A hypersaline microbial mat from the Pacific Atoll Kiritimati: Insight into composition and carbon fixation using biomarker analyses and a ^{13}C-labeling approach

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Abstract

Modern microbial mats are widely recognized as useful analogs for the study of biogeochemical processes relevant to paleoenvironmental reconstruction in the Precambrian. We combined microscopic observations and investigations of biomarker composition to investigate community structure and function in the upper layers of a thick phototrophic microbial mat system from a hypersaline lake on Kiritimati (Christmas Island) in the Northern Line Islands, Republic of Kiribati. In particular, an exploratory incubation experiment with ^{13}C-labeled bicarbonate was conducted to pinpoint biomarkers from organisms actively fixing carbon. A high relative abundance of the cyanobacterial taxa *Aphanocapsa* and *Aphanothece* was revealed by microscopic observation, and cyanobacterial fatty acids and hydrocarbons showed ^{13}C-uptake in the labeling experiment. Microscopic observations also revealed purple sulfur bacteria (PSB) in the deeper layers. A cyclic $C_{19:0}$ fatty acid and farnesol were attributed to this group that was also actively fixing carbon. Background isotopic values indicate Calvin-Benson cycle based autotrophy for *cyc*$C_{19:0}$ and farnesol producing PSBs. Biomarkers from sulfate reducing bacteria (SRB) in the top layer of the mat and their ^{13}C uptake patterns indicated a close coupling between SRBs and cyanobacteria. Archaeol, possibly from methanogens, was detected in all layers and was especially abundant near the surface where it contained substantial amounts of ^{13}C label. Intact glycosidic tetraether lipids detected in the deepest layer indicated other archaea. Large amounts of ornithine and betaine bearing intact polar lipids could be an indicator of a phosphate-limited ecosystem, where organisms that are able to substitute these for phospholipids may have a competitive advantage.

Introduction

Modern cyanobacterial mats are prevalent in diverse environments including desert crusts, coastal lagoons, hot springs and aquatic hypersaline settings (e.g., Potts, 1994; Jonkers *et al.*, 2003; Jahnke *et al.*, 2001; Wieland *et al.*, 2003). Extreme environmental conditions suppress the activity of grazing organisms (e.g., Cornee *et al.*, 1992; Fenchel, 1998) and enable development of mats that may reach gigantic proportions taking the minute scale of their main contributors, cyanobacteria, into account. Phototrophic microbial mats are characterized by daily fluctuations in redox status due to the physiology of the cyanobacteria and associated microorganisms (Canfield *et al.*, 2005). Diatoms are prevalent in the surface layers of many modern mats, possibly because they are well adapted to high irradiance during the day. Oxygen supersaturation is common within and above mats during daytime owing to high rates of photosynthesis (Jørgensen *et al.*, 1983). At night oxygen demand from cyanobacterial respiration and heterotrophy often results in anoxia (Fenchel, 1998). Anoxygenic phototrophs have been found at the oxygen-sulfide interface under certain light conditions (Pinckney & Paerl, 1997). Sulfate reduction is usually prevalent supplying sulfide for sulfide-oxidizers above (Canfield & Des Marais, 1991). Deeper in the mat cyanobacterial cell density decreases substantially and oxidation of sulfide and ammonia from underlying sediment dominate (Canfield *et al.*, 2005).

Cyanobacteria are widely considered the first oxygen-producing phototrophs on Earth and are responsible for oxygenating the atmosphere and oceans (e.g., Fischer, 1965; Holland, 2006). They represent a large fraction of marine primary production today (Lochte & Turley, 1988), and most likely since they evolved some 3.5 Ga (Summons *et al.*, 1999; Schopf, 2006; Knoll *et al.*, 2007). The timing of the inception of oxygenic photosynthesis is both uncertain and controversial. However, there is evidence in the form of stromatolites that complex microbial communities, possibly including cyanobacteria, existed in shallow seas as early as 3.5 Ga ago

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(Allwood *et al.*, 2006; Altermann *et al.*, 2006) and that, by the Neoarchaean (2.7-2.5 Ga), they were principal builders of wide carbonate platforms (Knoll, 2003). Living microbial mats can therefore be useful as modern analogues for early stromatolites to examine ancient processes such as their potential role in the production of reduced gases on early Earth (Hoehler *et al.*, 2001), quantification of oxygen and carbon cycling with respect to salinity (Canfield *et al.*, 2004) or the role of anoxygenic phototrophs on calcification (Bosak *et al.*, 2007).

Lipid analyses of microbial communities (e.g., Rajendran *et al.*, 1992; Rütters *et al.*, 2002) and their fossil counterparts (e.g., Summons *et al.*, 1996) offer valuable insights into microbiological diversity. The Archaean sedimentary record suffers from the scarcity of preserved and recognisable microbial remains (Altermann, 2004), so molecular fossils are a valuable source of information (e.g., Brocks *et al.*, 1999; Brocks & Summons, 2003). The in-depth study of the biomarker structure is therefore necessary to improve recognition of source organisms and interpretation of the early sedimentary record. The information gained from identifying biomarkers and their distributions can be further increased by simultaneous determination of the stable carbon isotope composition of these lipids. Natural abundance studies take advantage of the small difference in isotope ratios found in nature (Peterson, 1999; Hayes, 2001), due to the fact that enzyme-catalyzed reactions discriminate against ^{13}C. Stable isotopic data can be useful for gaining inferences about ancient biochemistries (e.g., Schidlowski *et al.*, 1983), in identifying trophic relationships and in determining the mode of carbon fixation (e.g., Hayes, 2001). Additionally, ^{13}C-labeled substrates can be used to decipher carbon flows in microbial systems. In such studies, a portion of the stable isotope tracer is incorporated into the biomass of organisms that actively metabolize the labeled substrate (e.g., Middelburg *et al.*, 2000; van der Meer *et al.*, 2007).

In this study, a hypersaline microbial mat system from a lake on the central Pacific island of Kiritimati was investigated for microscopic structure and lipid biomarkers including their C-isotopic compositions. A simple labeling experiment using ^{13}C-bicarbonate allowed us to identify carbon flow into lipid biomarkers. Our motivation was to characterize carbon fixation and turnover and to pinpoint lipids with potential to be specific for different groups of organisms and their physiologies.

Material and Methods:

Kiritimati Island

Kiritimati (01°52'N, 157°20'W) lies within the Northern Line Islands of the Republic of Kiribati (Figure 1a.). It is the largest coral atoll in the world with a surface area of ~360 km^2. Approximately one quarter of Kiritimatis surface is covered by brackish to hypersaline lakes, some of which connect to a large lagoon in the northwestern part of the island (Figure 1b.). The majority of these lakes represent basins of evaporating seawater trapped following a high sea level in the mid-Holocene (Valencia, 1977; Woodroffe & McLean, 1998). Even though Kiritimati experiences a large variability in precipitation (Saenger *et al.*, 2006 and references therein) that can change the lake salinities dramatically over short time scales, the overall climate is evaporative and microbial mats are well developed in many of the islands' lakes. Recharge of the lakes and ponds occurs primarily during El Niño Southern Oscillation (ENSO) events, when torrential rains and higher sea levels can cause flooding.

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Environmental setting

Figures 1c and 1d show the position and a panorama of Lake 2A on the Island of Kiritimati, covering an area of 0.02 km^2 and holding a maximum depth of 20 cm. Microbial mats were well

laminated (Figure 1e), easily accessible and had a uniform appearance over a large area. Salinity, pH and dissolved oxygen (DO) were measured using a portable YSI Sonde 6600 connected to an YSI 650 MDS data logger. On the day of sampling, the temperature varied around 30°C, the pH of the water was 7.9 and the salinity 116. The water was oxygen-supersaturated (105% in the morning and 126% in early afternoon); the DO was 3.8 mg L^{-1} at 9 am and 4.8 mg L^{-1} at 1 pm. The top 5-7 cm comprised the actively growing microbial community (Figure 1e), underlain by approximately 80 cm of sediment, rich in calcite and halite, above a carbonate hard ground.

Labeling Experiment

Representative mat sections, 7 cm deep with an area of approx. 100 cm^2, were placed in plastic containers of 1 L filled with lake water. Sodium bicarbonate (^{13}C: 99%, Cambridge Isotope Laboratories, Inc) was pre-dissolved in a few ml of distilled water and added to the incubations such that the final concentrations reached approximately 200 µM. Following duplicate labeled incubations of 6 hr duration, and a control experiment in which no label was added, the mat edges were excised to exclude the possibility of lateral incorporation and the samples were sectioned into four layers on the basis of color and consistency. These comprised an upper layer (~5 mm) of orange, fluffy-gelatinous organic material with halite crystals, a second layer (~5-15 mm) of denser green/orange biomass, also mixed with halite, a third layer (~15-40 mm) of solid gelatinous, orange-purple biomass and a fourth (~40-60 mm) layer of solid gelatinous yellow/orange material. Distinction between the layers was incomplete because of diffuse boundaries and surface irregularities. Sections were then sealed in plastic bags, frozen and transported to MIT in a cooler. Inspection of samples after arrival showed that these were partially thawed. However, there is no evidence that the lipid distribution was altered by degradation. We also attempted a killed control experiment adding Lugol's solution (I_2 / KI) to

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the overlying water, where considerable label uptake at the end of the experiment revealed that it was unsuccessful.

Only layer 1 was used for investigating ^{13}C-uptake into lipid biomarkers, because almost no label was detected in layer 2.

Microscopy

Microscopy was performed with a Zeiss Axioimager A1 microscope (Carl Zeiss GmbH, Göttingen, Germany) and Zeiss Axiocam MRc Imaging system and software. Enumeration was performed at 40x magnification. Identification of morphotypes was in accordance with traditional phycological manuals (Geitler, 1932; Komárek & Anagnostidis, 1999; Komárek & Anagnostidis, 2005) and Bergey's Manual of Systematic Bacteriology (Castenholz, 2001) as well as by consulting current published work on microbial ecology (Campbell & Golubic, 1985; Garcia-Pichel *et al.*, 1998; Richert *et al.*, 2006).

Lipid extraction, derivatization and detection

Samples were freeze dried prior to extraction, spiked with internal standard (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine; PAF), and were extracted ultrasonically using the method for intact polar lipids (IPLs) after Sturt *et al.* (2004). Lipids were further separated on 2 g silica columns (deactivated with 5% water) using 15 ml hexane, 18 ml hexane/DCM (2:1), 18 ml DCM/acetone (9:1), and 20 ml methanol to yield hydrocarbons, ketones + esters, alcohols, and polar lipids, respectively.

The polar lipids were transesterfied over night at 70°C using 1 ml of acetyl chloride and 9 ml of anhydrous methanol under a nitrogen atmosphere. Alcohols were converted to trimethylsilyl (TMS) ether derivatives with 50 µl BSTFA (bis-trimethylsilyltrifluoroacetamide) and 100 µl pyridine at 80°C for 1 h.

Compound quantifications and identifications were performed using Agilent 6890 Series II gas chromatographs, one equipped with a flame ionization detector (GC-FID), the other equipped with an Agilent 5973 Mass Selective Detector (GC-MSD) operated at 70 eV. Both instruments used programmable temperature vaporization (PTV) inlets, 60 m Varian Chrompak CP-Sil 5 capillary columns (0.32 mm i.d.; 0.25 μm film thickness), and helium as the carrier gas. For GC-MSD, conditions were as follows: injection at 60ºC while the oven was held at the same temperature for 1 min. Subsequently, the oven temperature was raised to 150°C (120°C) at 10°C min^{-1} (20°C min^{-1}) followed by a temperature increase of 4°C min^{-1} to 320°C, and an 15 (25)-min isothermal period for fatty acids/alcohols (for hydrocarbons). Quantification was conducted using internal standards: nonadecanoic acid, nonadecanol, and *n*-hexatriacontane. GC-FID conditions were the same as for GC-MSD. Identification was performed by comparison of mass spectra with those reported in the literature, while in some cases use was also made of relative retention times, as for *cyc*$C_{19:0}$.

Isotopic composition of fatty acids, alcohols and hydrocarbons were determined using coupled gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). At Woods Hole Oceanographic Institution, an Agilent model 6890 GC equipped with a DB-5 (60 m, 0.32-mm inner diameter, and 0.25-μm film thickness column was coupled via a Combustion Interface III to a ThermoFinnigan Delta Plus mass spectrometer (ThermoFisher, Bremen, Germany). At MIT, a similar set-up comprised a ThermoFinnigan Trace GC equipped with a J&W DB-5MS column (60m x 0.32 mm, 0.25 mm film) and coupled to a combustion furnace interfaced to a Finnigan MAT DeltaPlus XP isotope ratio monitoring mass spectrometer. Both were operated with Isodat 2.0 software. Chromatographic conditions were initially 60ºC for 2 min, then 60-320ºC at 6 or 8ºC min^{-1}. Samples were analyzed in duplicates. The accuracy of

isotope results was monitored routinely with standards (obtained from A. Schimmelmann, Indiana University) and found to be 0.3‰ or lower. Sample replicates produced standard errors less than 0.5‰ vs. VPDB. Isotope results were corrected for the introduction of the additional carbon atoms during derivatization with acidic methanol or BSTFA (Abrajano et al., 1994). Internal standards for quantification were added prior to extraction.

HPLC-MS analysis was performed at the University of Bremen using the same settings as described by Sturt *et al.* (2004). Relative concentrations of IPLs were calculated based on MS response of molecular ions relative to that of known amounts of the internal standard. The values of the phosphatidylglycerol (PG), phosphatidylethanolamine (PE), Monoglycosyldiacylglycerol (1Gly-DG), Phosphatidylcholine (PC), and glyceroldialkylglyceroltetraether (2Gly-GDGT) were corrected for their response factor determined using commercially available standards. Lack of authentic standards for some IPL types prevented determination of the response factor, therefore we used an average value for typical IPLs.

Bacteriohopanepolyols (BHPs) were prepared via acetic hydrolysis with acetic acid in methanol (0.1 vol%) for 1 h at 70 °C and subsequent acetylation and analyses via GC-MS, applying the method of Talbot & Farrimond (2007).

Data analysis

Carbon isotopic ratios (^{13}C / ^{12}C) are expressed in the delta notation ($\delta^{13}C$) relative to Vienna Pee Dee Belemnite Standard (^{13}C / $^{12}C_{VPDB}$ = 0.0112372=R_{VPDB}): $\delta^{13}C$ (‰) = [(R_{sample} / R_{std})-1)] × 1000, where R_{sample} and R_{std} are the ^{13}C / ^{12}C of sample and standard (Craig, 1957). Incorporation as ^{13}C is reflected as excess (above background samples) ^{13}C and is expressed in terms of total uptake (*I*) as well as specific uptake (i.e. $\Delta\delta^{13}C = \delta^{13}C_{sample} - \delta^{13}C_{control}$) after Middelburg *et al.*, 2000). Total uptake *I* of ^{13}C in lipids was calculated as the product of excess ^{13}C (*E*) and

concentration of the respective compound. *E* is the difference between the fraction *F* of the sample and background: $E = F_{sample} - F_{background}$, where $F = {}^{13}C / ({}^{13}C/{}^{12}C) = R / (R + 1)$ and $R = (\delta^{13}C / 1000 + 1) \times R_{VPDB}$.

Results

Microscopic observations

Visual impression of the microscopic observations is given in Figure 1f-g. The microscopic observations are given as abundance scores from 0 to 5 for the different groups in Figure 2 (0 – not present; 1 – 0-10%; 2 – 10-25%; 3 – 25-50%; 4 – 50-75%; 5 – 75-100%). In the top layer the cyanobacterium *Aphanocapsa* sp. was the largest contributor in terms of abundances. We also found considerable amounts of *Aphanothece* spp. and the diatom *Nitzschia* sp. Minor amounts of the cyanobacterium *Entophysalis* sp. and the filamentous cyanobacterium *Leptolyngbya* were present, mostly as trichomes inside a distinct sheath. In the second layer *Entophysalis* and *Aphanocapsa* dominated, followed by *Aphanothece* and *Leptolyngbya*. In the deeper layers empty sheaths of *Leptolyngbya* were abundant, possibly due to higher resistance to degradation. Microscopically, purple sulfur bacteria (PSB) could only be observed in the third and fourth layer. In the third layer the PSBs were accompanied by *Aphanocapsa*, whereas in the fourth layer, beside *Aphanocapsa* and PSBs, filaments of *Leptolyngbya* were also abundant.

Fatty acid composition

Compound compositions of the different layers are given in Table 1 and Figure 3.

The phospholipid-derived fatty acids (PLFAs) were clustered into eight groups (Figure 3a.). In the first layer 28% of the PLFAs were saturated, followed by 19% for $C_{16:1\omega7} + C_{18:1\omega9}$ PLFAs, whereas only 3% can be attributed to *cyc*$C_{19:0}$. In the second layer the saturated fatty acids were

the most abundant group (39%), followed by $C_{16:1\omega7}$ + $C_{18:1\omega9}$ (16%). *Cyc*$C_{19:0}$ increased to 7% of the total. In the third and fourth layers the saturated fatty acids (45% and 38%) were again the most important group of PLFAs. *Cyc*$C_{19:0}$ gained greater importance in the third layer, reaching 9% of the fatty acids. In the fourth layer the *iso*-/*anteiso*- PLFAs reached 22%.

The natural abundance isotopic compositions ($\delta^{13}C$) of the fatty acids from the first layer were mainly between −20 and −24‰ (Figure 4a.). The fatty acids $C_{16:1\omega7}$, *cyc*$C_{17:0}$, and $C_{18:1\omega7}$ were slightly heavier with values around −18‰. The heaviest value was detected for $C_{16:1\omega5}$ (−16.3‰).

Alcohol composition

The distribution of alcohols is displayed in Figure 3b. In the first layer the saturated alcohols accounted for 33% of the total alcohols, followed by archaeol (bis-*O*-phytanylglyceroldiether) with 28% (Figure 3b.). In the deeper layers saturated alcohols became more prominent, reaching 66% of the alcohols. Different sterols (cholesterol, ergosterol, sitosterol) and their saturated counterparts were found in varying amount (between 2 and 8%) in each layer. Farnesol was present in minor amounts in all layers, reaching its maximum with ca. 5% in the third layer.

$\delta^{13}C$ values of the alcohols in the first layer were between −20 and −24‰ for most of the investigated components (Figure 4b.). Some components were more depleted in ^{13}C including one OH-$C_{17:1}$ isomer (−27.9‰), OH-$C_{18:0}$ (−25.8‰) and diplopterol (−30.3‰). Sitosterol and sitostanol were slightly more enriched than the average (−19.1‰ each) and cholesterol and cholestanol had values of −19.4 and −19.8‰, respectively.

Hydrocarbon composition

The distribution of hydrocarbons (Figure 3c.) revealed a clear dominance of *n*-C_{17}, comprising up to 80% of total hydrocarbons in the top layer. *n*-C_{18} was prominent in the third layer, reaching 32%. Phytenes and phytane were abundant in the deepest layer, comprising 20% of the hydrocarbons. Their isotopic compositions are displayed in Figure 4c. As with the sterols, the

majority of the measured hydrocarbons have $\delta^{13}C$ values between −20 and −24‰. Lighter values were observed for squalene (−32.1‰), hop-17(21)-ene (−30.9‰) and diploptene (−31.5‰).

Intact polar lipid (IPL) composition

IPL distributions are displayed in Figure 3d. In the first layer, ornithine lipids were most abundant, comprising 33% of total IPLs, followed by the betaine lipids with 14%. The betaine lipids were the most abundant IPLs in the second and third layers, with 23% and 33%, respectively. Ornithine lipids and phosphatidylglycerol (PG), were also abundant in the second layer (20% and 14%, respectively). Monoglycosyldiacylglycerol (1Gly-DG) was an abundant component in all layers, contributing with 10%, 11%, 12%, and 8% from the first to the fourth layer, respectively. Phosphatidylcholine (PC) was the major IPL only in the deepest layer (37%). Phosphatidylserine (PS) and the archaeal components, diglycosyldiacylglycerol (2Gly-GDGT) and glyceroldialkylnonitoltetraether (2Gly-GDNT), both types attached to diglycosidic polar headgroups, were only detected in the deepest layer (0.4%, 0.5% and 1.2%, respectively). 2Gly-GDGTs occurred with 1 or 0 pentacyclic rings. 2Gly-GDNT was identified via the characteristic loss of one sugar head group and a daughter ion of m/z 1456, indicative of four pentacyclic rings in the biphytane chains.

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Bacteriohopanepolyols (BHPs)

We found traces of 17β(*H*), 21β(*H*)-bacteriohopanetetrol (BHT) in the third layer. In all four layer, traces of 17β(*H*), 21β(*H*)-32-hopanoic acid were found, whereas 17β(*H*), 21 β(*H*)-30-hopanol and 17β(*H*), 21β(*H*)-33-hopanoic acid could only be detected in the third and fourth layer.

^{13}C-Labeling experiment

The results of the C-isotopic labeling experiment suggested an active photosynthetic carbon-fixing community. Label uptake was apparently confined to the surface layers since we found only minor uptake in the second and no isotopic enrichment in layers 3-4. Dissolved inorganic carbon (DIC) isotopic composition in the overlying water was not determined. However, since the lake was shallow and well-mixed, equilibrium with atmospheric CO_2 can be assumed, i.e. a DI^{13}C value close to 1‰, similar to that of the Pacific (Takahashi *et al.*, 2000).

a. fatty acids

The uptake of the labeled ^{13}C-bicarbonate showed remarkable differences for the different PLFAs (Figure 5a. and 5b.). The specific uptake was highest in $C_{14:0}$, $C_{16:0}$, and $C_{18:0}$, as indicated by the high $\Delta\delta^{13}C$ values of these PLFAs: 28‰, 17‰, and 15‰, respectively (Figure 5a.). The total uptake accounts for the concentration of each analyte. The highest total uptake was detected for $C_{16:0}$, reaching values as high as 1.0 µg ^{13}C g^{-1} dry mat. Considerable uptake also took place in $C_{18:1\omega7}$ and $C_{18:0}$, with mean values around 0.2 µg ^{13}C g^{-1} dry mat. The total uptake into all fatty acids for the two replicates were 3.2 µg ^{13}C and 1.2 µg ^{13}C g^{-1} dry mat.

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b. alcohols

The specific uptake was highest for $C_{15:0}$-OH (72‰) and $C_{17:0}$-OH (35‰) (Figure 6a.). The total incorporation into alcohols was generally lower than into PLFAs (Figure 6b.) owing to their generally lower concentrations. The highest uptake was associated with $C_{15:0}$-OH and archaeaol with 1.0, and $0.6 \cdot 10^{-3}$ µg ^{13}C g^{-1} dry mat, respectively. In the two parallel incubations the total uptake into alcohols was 6.1 and $4.5 \cdot 10^{-3}$ µg ^{13}C g^{-1} dry mat, respectively.

c. hydrocarbons

High specific uptake was measured for diploptene (13‰), followed by one *n*-$C_{17:1}$-isomer (10‰), *n*-$C_{16:0}$ (8‰), and hop-17(21)-ene (7.5‰) (Figure 7a.). The highest total uptake was determined

for *n*-$C_{17:0}$, with a mean value of $0.8 \cdot 10^{-3}$ µg ^{13}C g^{-1} dry mat (Figure 7b.). Diploptene was also characterized by high total uptake, reaching $0.4 \cdot 10^{-3}$ µg ^{13}C g^{-1} dry mat (Figure 7b.). The values for the total uptake in all hydrocarbons were 1.9 and $0.8 \cdot 10^{-3}$ µg ^{13}C g^{-1} dry mat for the two replicate incubations.

Discussion

This study of a hypersaline microbial mat from Kiritimati was designed in order to characterize the biomarkers with potential to identify active microbial groups and physiologies using a combination of microscopic investigations together with biomarker characterization including labeling techniques. The layers of the mat revealed considerable differences, regarding the microscopically determined microbial distribution, as well as the biomarker structure. Biomarkers could be assigned to different organisms, comparing our microscopic investigations with the biomarker abundances and isotopic compositions. Our interpretation of the ^{13}C labeling experiment is summarized in Figure 8, revealing that uptake over the 6 h course of the experiment was not confined to photoautotrophs but also involved re-mineralization of the newly fixed carbon by organotrophic bacteria and incorporation by archaea.

Microscopic observations revealed the dominance of cyanobacteria in this hypersaline mat system (Figure 2). The top layer was dominated by intact cells of *Aphanocapsa* and *Aphanothece* spp., suggesting a vital cyanobacterial community. Species of these genera are often described as the principle contributors to hypersaline microbial mat systems at numerous localities (e.g., Javor, 2002). The terminology describing these coccoid halophilic morphotypes in the literature is not consistent; they are sometimes used interchangeably or named *Synechococcus, Cyanothece* and *Halothece* (see Garcia-Pichel *et al.*, 1998). In addition to cyanobacteria, the diatom *Nitzschia*

was abundant in the surface layer. Diatoms are known to regularly contribute to the top layers of microbial mats (e.g., Mir *et al.*, 1991).

Based on fatty acids alone it can be difficult to discriminate between cyanobacteria and algae, especially because no long-chain PUFA could be detected in our samples, which would be a clear indicator for diatoms. We attributed the polyunsaturated PLFA (PUFA) (e.g., Dunstan *et al.*, 1994), the fatty acids $C_{16:1\omega7}$ + $C_{18:1\omega9}$ and the C_{17} hydrocarbons to the group cyanobacteria and algae (Figure 8). We viewed $C_{16:1\omega7}$ + $C_{18:1\omega9}$ primarily as cyanobacterial markers (Caudales *et al.*, 2000; Gugger *et al.*, 2002; de Oteyza *et al.*, 2004) based on the fact that cyanobacteria were the most abundant group in the mat and that these fatty acids occurred in all layers, decreasing in abundance with depth (Figure 3a.). The labeling experiment also revealed considerable uptake into PLFAs attributed to cyanobacteria, accounting for at least 13% of the ^{13}C incorporation into PLFAs (Figure 5c.). This is a potential underestimation, as the ubiquitous $C_{16:0}$ is prevalent in cyanobacteria (e.g. Kenyon *et al.*, 1972). The cyanobacterial hydrocarbons *n*-$C_{17:0}$ and *n*-$C_{17:1}$ (Chuecas & Riley, 1969; Grimalt *et al.*, 1991) accounted for up to 80% of the total hydrocarbons (Figure 3c.), consistent with the importance of cyanobacteria in this mat system. The rapid ^{13}C uptake into C_{17} hydrocarbons and diploptene (Figure 7) is in accordance with this interpretation. Phytene and phytane were important components in the third and fourth layers of the mat (Figure 3c.). They are diagenetic products and probably derived from the phytol side chain of chlorophyll or bacteriochlorophyll (Volkman & Maxwell, 1986). Diplopterol was present in all depth layers in our mat, its abundance increased with depth (Figure 3b.). Diplopterol can be attributed to a variety of bacteria including cyanobacteria (Rohmer *et al.*, 1984), purple non-sulfur bacteria (Ourisson *et al.*, 1987), methylotrophs (Summons *et al.*, 1994), and *Desulfovibrio* (Blumenberg *et al.*, 2006). The labeling experiment revealed no label uptake into this compound (Figure 6), which makes a phototrophic source in our setting unlikely. Noteworthy is a considerable uptake

of ^{13}C into diploptene (Figure 7), indicating a different biological source for this compound than for diplopterol. Other studies also revealed that in some bacterial strains diploptene was present but diplopterol not (Joyoux *et al.*, 2004; Härtner *et al.*, 2005).

Cyanobacteria are known to be one of the most prolific sources of BHPs (Talbot *et al.*, 2008). Traces of Bacteriohopanetetrol (BHT) were detected in the third layer of the mat, where *Aphanocapsa* was the dominating cyanobacterium (Figure 2). BHT has been detected in different members of the Chroococcales and in other cyanobacterial mats (Talbot *et al.*, 2008), but is also widespread in the bacterial domain (e.g., Rosa-Putra *et al.*, 2001; Blumenberg *et al.*, 2006) and often found in sedimentary environments (e.g., Talbot & Farrimond, 2007).

Cholesterol is a general marker for eukaryotes (Volkman, 1986) and may therefore derive from diatoms in the Kiritimati mats, notwithstanding their minimal uptake of ^{13}C label at the time of the experiment (Figure 6). The lower uptake into diatom-derived lipids may be due to the longer generation time of diatoms compared to cyanobacteria.

The common cyanobacterial and chloroplast intact polar lipids (IPLs) 1Gly-DG, 2Gly-DG, SQ-DG and PG (Kiseleva *et al.*, 1999; Okazaki *et al.*, 2006) were abundant in this mat. Ritter & Yopp, 1993) found 1Gly-DG, 2Gly-DG and PG in *Aphanothece halophytica*, but not SQ-DG. In addition to these IPLs, betaine lipids are abundant in the thylacoid membranes of eukaryotic algae (Dembitsky, 1996). PC, PE, and PS are generally not found in cyanobacteria (Wada & Murata, 1998).

The second important group of phototrophs in this system are the anoxygenic phototrophs, which are probably mainly represented by PSB. Typical biomarkers for PSB are *cyc*$C_{19:0}$ and farnesol (Figure 8), even though we can not exclude a different source in our setting, because our biomarker data do not match the microscopic observation. The *cyc*$C_{19:0}$ fatty acid occurs abundantly in the lipids of PSB, such as *Ectothiorhodospira* (Grimalt *et al.*, 1991). The

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relative abundances of this PLFA increased slightly with depth, peaking in the third layer (Figure 3a.). Further, its $\delta^{13}C$ value of −22.1‰ is consistent with autotrophic carbon fixation via the Calvin-Benson cycle. From the labeling experiment we calculated a contribution of 1% to total carbon-fixation for *cyc*$C_{19:0}$ fatty acid in the first layer (Figure 5c.). This small contribution to carbon fixation in the first layer matches the microscopic data that revealed no detectable PSBs at this level and their known preference for low-light environments and low oxygen tension (e.g., Hirschler-Rea *et al.*, 2003). The alcohol fraction contained farnesol, the primary ester-bound alcohol side-chain of bacteriochlorophyll (Bchl) e, d and g (Airs *et al.*, 2001), and $C_{16:0}$-OH, another Bchl side-chain (Glaeser & Overmann, 2003). These Bchls are the photosynthetic pigment of both PSB (Hirschler-Rea *et al.*, 2003) and purple non-sulfur bacteria, as well as green sulfur bacteria and green nonsulfur bacteria (filamentous anoxygenic phototrophs) (Permentier *et al.*, 2000; Glaeser & Overmann, 2003; Nübel *et al.*, 2002). An origin of farnesol from green sulfur bacteria is unlikely because these organisms use the reverse citric acid cycle, which causes relatively small carbon isotope fractionation resulting in $\delta^{13}C$ values of ca. −12‰ (e.g., Manske *et al.*, 2005). A $\delta^{13}C$ value of −21.9‰ for farnesol (Figure 4b.) points to PSB as the most likely source, and ^{13}C-uptake indicates the PSBs were actively fixing carbon (Figure 6a). PSBs produce a variety of IPLs, with 1Gly-DG, 2Gly-DG, PC, PE, and PG being particularly common (Linscheid *et al.*, 1997), all were present in our mat. Purple non-sulfur bacteria and several other gram-negative bacteria accumulate betaine and ornithine lipids (Imhoff *et al.*, 1982) under phosphate-limiting conditions (Benning *et al.*, 1995). Abundant ornithine lipids suggest phosphate may therefore have been limiting mat growth in Lake 2A (Figure 3d.).

SRBs are prevalent in microbial mats and their activity is tightly coupled to cyanobacterial carbon fixation (Canfield & Des Marais, 1991). They also promote lithification

and may be instrumental for mat preservation in the geological record (reviewed by Baumgartner *et al.*, 2006). C_{17} PLFAs are typical constituents of SRBs (Boschker & Middelburg, 2002; Figure 8), which were present at all depths (Figure 3a.). The uptake into C_{17} fatty acids in the first layer of the mat accounted for 6% of the total uptake into PLFAs (Figure 5). The relatively high ^{13}C uptake suggests a close coupling between SRBs and phototrophs (Figure 8), as also reported by other authors (e.g., Frund & Cohen, 1992; Decho *et al.*, 2005). The close coupling is possible only if SRBs can tolerate oxygen exposure (e.g., Postgate, 1959), a phenomenon demonstrated by Cypionka *et al.* (1985). Krekeler *et al.* (1998) reported different oxygen-escaping strategies for SRBs in microbial mats, like migration to anoxic zones, formation of clumps, and oxygen removal by active respiration in bands. They also found 20 times lower most-probable-number counts of SRBs during the day under oxic conditions than at night. In contrast, Canfield & Des Marais (1991) reported highest rates of sulfate reduction in the oxic zone of a hypersaline mat system from Guerrero Negro, Baja California, Mexico. Our results suggest the presence of an active sulfate reducing community operating during the day in the upper, generally oxic zone of the microbial mat.

Gram-positive bacteria are abundant in hypersaline environments (Caton *et al.*, 2004; Ghozlan *et al.*, 2006) and stromatolites (Burns *et al.*, 2004). Branched saturated fatty acids (mainly $iC_{15:0}$, $aiC_{15:0}$, and $iC_{16:0}$ in our setting) are often their primary fatty acids (Lechevalier & Lechevalier, 1988; Romano *et al.*, 2008), even though an origin in SRB can not be excluded (e.g. Rütters *et al.*, 2002). The relative abundance of the *iso*-/*anteiso*- group was high in all depth layers (Figure 3a.), reaching a maximum of ca. 22% in the deepest layer. 8% of the total PLFA ^{13}C -label uptake occurred into $iC_{15:0}$, $aiC_{15:0}$, and $iC_{16:0}$ (Figure 5b.), indicating a tightly coupled carbon cycle in the mat (Figure 8). Different Gram-positive bacteria were investigated for their IPL composition by Schubotz, (2005) who found PE, PG, and diphosphatidylglyerol (DPG) in all

isolates. With PE representing only a minor constituent and DPG being absent in our mat, we cannot exclude a different origin for the branched saturated fatty acids we observed.

Archaea, affiliated with the *Methanosarcinales*, the *Halobacteriales*, and uncultured *Euryarchaeota*, mainly from the marine benthic group D (MBGD), were detected by Sørensen *et al.* (2005) in a hypersaline mat from Eilat, Israel. Crenarchaeota and euryarchaeota were detected in hypersaline stromatolites by Papineau *et al.* (2005), while Burns *et al.* (2004) also observed methanogenic archaea of the *Methanosarcinales* group in stromatolites.

Archaeol is a biomarker that derives from archaea (Koga *et al.*, 1998). It was detected in all layers and was especially abundant in the top layer (Figure 3b). In our experiment, it accounted for a considerable label uptake (Figure 6, Figure 8). Like SRB, methanogenic archaea can be autotrophic in their use of CO_2 and H_2 (Hoehler *et al.*, 2001) and this could explain the observed ^{13}C-uptake into both groups. Another possible origin of the labeled archaeol could be methanogens taking up non-competitive substrates, like trimethylamines (TMA), formerly produced by carbon-fixing cyanobacteria (King, 1988; Orphan *et al.*, 2008). A vital community of methylotrophic methanogens would also be supported by our observation that archaeol concentrations were maximal in the top layer of the mat. But uptake of TMA in the natural system would probably lead to isotopic values much more depleted than the ones we found (Summons *et al.*, 1998). Alternatively, heterotrophic halophilic archaea could have assimilated cyanobacterial exudates in the sunlit surface layer (Burns *et al.*, 2004).

We also detected intact tetraether lipids that derive from archaea (Sturt *et al.*, 2004) in the deepest layer. Jahnke *et al.* (2008) found archaea even dominating above bacteria in the deeper layers of a hypersaline microbial mat of Baja California. GDGTs occur in a variety of archaea (e.g., Hopmans *et al.*, 2000; Sinninghe Damste *et al.*, 2002; Sturt *et al.*, 2004) in several environments, including the deep biosphere (Biddle *et al.*, 2006; Lipp *et al.*, 2008). GDNTs were

so far only attributed to the hyperthermophile crenarchaeon Sulfolobales (Hanford & Peeples, 2002; Sturt *et al.*, 2004), which is an unlikely source in our setting. Biddle *et al.*, (2006) and Lipp *et al.*, (2008) found GDNT in the deep biosphere, which is an additional indicator suggesting a more widespread occurrence of this compound, maybe indicating an organism related to the sulphur cycle (suggested by Sturt *et al.* 2004).

Non-phosphatidyl polar lipids accounted for 44 to 75% of total IPLs in different layers of our mat (Figure 3d). Van Mooy *et al.* (2006) found membrane lipids devoid of P accounting for over 90% of IPLs in picocyanobacteria in the North Pacific tropical gyre and hypothesized that substitution of S for P in membrane lipids could offer a competitive advantage for organisms living in phosphate-limited environments. Besides abundant 1Gly-DG, 2Gly-DG, and SQ-DG, we also found other non-phosphatidyl lipids including betaine and ornithine lipids. Betaine lipids are abundant in many eukarya including algae, bryophytes, fungi and some protozoa (Dembitsky, 1996). These lipids have also been discovered in the photosynthetic purple bacterium *Rhodobacter sphaeroides* (Benning *et al.*, 1995). They resemble the more commonly known PC in molecular geometry and charge distribution, and probably substitute this substance in membranes under phosphate-limiting growth conditions (Araki *et al.*, 1991). Since the physiological importance of non-phosphatidyl polar lipids is currently not understood, it is also possible that the high content is an adaptation to environmental factors, like high salinity. Lipids that derive from the amino acid ornithine are widespread among bacteria (Lopez-Lara *et al.*, 2003), especially in the gram-negative bacteria (Ratledge & Wilkinson, 1988), but have not been found in eukarya or archaea. These lipids are also described as being produced in some bacteria under phosphorus-limiting conditions (Benning *et al.*, 1995; Weissenmayer *et al.*, 2002; Choma & Komaniecka, 2003). Aygun-Sunar *et al.* (2006) showed that ornithine lipids are

required for maintaining optimal steady-state amounts of some extracytoplasmic proteins, important for various cellular processes, including electron transport.

The sterol ratio $C_{27\text{-}29\Delta0}/C_{27\text{-}29\Delta5}$ increased from 0.2 for the top three layers to 0.5 in the deepest layer. This is in agreement with a sterol production only in the upper layers of the mat becoming gradually buried by upward growth of the phototrophic community. Longer exposure to the increasingly reducing conditions promotes diagenetic alterations of the sterols. However, uptake of the label into several stanols may also be consistent with these lipids having a functional role in the mat community.

Conclusions

Based on microscopic and organic-geochemical analyses, cyanobacteria (*Aphanocapsa* and *Aphanothece*) were found to dominate the hypersaline mat system on Kiritimati, represented by different fatty acids and hydrocarbons with considerable label uptake. Anoxygenic phototrophs were represented by the fatty acid *cyc*$C_{19:0}$ and the alcohol farnesol, with an isotopic composition revealing carbon fixation via the Calvin-Benson cycle. Label uptake by SRBs indicated close coupling with carbon fixing organisms. Archaeol was detected and responsible for considerable label uptake after 6 h of incubation, indicating active archaea in the top layer of the mat. Our approach combining lipid analyses with a simple labeling experiment and microscopic investigations of a recent microbial mat successfully revealed that different prokaryotic groups prosper in close proximity and mutual dependence. Many of the groups that we found are of great geobiological relevance, because they are thought to be the main constructors of analogue ancient microbial mat systems (Allwood *et al.*, 2006; Altermann *et al.*, 2006). Our results may aid in tracing microbial evolution in the geological record, but more lipid-biomarker-based mat studies are needed to get a representative overview of the diversity in modern systems.

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1 Tables

2 Table 1: Lipid composition of different layers of the microbial mat

Depth interval	Lipid component [μg g^{-1} dry mat]		
	fatty acids	alcohols	hydrocarbons
Layer 1	$44*10^3$	220	31
Layer 2	$35*10^3$	140	21
Layer 3	$43*10^3$	280	24
Layer 4	$35*10^3$	270	27

Figure captions

Figure 1: (a) Geographic position of (b) Kiritimati Island (formerly known as Christmas Island) in the Northern Line Islands, Republic of Kiribati, based on Saenger *et al.*, 2006) with the frame (c) showing the position of the Lake 2A; (d) Lake 2A on Kiritimati Island; (e) Vertical section through the mat with the four investigated layers magnified in the insert; (f) Photomicrograph from the upper gelatinous layer (magnification: 100x), A - *Nitzschia*; B - *Aphanocapsa*; C - *Aphanothece*; D - *Entophysalis*; E - sheaths von *Phormidium*/*Leptolyngbya*; (g) Photomicrograph from the fourth layer (magnification: 63x): A - PSB.

Figure 2: Relative abundance of microorganisms in the layers of the mat based on microscopic observations, the assessment is given in scores of 1-5 of their relative abundance in % of total biomass (5: 100-75%; 4: 75-50%; 3: 50-25%; 2: 25-10%, 1: 10-0.1%, score 1 means that there was at least one specimen found).

Figure 3: lipid biomarker distribution over depth: (a) PLFAs (*iso-/anteiso*-PLFA: *i*$C_{14:0}$, *i*$C_{15:0}$, *ai*$C_{15:0}$, *i*$C_{16:0}$; C_{17} PLFA: *i*$C_{17:1}$, *i*$C_{17:0}$, *ai*$C_{17:0}$, $C_{17:1\omega8}$, $C_{17:1\omega6}$, $C_{17:0}$; polyunsaturated PLFA: $C_{16:2}$ and $C_{18:2}$; saturated PLFA: $C_{14:0}$, $C_{15:0}$, $C_{16:0}$, $C_{18:0}$; hydroxy-PLFA: 12*OH*-$C_{19:0}$, 14*OH*-$C_{21:0}$, 12*OH*-$C_{21:0}$); (b) alcohols (saturated alcohols: $C_{15:0}$-OH, $C_{16:0}$-OH, $C_{17:0}$-OH, $C_{18:0}$-OH, $C_{20:0}$-OH to $C_{28:0}$-OH, and $C_{30:0}$-OH; unsaturated alcohols: $C_{17:2}$-OH, $C_{17:1}$-OH, $C_{18:1}$-OH, $C_{20:1}$-OH, $C_{22:1}$-OH); (c) hydrocarbons (*n*-C_{17}: *n*-$C_{17:0}$ and two *n*-$C_{17:1}$ isomers; hopenes: hop-17(21)-ene and hop-21-ene), (d) IPLs (1Gly-DG: monoglycosyldiacylglycerol, SQ-DG: sulfoquinovosyldiacylglycerol; 2Gly-DG: diglycosyldiacylglycerol, betaines: betaine lipids, ornithines: ornithine lipids, PG: phosphatidylglycerol, PC: phosphatidylcholine, PE: phosphatidylethanolamine, PS: phosphatidylserine, 2Gly-GDGT: glyceroldialkylglyceroltetraether, 2Gly-GDNT: glyceroldialkylnonitoltetraether).

Figure 4: stable carbon isotopic composition ($\delta^{13}C$) of lipids extracted from the first layer of an untreated microbial mat of: (a) PLFAs; (b) alcohols; (c) hydrocarbons.

Figure 5: (a) specific uptake ($\Delta\delta^{13}C$) into PLFAs of the first layer; (b) total uptake into PLFAs of the first layer; (c) total uptake into grouped PLFAs (cyanobacteria + algae: polyunsaturated PLFAs and $C_{16:1\omega7}$ + $C_{18:1\omega9}$; sulfate-reducing bacteria (SRBs): C_{17} PLFAs; gram-positive: branched saturated PLFAs; purple sulfur bacteria (PSBs): *cyc*$C_{19:0}$) after 6 h of incubation. Error bars indicate range of two replicate incubations.

Figure 6: (a) specific uptake ($\Delta\delta^{13}C$) into alcohols of the first layer; (b) total uptake into alcohols of the first layer after 6 h of incubation; (c) total uptake into grouped alcohols (archaea: archaeol; eukaryotes: ergosterol + ergostanol, sitosterol + sitostanol; cyanobacteria: C_{17}-ols; purple sulfur bacteria (PSBs): farnesol) after 6 h of incubation. Error bars indicate range of two replicate incubations.

Figure 7: (a) specific uptake ($\Delta\delta^{13}C$) into hydrocarbons of the first layer; (b) total uptake into hydrocarbons of the first layer after 6 h of incubation. Error bars indicate range of two replicate incubations.

Figure 8: working model for the carbon flow under light conditions in the hypersaline microbial mat, solid arrows represent autotrophy (green: photoautotrophy, red: chemoautotrophy), whereas dashed lines represent heterotrophy.

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