularia and Calothrix (Clusters 1 and 2), from Mammoth hot spring mats in
Yellowstone National Park (Cluster 3), from a filamentous, non-heterocystous genera *Phormidium* and *Leptolyngbya* (Cluster 7), and from the coccoid *Synechocystis* sp. PCC6702 (Cluster 12). The sequences from Clusters 1 and 2, morphologically and genetically resembled the dominant *Dichothrix* sp. isolated from the button thrombolitic mats. Six of the 17 clusters were unique to the Highborne Cay microbialites (Fig. 7 hashed clusters).
Table 3 ARISA profile of button thrombolitic mats

<table>
<thead>
<tr>
<th>Peak size (bp)</th>
<th>No. replicates containing peak</th>
<th>Mean peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td>418</td>
<td>4</td>
<td>38.5</td>
</tr>
<tr>
<td>439</td>
<td>2</td>
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<td>465</td>
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<td>63.6</td>
</tr>
<tr>
<td>474</td>
<td>9</td>
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</tr>
<tr>
<td>499</td>
<td>10</td>
<td>115.7</td>
</tr>
<tr>
<td>550</td>
<td>9</td>
<td>85.1</td>
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<td>6</td>
<td>56.4</td>
</tr>
<tr>
<td>742</td>
<td>3</td>
<td>134.5</td>
</tr>
<tr>
<td>753</td>
<td>1</td>
<td>31.5</td>
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<td>769</td>
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<td>41</td>
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<td>783</td>
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<td>799</td>
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<td>76.7</td>
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<tr>
<td>814</td>
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<td>824</td>
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<td>141.1</td>
</tr>
<tr>
<td>1074</td>
<td>1</td>
<td>36.5</td>
</tr>
</tbody>
</table>

*Only those peaks >20-fluorescent unit threshold were reported.

*Band sizes selected for sequencing and analysis.

ARISA profiling in thrombolitic mats

To complement the 16S rRNA gene phylogenetic analyses, the dominant button thrombolitic mats were analyzed with the ARISA method. The amplification of the ITS region between the 16S and 23S rRNA gene generated 22 peaks (i.e. >20 fluorescent units), but only 14 occurred in two or more of the 10 replicates. The ARISA profile of the button mats including the peak size and area are listed in Table 3. Of the 14 significant peaks, three peaks (474, 589, and 656 bp) were selected for further analyses as they represented the largest peak areas in the button mats and were found in most replicates (Table 3). For comparison purposes, we also chose two more rare peaks (814 and 824 bp) for sequencing analyses that were found in only three of the 10 replicates. Although not as abundant as the other peaks, the mean area of these two peaks combined were the highest of the larger ITS regions. These peaks were combined for sequencing analyses due to their close proximity in size. At least 24 clones of each band were sequenced and assigned a putative name based on the BLAST hits for the ITS region. A selected subset of these sequenced ITS regions from the button mats are listed in Table S1 along with their closest BLAST hit, accession number, and percent similarity to the top BLAST hit.

In all four of the ITS bands examined, the highest levels of sequence identity (98–100%) were to ITS clones isolated from an artificial microbialite (AM) model derived from Type 2 stromatolites collected from Highborne Cay, Bahamas (Havermann & Foster, 2008). Most of the non-AM sequences were highly novel with the majority of the sequences sharing sequence similarity of <94% to anything in the known database. Each band represented a heterogenic population comprised multiple phyla with the Proteobacteria being the most dominant just as in the 16S rRNA clone libraries. In the 474-bp band, most of the recovered clones (n = 11) from this peak were similar (92% max identity) to a clone isolated from the cyanobacterial layer of the AM model that shares affiliation to Alphaproteobacteria. The other two dominant sequences recovered from the 474-bp band shared sequence similarity with the spirochete Treponema denticola ATCC 35405 (96%) and the sulfate reducing bacterium (SRB) Desulfovibrio desulfuricans. The sequence similarity of the Desulfovibrio was, however, very low (83%) and most probably reflects a novel organism. The remaining bands also showed similar heterogenic results, with most of the clones sharing sequence similarity (98–100%) to the cyanobacterial and calcified crust layers of the AM models. Interestingly, in the 814/824 bp bands there was a 100% identity match to Xanthomonas fragariae, a fastidious plan pathogen and a 97% match to the anaerobic cellulose-degrading Clostridium cellulolyticum. The other non-AM bands had low identity (≤93%) to the known ITS regions in the GenBank database.

Eukaryotic diversity in thrombolitic mats.

Clone libraries of domain-level eukaryotic 18S rRNA gene sequences were generated for each thrombolite type. Diversity indices for the libraries were generated (Table 2), and an overview of the taxonomic affiliation of the sequences is summarized in Fig. 8. Relative to the diversity of bacteria, the eukaryotic diversity of these samples was quite low. The clone libraries from the button thrombolites were less diverse than the pink thrombolites, and were primarily comprised sequences form the phylum Nematoda, including the families Rhabdolaimidae (46%), Leptolaimidae (11%), Heteroderidae (6%), Monohysteridae (6%), Enoploidea (1%) and Desmodoridae (1%). The clone library generated from the pink thrombolites was also dominated by sequences from the phylum Nematoda (Fig. 8). In both mat thrombolitic mat types, the most abundant recovered sequence (20%) shared similarity (90–99%) to the marine organism Syringolaimus striatocaudatus. Sequences from phyla Alveolata (27%) and Chlorophyta (7%) were substantially more abundant in the clone libraries generated from the pink thrombolitic mats. In both mat types (button, 14%; pink 9%), there was an extensive number of recovered sequences that shared sequence similarity (88–99%) to the unaffiliated GNEG cluster derived from the hypersaline mats of Guerrero Negro and the HAVO cave mats found in Kilauea Crater, Hawai‘i.

DISCUSSION

The biogeochemistry and microbial ecology of the lithifying microbialitic mats at Highborne Cay have been the subject of several investigations in recent years (e.g. Visscher et al.,
The unusual shape of the O2 profiles in mats may be the product of differences detected in geochemically different microstructures (Fig. 2c,f) in the thrombolitic et al. (Reid 2, 3) that are classified based on the microbial composition In stromatolites, there are three dominant mat types (Type 1, from the classic mat types seen in the adjacent stromatolites. distinctive community structures, which differ significantly of the microbial mat communities associated with the accretion thrombolites of Highborne Cay, Bahamas. At each of the thrombolitic microbialite collection sites, two dominant mat types were detected, referred to in this study as button and pink thrombolitic mats. Microscopic comparisons revealed that the button and pink mats have very distinctive community structures, which differ significantly from the classic mat types seen in the adjacent stromatolites. In stromatolites, there are three dominant mat types (Type 1, 2, 3) that are classified based on the microbial composition and activity, EPS concentrations, and extent of lithification (Reid et al., 2000; Stolz et al., 2009). The presence of distinctly different microstructures (Fig. 2c,f) in the thrombolitic mats may be the product of differences detected in geochemical pathways and community composition; however, this remains to be verified. The unusual shape of the O2 profiles in the button mats, with multiple peaks at varying depths, corroborated the clotted mesostructure found in the thrombolitic mats indicating clusters of very high photosynthetic activity. In addition to gross morphological differences between the button and pink thrombolitic mats, there were also disparities in the extent of DO production even when normalized to Chla content (data not shown). In full light conditions all of the mat types, with the exception of buttons, remain slightly undersaturated with respect to DO throughout the day. The undersaturation may have been in part due to the lack of photosynthesis when respiration was occurring, thereby drawing O2 from the water column, or it may simply reflect the release of oxidizing labile carbon when the samples were processed. Together these results suggest that oxygen production and aerobic respiration are nearly in balance and that the majority of the oxygen generated was consumed immediately by heterotrophs and sulfate-oxidizing bacteria and never made it into the overlying water column.

The button mat type, however, was marked by high levels of DO and relatively low levels of DIC, which tightly correlated to the light regime of the diel cycle. If the sharp increase of DO in the flux chambers resulted from a very high production rate (P) and much lower respiration rate (R), then we would have expected to see a decrease in DIC, even though seawater is well buffered with respect to DIC. These results, however, suggest that those respiration processes that do not utilize O2 as an electron acceptor may be playing a role in the CO2 production during photosynthetically active periods. Alternative (i.e. anaerobic) respiration processes that could be present include fermentation, denitrification, sulfate reduction and methanogenesis, all of which would contribute to the CO2 production. Clone libraries generated to the button and pink thrombolitic mats did recover several 16S rRNA sequences that shared homology to organisms (e.g. Desulfosarcina spp. and Geobacter spp.) that typically exhibit those metabolisms. Nitrate and iron (Paerl et al., 1993; Millero et al., 1995) are present in low concentrations in Bahamian water, so most likely, SRB play a pivotal role in the thrombolitic mat community, similar to what has been suggested for stromatolites (Visscher et al., 2000). Interestingly, sulfate reduction impacts the DO dynamics indirectly; the sulfide produced by the SRB is typically reoxidized, thereby consuming O2 in the process. Both cyanobacteria and SRB facilitate calcium carbonate precipitation through metabolic activity and by producing EPS (Dupraz & Visscher, 2005; Braissant et al., 2007).
The peak of oxygen productivity (Fig. 4) in the uppermost 6 mm correlated directly to the spatial location of the vertically orientated calcified Dichothrix sp. (Fig. 2b). These results suggest that oxygenic photosynthesis, just like the adjacent stromatolites, is a driving force of the biogeochemical cycling in thrombolitic mats (Visscher et al., 1998). It is noteworthy that the button mats seem to have a much higher rate of oxygen production than the stromatolitic mats. Pinckney et al. (1995) hypothesized that a high photosynthesis to aerobic respiration ratio (P:R) was necessary for lithification in stromatolites (i.e. creating excess alkalinity), but successive work (Pinckney & Reid, 1997; Paerl et al., 2001) was unable to confirm this hypothesis in stromatolitic mats. Our observations in the pink mats appear to be in agreement with the P:R hypothesis. The high diel DO fluctuations observed in button mats are not reflected in a similar DIC pattern. In contrast, diel DIC fluctuations are much lower and similar to that of the other thrombolitic and stromatolitic mats, indicating rapid internal recycling of DIC in the button mat types. When the light intensity is reduced to c. 50%, the DO flux is close to zero in all mat types (Fig 2), indicating that P and R are almost in balance and that the clusters of Dichothrix sp. are highly light limited. This may at least partially explain why button mat types are rarely found immediately against the shoreline, and instead they are found further seaward on the thrombolitic platforms. Near shore areas of the platforms are periodically buried by surrounding sediment, which leads to light limiting conditions.

The accompanying DIC pattern at 50% light, on the other hand, is much more dynamic than that at full daylight. The early daytime decrease of DIC could reflect a combination of photosynthetic uptake and possible CaCO$_3$ precipitation. This light-dependent change in community metabolism requires further investigations. It is clear that as in stromatolite mats, light availability (possibly coupled to burial and/or exposure to the atmosphere at low tide) is a driving force in thrombolitic mat biogeochemistry.

Previous work on the stromatolites at Highborne Cay determined that in microbially mediated precipitation, the heterotrophs are reliant on the photosynthetic community as their main source of organic matter (Paerl et al., 2001). In the button mats, the clusters of the Dichothrix sp. may be forming close associations with cooperative micro-organisms, which may be utilizing the photosynthetically derived O$_2$ as well as other alternative electron acceptors. Their combined metabolic activities may lead to precipitation of CaCO$_3$ and eventually, the clotted texture of the internal structure. Additional work on the internal composition, especially that of the clotted structures is needed to further examine the specific roles of the microbial community.

**Microbial diversity within thrombolitic microbialites**

The Proteobacteria, most notably the class Alphaproteobacteria, were the most abundant taxon in both thrombolitic mat types. The prevalence of Alphaproteobacteria in lithifying microbial mats results is consistent with all known extant stromatolitic microbialites and represent at least half of all recovered 16S rRNA sequences from microbialites (Burns et al., 2004; Papineau et al., 2005; Baumgartner et al., 2009; Foster & Green, 2010). Despite the richness of Alphaproteobacteria diversity in the thrombolitic mats, the results of this study suggest that this group of organisms does not play a critical role in thrombolite formation. Most of the recovered Alphaproteobacteria, such as Rhodobacter spp. and Roseobacter spp., while present in microbialites (Foster & Green, 2010), are also cosmopolitan in non-lithifying habitats such as the coastal and open waters (DeLong & Karl, 2005). Furthermore, analyses of the recovered OTUs revealed that when comparing the button and pink mats together only one OTU accounted for more than 5% of recovered sequences (Table 2). This result contrasts significantly to the phylum Cyanobacteria where 7 OTUs account for more than 58% of the recovered sequence suggesting that the cyanobacteria may be playing a more critical role within the community. Although it is likely that Alphaproteobacteria do contribute to the nutrient cycling in the thrombolitic communities it is, however, unlikely that they are essential for thrombolite formation and accretion.

**Cyanobacterial diversity in thrombolitic mats**

Although a morphologically and molecularly diverse community of cyanobacteria is ubiquitous in all known stromatolite ecosystems (Foster & Green, 2010), the abundance of calcified cyanobacterial filaments, however, appears to be a unique feature of the Highborne Cay thrombolitic mats. These calcified cyanobacterial filaments, which spatially correlate to the peak of midday oxygen production, suggest that calcification is occurring in the sheaths of living cyanobacteria and are not the by-product of dead or dying cells (Planavsky et al., 2009). The calcifying filamentous cyanobacteria have been morphologically classified to the genus Dichothrix (Rippka et al., 2001; Planavsky & Ginsburg, 2009). However, since there were no cultivated Dichothrix spp. isolates in GenBank prior to this study, phylogenetic analyses of the 16S rRNA gene derived from dissected Dichothrix filaments most closely related the genera Calothrix sp. (97%) and Rivularia sp. (96%).

The prevalence of Nostocales in the thrombolites represents one of the most significant differences between the thrombolitic and stromatolitic mat diversity. The presence of heterocystous Nostocales is rare in Type 1, 2, and 3 stromatolites. In a recent comparative analysis of all known 16S rRNA sequences from stromatolites, only three Calothrix-like sequences were detected out of 1497 sequences recovered (Foster & Green, 2010) and these sequences were derived from the hypersaline stromatolites in Shark Bay (Goh et al., 2009), not at Highborne Cay. The elevated numbers of heterocystous cyanobacteria in thrombolites, specifically those fil-

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Eukaryotic diversity in thrombolitic microbialites

Although the ribosomal gene analyses and ARISA profiling suggest a complex and metabolically diverse community of bacteria within the thrombolitic mats, the eukaryotic diversity, however, was remarkably low. Clone libraries generated to the 18S rRNA gene, recovered only 26 unique eukaryotic ecotypes in the thrombolitic mats types. While this was a relatively limited sampling of the eukaryotic diversity, the lack of eukaryotic diversity in the system results in a fairly robust coverage of the eukaryotes. The vast majority of recovered eukaryotic sequences from the thrombolitic mats were affiliated with the phylum Nematoda, with 28% of the recovered clones in button mats and 20% of the pink mats sharing sequence similarity to the species *Syringolaimus* sp. of the Ironidae family. *Syringolaimus* sp., as well as many of the other recovered Nematoda ecotypes are commonly found in high-energy sandy beach environments and can withstand intense wave action (Nicholas & Hodda, 1999). Nematoda, specifically the families of Monhysteridae and Rhabdolaimidae, are the dominant eukaryote found in the hypersaline mats of the Guerrero Negro (Feazel *et al.*, 2008) and thestromatolites of Shark Bay, Australia (Allen *et al.*, 2009). The enrichment of nematode sequences in the thrombolitic mats may reflect the ability of these organisms to withstand, extensive desiccation, high sulfide and anaerobic conditions typically found in microbial mat ecosystems (Feazel *et al.*, 2008; Baumgartner *et al.*, 2009). The ecological role of the nematodes within the thrombolitic mats is unclear, however, previous studies have associated meiofaunal organisms such as nematodes with grazing and bioturbation (Walter & Heys, 1985; Farmer, 1992). The nematodes may be drawn to the thrombolitic mats through chemotaxis. Previous studies have shown that nematodes are attracted to volatile organic compounds produced by cyanobacterial-dominated biofilms (Höckelmann *et al.*, 2004), which may facilitate the nematodes search for nutrient-rich food sources within the mats. Although nematode activity may result in some bioturbation of the thrombolitic mats, the diversity and productivity results of this study suggest that the thrombolitic button mats are discrete communities that are distinctive from the adjacent stromatolites and are not simply ‘bioturbated stromatolites’ as was once proposed (Walter & Heys, 1985).

The lack of eukaryotic diversity in the Bahamian thrombolitic mats may be the result of two important factors. First, thrombolitic microbialites much like their stromatolitic counter parts are continually undergoing periodic burial events of oolitic sands, which can last for days, weeks or even months (Riding, 1991; Reid *et al.*, 2000). Previous studies of the adjacent stromatolites have shown that even after these lengthy burial events, cyanobacteria within the microbialitic mats reivate photosystem II upon exposure to oxygen and light (Kromkamp *et al.*, 2007; Perkins *et al.*, 2007). The photosynthetic eukaryotes detected on the surfaces of the stromatolites do not, however, recover from extended burial events (Perkins *et al.*, 2007). Second, the metabolic diversity of thrombolitic eukaryotes is far more limited than that found in the bacterial communities and would suggest more of a grazing, bacterivorous role in the thrombolitic mats. In contrast, the diverse nature of the bacteria recovered from the thrombolitic mats, suggest the ability to occupy a broader range of metabolic and biochemical niches within the thrombolitic microbialites.

Role of microbes in thrombolite accretion

Based on morphology, biochemical activity and microbial composition, the two characterized thrombolitic mat types exhibited pronounced differences from the three classic mat types of the adjacent stromatolites. In stromatolites, the three mat types reflect alternate stable states that fluctuate based on changing environmental conditions (Stolz *et al.*, 2009). The thrombolitic mat types, while different in structure and diversity, may also reflect stable states (May, 1973). The prevalence of each state may depend on the water temperature, tides, light flux, or other environmental conditions. The presence of...
a few *Dichotrix*-like sequences in the pink thrombolites may suggest an alternation between mat types. However, another possibility is that the two mat types may reflect a successive development of thrombolitic microbialites. The pink mats are typically located closer to the shore and may experience longer burial events than the button counterparts. Once exposed these pink mat surfaces, which are covered in an EPS-rich biofilm, may undergo similar trapping and binding events similar to that in Type 1 stromatolitic mats resulting in the subsequent colonization by the button mat ecotypes. Additional work will be needed to monitor growth and accretion of these communities and mat types and monitor spatial vari-
ances between button and pink populations.

Overall, the biochemical and diversity characteristics of the thrombolitic mats all suggest the accretion of these communities are primarily the result of bacterial activities and not euakaryotic. The limited metabolic range of the recovered eukaryotes to respiration and fermentation may restrict the euakaryotic contributions to thrombolitic mats formation. Together, these results suggest an alternative mechanism of thrombolite development in Highborne Cay, one that significantly differs previous eukaryote-centric models of thrombo-
lite formation. The coupling of a diverse consortium of bacteria with an abundant population of calcified filamentous cyanobacteria in the button mats confirms the biocentric role microbes play in thrombolite formation and lithification.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article:

**Table S1** Selected BLAST results of cloned ARISA intergenic spacer bands amplified from button thrombolitic microbial mats

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