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Bacterially mediated precipitation in marine stromatolites

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Summary

Stromatolites are laminated, lithified ($CaCO_3$) sedimentary deposits formed by precipitation and/or sediment accretion by cyanobacterial–bacterial mat communities. Stromatolites have been associated with these communities as far back as the Precambrian era some 2+ billion years ago. The means by which microbial communities mediate the precipitation processes have remained unclear, and are the subject of considerable debate and speculation. Two alternative explanations for microbially mediated precipitation include: (i) cyanobacterial photosynthesis increases pH in a system supersaturated in respect of $CaCO_3$, resulting in $CaCO_3$ precipitation and then laminated lithification, and (ii) decomposition of cyanobacterial extracellular organic matter (e.g. sheaths, mucilage and organic acids) by microheterotrophs leads to release of organic-bound Ca^{2+} ions and $CaCO_3$ precipitation. We evaluated these explanations by examining metabolically active, lithifying stromatolitic mat communities from Highborne Cay, Bahamas, using microautoradiography. Microautoradiographic detection of $^{14}CO_2$ fixation and ^3H organic matter (D-glucose and an amino acid mixture) utilization by photosynthetically active cyanobacteria and microheterotrophs, combined with community-level uptake experiments, indicate that bacteria, rather than cyanobacteria are the dominant sites of $CaCO_3$ deposition. In the oligotrophic waters in which stromatolites exist, microheterotrophs are reliant on the photosynthetic community as a main source of organic matter. Therefore, autotrophic production indirectly controls microbially mediated precipitation and stromatolite formation in these shallow marine environments.

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Introduction

Stromatolites are lithified, laminated, calcareous deposits formed by precipitation and/or trapping and binding of sediment by microorganisms. The upper layers of modern stromatolites contain a taxonomically and physiologically diverse, actively growing microbial community, dominated by cyanobacteria, heterotrophic and chemolithotrophic bacteria (Golubic, 1991; Paerl and Pinckney, 1996). Fossil stromatolites dating back more that 2.5 billion years are evidence that these structures may have represented some of the oldest life forms on Earth (Walter, 1983; Schopf and Packer, 1987). Modern-day analogues of these ancient microbial systems can be found in select tropical shallow environments, notably at Shark Bay, Australia (Logan, 1961) and the Bahamian islands (Monty, 1967; Dravis, 1983; Dill *et al*., 1986; Reid *et al*., 1995), which was the setting for this study.

Despite numerous studies on stromatolites during the past 100 years, mechanisms of stromatolite accretion are still poorly understood (Grotzinger and Knoll, 1999). Until recently, it was believed that precipitation of microcrystalline carbonate in stromatolites mainly resulted from high rates of photosynthetic activity (CO_2 fixation) of resident cyanobacteria (Riding, 1982; Pentecost and Riding, 1986). According to this scenario, photosynthetic CO_2 uptake leads to localized CO_2 depletion which, in turn, causes a shift in the inorganic C equilibrium (towards CO_3^{2-}), a concomitant rise in pH and deposition of CO_3^{2-}. This sequence of events leads to the production of layers of $CaCO_3$ in mat regions supporting the photosynthetically active community.

Cyanobacteria are not, however, the sole microbial constituents of microbial mats and recent work has suggested that they may not be the primary agents of precipitation. Microbial mats typically contain a productive, functionally and phylogenetically diverse heterotrophic bacterial community (Ramsing *et al*., 1993; Zehr *et al*., 1995; Steppe *et al*., 1996; Visscher *et al*., 1998). Microheterotrophs may be closely associated with cyanobacteria, either as attached epibionts or embedded in sheaths (Paerl *et al*., 1993). Recent studies suggest that this microheterotrophic community may play a far more important and direct role in $CaCO_3$ precipitation and lithification of stromatolites than previously assumed (Chafetz and Buczynski, 1992; Bartley *et al*., 2000; Reid *et al*., 2000). The proposed mechanism is as follows:

actively growing microheterotrophs utilize cyanobacterial exudates, including mucilage and sheath materials, as carbon and energy sources. These polysaccharide exudates are capable of binding Ca^{2+} ions (Decho, 1990; 2000), thereby preventing their precipitation as $CaCO_3$. As the exudates are metabolized by microheterotrophs, Ca^{2+} is released in solution, facilitating $CaCO_3$ precipitation. If operative, this process should be associated with actively growing heterotrophic microorganisms, serving as the sites of localized $CaCO_3$ deposition (Westbroek *et al*., 1994; Stal, 2000).

In the present study, microautoradiography was used to microscopically detect and visualize metabolically active microbes in stromatolitic mats. We observed both heterotrophic and autothrophic (photosynthetic) components of the community in relation to precipitation and lithification patterns in mats. This enabled us to evaluate the proposed mechanisms of biotic precipitation and lithification. Implications for marine stromatolite formation are discussed.

Results

Description of stromatolitic mats

The Highborne Cay stromatolitic mats consist of several coloured laminae, each dominated by a distinct microbial assemblage (Fig. 1). The mat surface was comprised of a firm, caramel-coloured layer approximately 0.5 mm thick. This layer was dominated by condensed polysaccharides (Decho, 2000), which were presumed to be mucus and sheath materials produced by the dominant filamentous cyanobacterium *Schizothrix* sp. In a few instances, intact *Schizothrix* filaments were observed inside sheaths. Other cyanobacteria included *Oscillatoria* spp., *Lyngbya* sp. and aggregated coccoid forms (*Synechococcus, Synechocystis*). Pennate diatoms (e.g. *Navicula* spp.) were present in this layer, but diatom density was quite low ($<$ 10% of autotrophic biomass, determined microscopically). This layer was rich in bacteria, including numerous rod, filamentous and coccoid forms. Bacteria were frequently associated with mucilaginous cyanobacterial sheaths as well as carbonate sand grains that were incorporated in the layer.

Immediately below the surface layer was a light green layer ranging from $\approx$0.1–0.3 cm in width (Fig. 1). This layer was largely comprised of actively growing *Schizothrix* filaments in sheaths (Fig. 2). Other cyanobacterial genera included non-heterocystous types (*Lyngbya*, *Oscillatoria*) and heterocystous types (*Dichotrix and Calothrix*) (Fig. 2). Some pennate diatom species were also observed. Bacteria were abundant and widely distributed, with coccoid, rod-shaped and filamentous bacteria associated with cyanobacterial filaments and sheaths. Most bacteria were non-pigmented, based on the absence of colour and detectable fluorescence when examined microscopically, as well as a lack of high-performance liquid chromatography (HPLC)-determined bacteriochlorophylls (Pinckney and Paerl, 1997). A substantial number of filamentous, rod-shaped and coccoid bacterial cells were closely associated with fine precipitates (Fig. 2). In contrast, cyanobacterial filaments and sheaths were virtually free of these precipitates (Fig. 2). They were assumed to be aragonite, based on scanning electron microscopy (SEM) observations of needle-shaped morphology and energy-dispersive X-ray elemental analyses of equivalent samples (R. P. Reid, unpublished data).

Fig. 1. Top: view of the stromatolites in the intertidal zone (W. Atlantic Ocean) of Highborne Cay, Exumas Islands, Bahamas. Middle: cross-section of a stromatolite obtained from Highborne Cay. Note the laminated layers distinctive of stromatolitic deposits. Bottom: photomicrograph ($\times$ 400) showing the cyanobacterial (*Schizothrix* sp.) filaments comprising the 'fabric' of green laminae in the stromatolite shown above.

Microautoradiographic examinations

The surface and subsurface mat layers were examined by

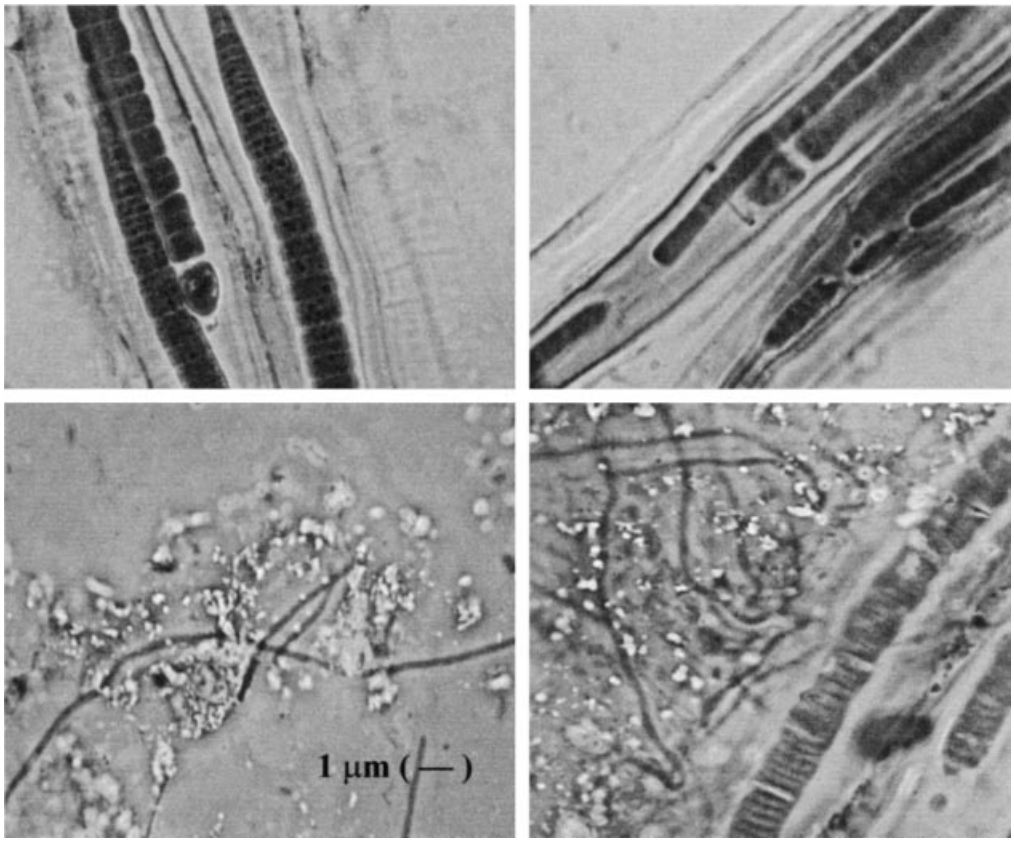

Fig. 2. Photomicrographs of the dominant microbial constituents of microbial mat layers of the Highborne Cay stromatolites. Top left: heterocystous filamentous cyanobacteria (*Dichothrix* sp.) and their surrounding mucilaginous sheath. Note the absence of $CaCO_3$ precipitates in the sheaths. Top right: non-heterocystous filamentous cyanobacteria (*Schizothrix* sp.). This cyanobacterium also has a distinct sheath, which is devoid of precipitates. Bottom left and right: filamentous and rod-shaped bacteria commonly associated with cyanobacteria. Note the presence of fine $CaCO_3$ precipitates associated with the bacterial cells, but absent around the cyanobacterial filament (lower right) to which the bacteria are attached.

microautoradiography, enabling us to determine shape, location and immediate microenvironments surrounding metabolically active bacteria and microalgae that had incorporated radiolabelled heterotrophic and autotrophic substrates (Fig. 3). ^{3}H-glucose and amino acid incorporation was widely distributed among bacterial morphotypes (Fig. 3). There were differences in silver grain density (exposure) and, hence, differences in uptake among members of the bacterial community. In general, maximum uptake was present in cells that also had fine $CaCO_3$ precipitates associated with them. Much lower amounts of uptake of these organics were associated with either cyanobacteria or eukaryotic microalgae in the mat; this indicated that bacteria were the prime utilizers of these heterotrophic compounds. ^{14}C-HCO_3 uptake was almost exclusively observed among cyanobacterial and eukaryotic microalgal components of the mat. In the caramel surface layer, *Schizothrix* filaments and diatoms were actively engaged in ^{14}C uptake. Photosynthetically active (i.e. ^{14}C-labelled) *Schizothrix* filaments were observed associated with $CaCO_3$ deposits; however, these deposits were located outside the mucilaginous sheaths (Fig. 4). Frequently, bacteria were found embedded in these deposits (Fig. 5). On several occasions, purple-pigmented bacteria were labelled with ^{14}C. Based on morphological observations, these bacteria resembled members of the genus *Chromatium.*

Liquid scintillation counts revealed that the addition of DCMU (3(3,4-dichlorophenyl)-1,1-dimethylurea) partially inhibited the uptake of ^{14}C-HCO_3 (Fig. 6). Microautoradiographs revealed that uptake was inhibited among both filamentous and coccoid cyanobacteria, while no reduction in uptake was observed among purple-pigmented bacteria. DCMU did not inhibit the uptake of either ^{3}H-amino acid mix or glucose (Fig. 6).

Based on phase-contrast microscopic observations of microautoradiographs, metabolically active (both ^{3}H-glucose and amino acid utilizing) heterotrophic bacteria proved to be the most common sites of $CaCO_3$ precipitation. Precipitates were present around bacteria either located in the amorphous mucilaginous mat matrix or more closely associated with cyanobacterial filaments.

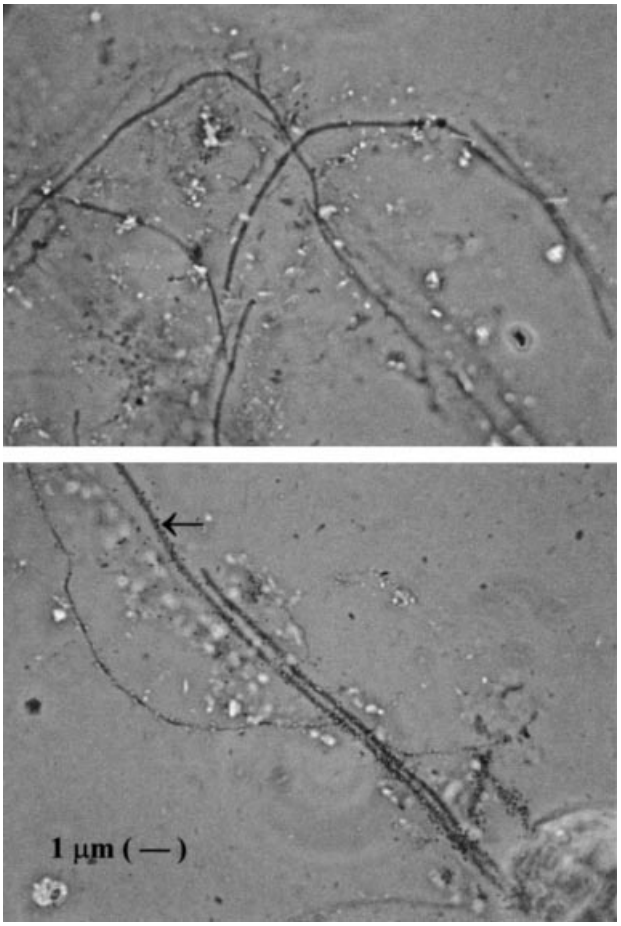

Fig. 3. Heterotrophic bacteria present in the surface mat matrix. These filamentous bacteria were capable of utilizing both glucose and an amino acid mix (note very fine silver dark grains, indicating radiolabelling, superimposed on the filament in the bottom frame and indicated by an arrow). Also present are the bright $CaCO_3$ precipitates commonly associated with these bacteria.

The filamentous non-heterocystous mat cyanobacteria, particularly *Schizothrix*, *Lyngbya and* some heterocystous genera (*Calothrix*, *Scytonema*, *Dichothrix*), made up a bulk of the phototrophic biomass in either the surface or subsurface layers. While they were photosynthetically active, cyanobacterial filaments were free of precipitates (Fig. 7). Well-defined sheaths and amorphous mucilagenous materials covering the filaments were also free of precipitates (Figs 2 and 7). Filamentous and coccoid bacteria that were embedded in this mucilaginous material were, however, frequently associated with fine $CaCO_3$ deposits (Fig. 7). These bacteria were metabolically active, as judged by ^{3}H-glucose and amino acid uptake.

Discussion

Metabolically active heterotrophic bacteria were major sites of $CaCO_3$ deposition in stromatolitic mats. These bacteria were morphologically and, in all likelihood, metabolically diverse. Although no efforts were made to isolate physiologically distinct populations, it is possible that several heterotrophic groups, previously identified as dominant microheterotrophs, were involved in the calcification process. These could potentially include: obligately and facultatively aerobic bacteria such as *Vibrio* spp., *Klebsiella* spp. and *Azotobacter* spp. (Zehr *et al.*, 1995; Olson *et al.*, 1999), facultative phototrophs such as *Rhodospirillum* spp. and *Rhodobacter* spp. (Steppe *et al.*, 2000, and obligate anaerobes including sulphate-reducing bacteria such as *Desulfovibrio* spp. and *Desulfobacter* spp. (Risatti *et al.*, 1994; Krekeler *et al.*, 1997; Visscher *et al.*, 1998). In addition, numerous, hitherto unidentified, filamentous and coccoid bacteria could have played a role in the observed calcification process. Work is currently underway to combine molecular characterization (probing) techniques with microautoradiography in

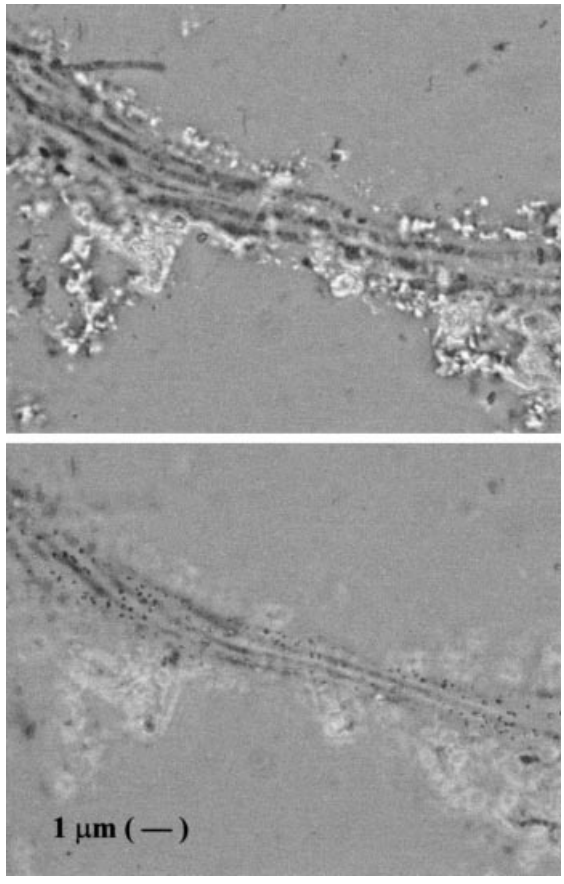

Fig. 4. Photomicrograph/microautoradiograph of *Schizothrix* sp. filaments residing in mucilaginous sheath. Sample is from the same material as described in Fig. 3. The sample was incubated with $NaH^{14}CO_3$ and examined for photosynthetic ^{14}C incorporation. Top: phase-contrast view, showing $CaCO_3$ deposits outside of the sheath. Bottom: phase-contrast view in a different focal plane, showing the exposed (dark) silver grains superimposed over the individual *Schizothrix* sp. filaments residing in the sheath.

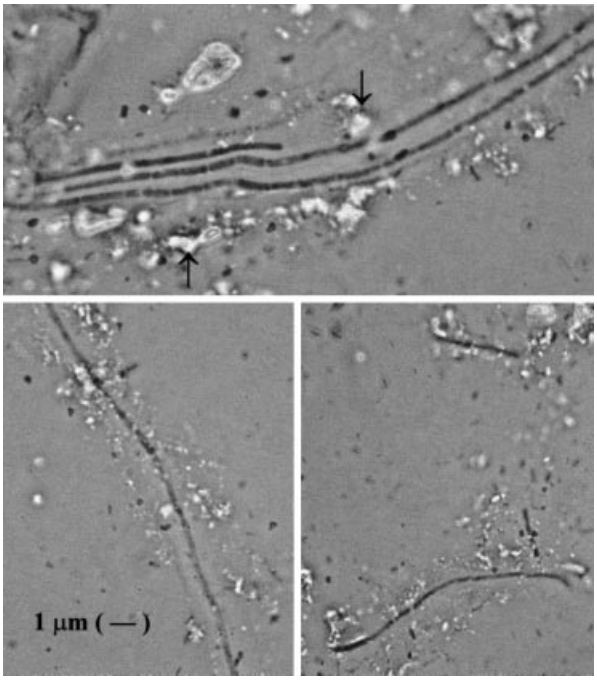

Fig. 5. Photomicrograph of rod-shaped and filamentous heterotrophic bacteria associated with *Schizothrix* sp. sheaths. Top: low magnification (×400) view, showing *Schizothrix* sp. filaments encased in sheaths and surrounded by bacteria and $CaCO_3$ precipitates (arrows). Bottom: higher magnification (×1000) view of same sample in which the epiphytic bacteria were separated from *Schizothrix* sp. bundles by mild sonication. Note fine $CaCO_3$ deposits closely associated with these bacteria.

order to more directly assign function to specific taxa in the complex microbial assemblages observed.

There are larger-scale, biogeochemical implications of these findings. If adequate supplies of organic matter are available, microheterophic utilization of these compounds should enhance the precipitation process. On a community 'net metabolism' basis, this means that periods of relatively high heterotrophic activity and metabolism (compared with autotrophy) should favour precipitation in these mat systems. Macro-scale observations at these field sites appear to substantiate this conclusion. For example, when stromatolitic mats are dominated by cyanobacterial communities during periods of rapid sediment accretion, associated lithification is minimal (Reid *et al*., 2000). Conversely, during periods when heterotrophic metabolism dominates C flow, as is the case when surface biofilms develop during interruptions in sediment accretion, massive precipitation events form micritic crusts (Reid *et al*., 2000).

While our results point to the importance of heterotrophically mediated $CaCO_3$ precipitation, this process is ultimately dependent on supplies of organic matter for calcification to proceed. Restated, without organic matter as the 'fuel', heterotrophic $CaCO_3$ precipitation could not proceed. In the oligotrophic waters in which stromatolites are found, external sources of organic matter are virtually absent, placing a heavy reliance on resident phototrophs to supply this 'fuel'. Virtually all the organic matter available for heterotrophic processes, as well as other metabolic processes, such as N_2 fixation, essential for maintaining fertility of this system, is supplied by cyanobacteria and microalgae residing in the surface mats (Bebout *et al*., 1993; Steppe *et al*., 2000). Therefore, rates of primary production by this phototrophic community are of fundamental importance for sustaining and promoting bacterial precipitation.

On the ecosystem level, precipitation depends on relatively high rates of primary production accompanied by locally intensive periods of heterotrophic activity. Therefore, the extent and magnitude of precipitation are ultimately dependent on autotrophic production. However, rather than exclusively involving autotrophs, the precipitation mechanisms are biologically complex and indirect, involving close metabolic coupling of a metabolically diverse microbial community, including cyanobacterial phototrophs and their heterotrophic consorts (Paerl and Pinckney, 1996). This also means that natural or human-induced environmental perturbations, including climatic, nutritional and habitat alterations, will have an impact on a wide spectrum of metabolically diverse and interacting microbes implicated in precipitation.

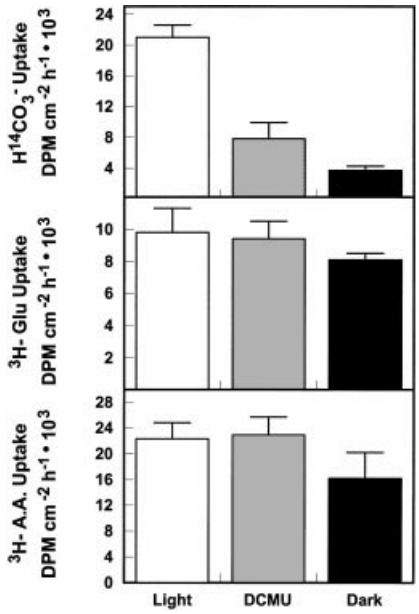

Fig. 6. Uptake of radiolabelled $^{14}CO_2$ (as $NaH^{14}CO_3$) (top), ^{3}H-glucose (Glu) (middle) and amino acid mixture (A.A.) (bottom) by the stromatolitic microbial mat community of Highborne Cay, Bahamas. Uptake is as DPM per cm^2 of mat h^{-1}. Samples were incubated under illuminated (light), DCMU-amended (DCMU) or dark conditions. Error bars show the standard deviation (SD) of quadruplicate samples for each treatment.

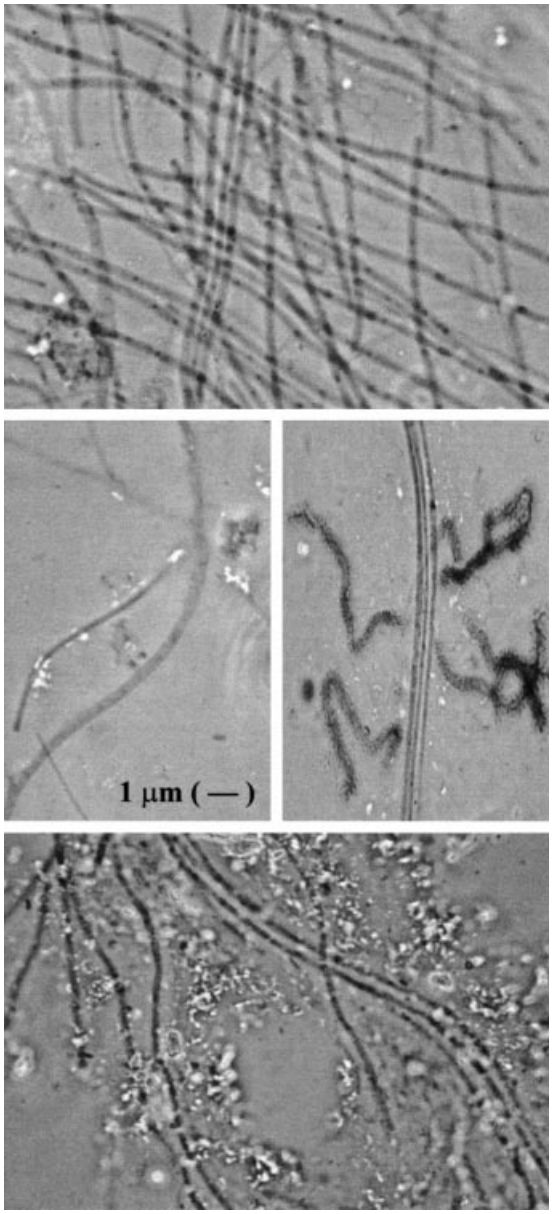

Fig. 7. Photomicrographs showing the *Schizothrix* sp. mat 'fabric' (top, ×200), associated filamentous bacteria and $CaCO_3$ precipitates (middle left), a microautoradiograph (middle right) showing heavily labelled (^{3}H-glucose uptake) filamentous bacteria associated with *Schizothrix* sp., and a high magnification view (×1000, bottom) of filamentous bacteria and closely associated $CaCO_3$ precipitates that were found in the mucilaginous matrix between the *Schizothrix* sp. filaments.

Experimental procedures

Research site

Stromatolite samples for this study were were collected at Highborne Cay, a small island near the north end of the Exuma Cays, Bahamas, approximately 50 km south-east of Nassau. Shallow subtidal and intertidal stromatolites are present in the lagoon of a fringing reef on the windward, eastern margin of Highborne Cay (Reid *et al.*, 1999).

Sampling and incubation procedure

Stromatolite pieces, ranging from 0.5 to 1 cm^2, were excised by scalpel or cored form the mat and incubated with various radiolabelled heterotrophic and photosynthetic substrates, all at trace concentrations. The substrates included: 6-^{3}H-D glucose (40 Ci mCi $mmol^{-1}$; ICN Radiochemicals Cat. no. 27020) added at 0.5 μCi ml^{-1} (2), ^{3}H-L-amino acid mix (210 mCi $mmol^{-1}$; ICN Radiochemicals Cat. no. 20063) added at 0.41 μCi ml^{-1} (3) and ^{14}C-Na HCO_3 (58 mCi mol^{-1}; ICN Radiochemicals Cat. no. 17441H) added at 0.35 μCi ml^{-1}. Dissected mat pieces were placed in 20 ml glass scintillation vials that were filled with ambient seawater. Radiolabelled substrates were then added to triplicate treatments which included illuminated and dark incubations, as well as illuminated $+2 \times 10^{-5}$M 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). The DCMU (Pfaltz and Bauer) was initially dissolved in 1 ml of 95% ethanol which was then diluted with 100 ml of deionized water to make a 10^{-2}M stock solution, which was then diluted in the seawater samples. Parallel DCMU-free additions containing ethanol only were run to test for the possible effects of ethanol; no observable differences between ethanol additions and ethanol-free controls were consistently observed. The DCMU treatment was carried out to examine photoheterotrophy and anoxic photosynthesis, neither of which are affected by this photosystem II inhibitor (Paerl, 1991; Pinckney and Paerl, 1997). In addition, chemical C precipitation, as $CaCO_3$, is possible and distinguishable (from photosynthetic CO_2 fixation) in DCMU-amended samples.

All samples were incubated in a circulating seawater bath maintained under natural irradiance and light on the deck of the R/V Calanus. Incubations lasted from 1 to 6 h and were terminated by the addition of 1.5% w/v seawater/borate-buffered formalin, after which samples were rinsed three times in filtered seawater (10 min per rinse) to remove unincorporated radioisotope. Mat samples were then sectioned and processed for liquid scintillation counting (LSC) and microautoradiography. For LSC, mat samples were air-dried and (in the case of $^{14}CO_2$ fixation) placed in an atmosphere of fuming concentrated HCl for 3 h to remove abiotically precipitated ^{14}C. Samples were then vented and placed in a tissue-solubilizing, biodegradable cocktail (e.g. Cytoscint; ICN). Quenching (owing to pigments, humic and other coloured substances in sediments) was corrected for by developing a quench curve using various amounts of unlabelled sediments amended with known quantities of calibrated ^{14}C-hexadecane or toluene (New England Nuclear).

Microautoradiography

Microautoradiography was used to visualize radiolabelled microorganisms in mat samples (Paerl, 1974). For details, see Paerl *et al.* (1993). Briefly, following radioisotope incubation, formalin-fixed samples were rinsed with 10–30 ml 0.01 M phosphate-buffered saline (PBS) to remove salts, while avoiding lysis of fragile cells. Washed and slurried mat samples were then gently settled onto 25 mm HA Millipore filters. Filters were then air dried for at least 4 h and optically cleared by placing them, face up, on clean microscope slides which were passed over the mouth of a 250 ml beaker

containing 50 ml of boiling (fuming) acetone. This step optically cleared the filters while attaching them to slides.

The following steps were conducted under complete darkness: slides containing attached filters were dipped in Kodak NTB-2 nuclear track emulsion, which was diluted 1:1 with deionized water and held at 40°C. Slides were vertically positioned in a slide holder for 20 min to allow them to partially dry. They were then placed in light-tight desiccant-containing slide boxes for 2 to 10 d to allow radioexposure of the emulsion.

Microautoradiographs were developed in Kodak D-19 for 2 min, transferred to a stop bath for 1 min, fixed for 5 min in Kodak rapid fixer and rinsed in gently flowing water for 15 min. Slides were then air dried overnight. Exposed microautoradiographs were observed using phase-contrast microscopy at ×400–1000, using a Nikon Optiphot microscope equipped with oil immersion achromatic objectives. Photomicrographs were recorded on Ilford Pan-F ultra fine grain film, which were then digitized.

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