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Lipid biomarker and isotopic study of community distribution and biomarker preservation in a laminated microbial mat from Shark Bay, Western Australia

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Summary

Modern microbial mats from Shark Bay present some structural similarities with ancient stromatolites; thus, the functionality of microbial communities and processes of diagenetic preservation of modern mats may provide an insight into ancient microbial assemblages and preservation. In this study, the vertical distribution of microbial communities was investigated in a well-laminated smooth mat from Shark Bay. Biolipid and compound specific isotopic analyses were performed to investigate the distribution of microbial communities in four distinct layers of the mat. Biomarkers indicative of cyanobacteria (e.g. *n*-$C_{17:1}$) were more abundant in the uppermost oxic layer. Diatom markers (e.g. C_{25} HBI alkene, $C_{20:4\omega6}$ and $C_{20:5\omega3}$ polar-lipid fatty acids (PLFAs)) were also detected in high abundance in the uppermost layer, but also in the deepest layer under conditions of permanent darkness and anoxia, where they probably used NO_3^- for respiration. *Cyc*$C_{19:0}$, an abundant PLFA of purple sulfur bacteria (PSB), was detected in all layers and presented the most ^{13}C-depleted values of all PLFAs, consistent with photoautotrophic PSB. Sulfur-bound aliphatic and aromatic biomarkers were detected in all layers, highlighting the occurrence of early sulfurisation which may be an important mechanism in the sedimentary preservation of functional biolipids in living, and thus also, ancient mats.

Keywords: Microbial mats, lipid biomarkers, stable isotopes, sulfate-reducing bacteria, cyanobacteria.

Introduction

Stromatolites are laminated sedimentary structures of biological origin. They extend throughout the geological record to 3.5 Ga and are recognised as the earliest visible traces of life on Earth [1–4]. However, the microfossils that built the ancient mats and provide detailed insights into the biogeochemistry of ancient mat-building populations are not often geologically preserved, hence little is known in detail about early life processes [5, 6] or the controls on, and pathways of, the preservation of these ancient microbial macrostructures.

Modern lithifying microbial mats can show close structural similarities to ancient stromatolites [6–8]. Consequently, studies of modern microbial mats and their preservation pathways may be helpful for discerning early microbial assemblages and complex, dynamic, elemental cycles. In modern laminated microbial mats, microbial communities orient themselves vertically along microscale physico-chemical gradients such as light, O_2, pH, E_h and nutrients in order to optimise metabolic processes [9]. However, although general vertical transitions are observed in microbial mats, a strong lateral heterogeneity resulting in mosaic-like distributions of porewater solutes has been previously reported in microbial mats [10, 11]. Diurnal changes in sunlit environments drive shifts in the dominant biogeochemical processes [12]. Cyanobacteria and diatoms are typically found in the upper mat layer and perform photosynthesis during the daytime that produces organic matter (OM) beneficial to other organisms. However, oxygen production favours chemoautotrophic and chemical oxidation of reduced species at the expense of anaerobic processes [9, 13, 14]. Thus, the oxic zone of the mat results from a dynamic balance between photosynthetic O_2 production and O_2 consumption by aerobic heterotrophic and chemoautotrophic bacteria. Permanently anoxic conditions deeper in the mat [10, 11, 15] support anaerobic microbes such as sulfate-reducing

bacteria (SRB) and, consequently, often contain high sulfide concentrations. Sulfur is a key element in modern microbial mats. However, high concentrations of sulfide can be lethally toxic to all microorganisms, including the SRB that produce it and other bacteria such as anoxygenic phototrophic bacteria and chemolithotrophic bacteria that rely on it [16].

Modern microbial mats, including laminated lithifying smooth mats, have been reported in the World Heritage listed Shark Bay, Western Australia [17, 18]. Previous studies on microbial mats from Hamelin Pool have revealed a high biodiversity in both smooth and pustular mats [19–21].

In this study, the zonation of microorganisms was investigated in a laminated microbial mat from Garden Point, a remote area of Shark Bay that favours the formation of coarse stratified mats [18]. This study investigated the vertical organisation of microbial groups in the mat using a combined biolipid (hydrocarbons and PLFAs) and isotopic approach, with a particular focus on biomarkers of cyanobacterial, diatom, sulfate-reducing and sulfur-oxidising groups. Similar lipid analyses have previously been used to provide important biological information on microbial mats [10, 11, 22–25]. For the first time in a modern stromatolite, the solvent extractable fraction released on desulfurisation of the OM was also analysed for evidence of organic sulfur compounds (OSCs) and potential insights into the role of sulfur in the preservation of stromatolite biolipids.

Material and Methods

Sampling site

Shark Bay is located in Western Australia, 800 km north of Perth. This area is known for the abundance and variety of microbial mats that occur, for example, within the tidal flats of Hamelin Pool [11, 21, 25–29]. Much less attention has been given to microbial mats from the Henry Freycinet embayment, located in the western part of Shark Bay. Smooth mat samples for this study were obtained from Garden Point located on the eastern area of the Freycinet embayment (see [18] for detailed maps). This area consists of a shallow tidal flat, characterised by a low, regular bottom gradient ranging from 20 to 80 cm km^{-1}. The embayment is often affected by southerly winds and longshore currents, supplying quartz sand to the sub-littoral and intertidal deposits [18]. A previous study on microbial mats from different Shark Bay sites highlighted unique lipid signatures in Garden Point mats including a relatively high proportion of markers attributed to sulfur-cycling organisms [10], making these mats highly suitable for biolipid preservation studies.

Sample description

The smooth mats sampled for this study contained small carbonate grains inter-bedded within organised laminae of microbes. These well-laminated stromatolites contained filamentous cyanobacteria that produce expolymers which trap sediments, favouring the formation of a flat surface [30]. The highly stratified mats contained 4 distinct layers (Fig. 1). Firstly, a beige and green layer close to the surface was suggestive of cyanobacterial dominance (2 mm thick). This overlies a purple-pink layer (5 mm thick) typical of purple sulfur bacteria. A third, dark green/brown layer (3 mm thick) overlies a fourth, black, permanently anoxic layer (6 mm thick) [31, 32].

Preparation of lipid samples

Sampling

The microbial mat was sampled in the field using an aluminium push core (inner diameter: 10 cm) and immediately frozen (-18 °C). Prior to extraction, the sample was defrosted and the four layers were separated using a metal spatula, carefully removing the edges of the sample that were in contact with the coring material. The spatula was carefully washed with dichloromethane (DCM) between each sample preparation. Each microbial mat layer was separated into two aliquots: one for hydrocarbon analysis and the other for PLFA analysis. Procedural blanks were performed throughout the entire process to confirm that the compounds identified were indigenous to the samples.

Extraction

For hydrocarbon analysis, dry aliquots (10 g) of the different microbial mat layers were ultrasonically extracted (5 h) using a 9:1 mixture of DCM: Methanol (MeOH). The solvent was filtered with pre-extracted cotton wool to remove particulates and the solvent was reduced to 2 mL by rotary evaporation. Activated copper turnings were added to remove elemental sulfur (72 h with stirring at room temperature). The syringe used to transfer the extracts was cleaned with *n*-hexane (≥ 20 times) between each sample preparation.

For PLFA analysis; a total lipid extract was obtained by ultrasonication (15 min) of dry mat material (2 g) in 50 mL of a chloroform ($CHCl_3$): MeOH: phosphate buffer (K_2HPO_4/ HCl) mixture (0.8:2:1; v/v/v) and isolated with additional $CHCl_3$ in the presence of double distilled water, following a previously reported procedure [33, 34].

144 Column Chromatography

145 Aliquots (500 µL) of all hydrocarbon extracts were separated using a small column
146 (5.5 cm x 0.5 cm i.d.) filled with activated silica gel (120 °C, 8 h). The aliphatic hydrocarbon
147 fraction was eluted with *n*-hexane (2 mL); the aromatic hydrocarbon fraction with a 1:3
148 mixture of DCM: *n*-hexane (2 mL) and the polar fraction with a mixture of 1:1 DCM: MeOH
149 (2 mL). The aliphatic and aromatic fractions were analysed by GC-MS.

150

151 The total-lipid extract was separated into neutral lipids, free fatty acids (FA) and
152 PLFA by successive elution through silica bonded columns (SPE-Si, Supelco, Poole, UK)
153 with $CHCl_3$ (2 mL), acetone (2 mL) and MeOH (1 mL).

154

155 PLFA methylation

156 The PLFA fraction was methylated by the addition of 0.2 M potassium hydroxide
157 (KOH) in MeOH (0.5 mL), and the mixture was heated to 75 ºC held for 5 min, then cooled
158 and neutralised with 0.2 M acetic acid (0.5 mL). Methylated PLFAs were subsequently
159 isolated with a 1:1 aqueous $CHCl_3$ mixture and analysed by GC-MS.

160

161 Cleavage of C-S bonds of polar fractions by Raney nickel

162 Aliquots of the polar fractions (ca. 20 mg) were desulfurised with Raney nickel. Each
163 fraction was dissolved in a 1:1 mixture of ethanol (EtOH): toluene (2 mL) together with a
164 suspension of Raney nickel (0.25 g in 0.5 mL EtOH) and refluxed under a N_2 stream (3 h).
165 The desulfurised products were subsequently extracted with DCM (5 mL, 3 times). The
166 organic phase was passed through a large column (20 cm x 0.9 cm i.d.) of anhydrous $MgSO_4$
167 to ensure H_2O removal. The extract was then concentrated and separated on a small activated
168 silica gel column to obtain saturate and aromatic fractions for GC-MS analysis. This method

has previously been shown to efficiently release sulfur-bound biomarkers from a large variety of organic samples [35–40].

Identification and isotopic characterisation of lipid biomarkers

Gas-Chromatography Mass-Spectrometry (GC-MS)

GC-MS analyses of the aliphatic hydrocarbon fractions were performed using an Agilent 6890 GC interfaced to an Agilent 5973 mass selective detector (MSD). An electronic pressure controlled (EPC) split/splitless injector (320ºC) was operated in the pulsed splitless mode. The GC was fitted with a 60 m x 0.25 mm i.d. WCOT fused silica capillary column coated with a 0.25 µm film (DB-5MS, JandW Scientific). The oven temperature was programmed from 40 to 325 °C (at 3° C min^{-1}) with the initial and final temperature hold times of 1 and 50 min, respectively. Ultra high purity He was used as carrier gas and maintained at a constant flow of 1.1 mL min^{-1}. 70 *eV* mass spectra were acquired in full scan mode, *m/z* 50-600 at ~ 4 scans per second and with a source temperature of 230 °C.

PLFA analysis was performed with an Agilent 6890/5975b GC-MS. The gas chromatograph was used in pulsed splitless mode, with a 60 m x 0.25 mm i.d. DB5-MS (JandW) column and with He as a carrier gas at a constant flow of 1.1 mL min^{-1} and an oven programme of 70 ºC (held isothermally for 1 min) to 140 ºC at 20 ºC min^{-1}, then to 290 ºC (held 15 min) at 4 ºC min^{-1}. Full scan (*m/z* 50–550) and selected ion data (*m/z* 55, 74, 87, 270, 284, 298 and 312) were simultaneously acquired. Product identifications were based on effective chain length values measured using relative retention times. Relative product abundances were measured by integration of total ion chromatogram (TIC) peak areas.

194 Multiple Reaction Monitoring (MRM) GC-MS

195 Sulfur-bound aliphatic hydrocarbons were analysed in MRM mode on a Micromass
196 Autospec Ultima mass spectrometer interfaced to an Agilent 6890N gas chromatograph with
197 an autosampler. MRM GC-MS affords a high signal to noise ratio and enhanced selectivity
198 for targeted lipid classes. The GC was fitted with a DB-5MS fused silica capillary column (60
199 m; 0.25 mm i.d.; 0.25 μm film thickness; JandW Scientific). He at a constant flow of 2 mL
200 min^{-1} was the carrier gas. The GC temperature program was 60 °C (2 min) to 150 °C at 10 °C
201 min^{-1}, then to 315 °C (held 24 min) at 3 °C min^{-1}. The source was operated in 70 eV electron
202 impact mode at 250 °C, with 8 kV accelerating voltage and predetermined precursor–product
203 reactions. Data were acquired and processed using MassLynx 4.0 (Micromass Ltd.).
204
205 Gas-Chromatography Isotope Ratio Mass-Spectrometry (GC-IRMS)
206 The aliphatic hydrocarbon and methylated-PLFA fractions were further analysed with
207 a Micromass IsoPrime isotope ratio - mass spectrometer coupled to an Agilent 6890 GC fitted
208 with a 60 m x 0.25 mm i.d., 0.25 μm thick DB-1 phase column to measure the $\delta^{13}C$
209 signatures of the major products. The samples were injected in pulsed splitless mode. The GC
210 oven was programmed as for the GC-MS analyses. The $\delta^{13}C$ values are reported in parts per
211 mil (‰) relative to the international Vienna Peedee belemnite (VPDB) standard. Isotopic
212 compositions were determined by integration of the *m/z* 44, 45 and 46 ion currents of the CO_2
213 peaks from each analyte and reported relative to CO_2 reference gas pulses of known $\delta^{13}C$.
214 Each sample was analysed at least in duplicate and all reported values had standard
215 deviations <0.3 ‰. To ensure optimal accuracy, in house standard solutions containing *n*-
216 alkanes and PLFAs of known isotopic composition were analysed after every second
217 hydrocarbon or PLFA fraction.
218

As the conversion of free PLFAs to their methyl ester analogues involved the addition of one methyl group per fatty acid molecule, the $\delta^{13}C$ values of the methylated PLFAs were corrected by taking into account the $\delta^{13}C$ value of the MeOH used in methylation and the fractional carbon contribution of the free fatty acid to the ester [41].

Results

Aliphatic hydrocarbons - molecular and $\delta^{13}C$ distributions

Aliphatic hydrocarbons were analysed to investigate the major bacterial, higher plant, or eukaryotic contributions to the mat. *N*-alkanes ranged from C_{15} to C_{34} with a predominance of short-chain *n*-alkanes (<*n*-C_{20}) (Fig. 2). *N*-C_{17} was the most abundant *n*-alkane in all layers, with the $C_{18:1}$ alkene also in high relative abundance in the deepest layer. Phytane, phytene and phytadiene isomers were very prominent in all layers, although less abundant in layer 1 (Fig. 3), but no pristane was detected, concordant with relatively reducing and hypersaline conditions [42]. An odd over even carbon-number preference for long-chain *n*-alkanes was evident from all four layers (i.e., C_{25}-C_{33} carbon preference indexes were between 1.8 and 2.4). In addition, the $\delta^{13}C$ values of the long chain *n*-alkanes (C_{24}-C_{33}) from the four layers were in the range -30 ‰ to -32 ‰ (Fig. 4 and Table S1). They were notably more depleted in ^{13}C than the short chain *n*-alkanes (C_{16}-C_{20}), which were between 9 ‰ and 12 ‰ more positive (Fig. 4).

The most prominent steroids were 5α(H),14α(H),17α(H) 20*R* isomers of cholest-2-ene (C_{27}), 24-methylcholest-2-ene (C_{28}) and 24-ethylcholest-2-ene (C_{29}) and all occurred in highest abundance in layer 4 (Fig. 2). A less abundant C_{27} sterene was also detected in layer 3. In addition, C_{27}, C_{28} and C_{29} sterenes presented more positive $\delta^{13}C$ values (-16.4 ‰, -13.3

243 ‰ and -15.2 ‰, respectively, for layer 4) than other biomarkers, suggestive of a specific ^{13}C-
244 rich source (Fig. 4 and Table S1).
245
246 Hop-22(29)-ene (i.e. diploptene) was detected in all layers and 22,29,30-trisnorhop-
247 17(21)-ene and 17β,21β-homohopane, a metabolite of aminobacteriohopanetetrol, were also
248 detected in layer 4 (Fig. 2). These terpenoids may be derived from cyanobacteria [43, 44], but
249 can also be sourced from SRB (e.g., *Desulfovibrio*; [45]) or other microbes [44, 46].
250 22,29,30-trisnorhop-17(21)-ene and diploptene also presented relatively more positive δ^{13}C
251 values (-19.9 ‰ and -16.3 ‰ for layer 3 and -17.3 ‰ and -15.6 ‰ for layer 4, respectively),
252 whilst C_{31} 17β, 21β-homohopane had notably lighter δ^{13}C signature (-32.6 ‰ for layer 3 and
253 – 31.2 ‰ for layer 4) indicative of a separate source (Fig. 4 and Table S1).
254
255 *Polar lipid fatty acids - molecular and δ^{13}C distributions*
256 Straight-chain saturated and monounsaturated acids (Table 1) were the most abundant
257 (methyl ester) PLFAs observed in the four layers of the mats. PLFAs potentially indicative
258 of cyanobacteria (i.e., 16:1ω7, 17:1ω5, 18:4ω3, 18:2ω6 and 18:1ω9) [47, 48] were detected
259 in relatively high abundance in all layers.
260
261 Potential diatom markers (i.e., 16:4ω1, 20:4ω6 and 20:5ω3 [49, 50]) were also
262 observed in all layers, although their relative abundance varied with depth (Fig. 5). These
263 PLFAs showed the most ^{13}C enriched values. For example, the δ^{13}C value of 16:4ω1 in the
264 four layers ranged from -11.1 to -15.1 ‰ and 20:5ω3 from -10.1 to -15.7 ‰ (Fig. 6).
265

Cyclopropyl-19:0 (*cyc*19:0) was detected in all layers, but was of clearly highest abundance in layer 2 (Fig. 5). *Cyc*19:0 (-19.9 ‰ to -29.4 ‰) was consistently the most ^{13}C depleted of all the PLFAs.

Methylhexadecanoic acid, a common SRB marker, was most prominent in the deepest layer of the mat. However, other PLFAs generally associated with SRB, including *iso*- and *anteiso*-15:0 and 17:0, 17:1ω8 and 17:1ω5 previously detected in *Desulfovibrio* [51–53], were common to all layers.

Whilst Gram-positive *Actinomycetes* were previously reported from *r*RNA analyses of smooth Shark Bay mats [21], no PLFA specifically associated with these bacteria (e.g., methyloctadecanoic acid [54]) was detected. The deepest layer of the mat presented the highest abundance of C_{14} – C_{18} hydroxy fatty acids (OH-FAs). These PLFAs are generally attributed to Gram negative bacteria [54], although *Actinomycetes* and some fungi can also produce hydroxy fatty acids [55].

Very long-chain fatty acids (up to C_{31}) displaying an even-over-odd carbon-number preference were consistently detected. These PLFAs were more ^{13}C-enriched (by on average of 7.7 ‰ in layer 4) than *n*-alkanes of similar carbon number range (Nb. showing odd carbon number preference) (Fig. 4 and 6), indicative of a separate source.

Bound hydrocarbons

To investigate the role of sulfur in the preservation of biolipids in the studied modern stromatolite, C-S bound biomarkers were released by Raney nickel treatment and analysed by

290 GC-MS. The hydrocarbons detected were compared with the lipid biomarkers identified in
291 the free hydrocarbon fractions.
292
293 The sulfur-bound aliphatic hydrocarbons from layer 4 (Fig. 7) showed a quite
294 different distribution to that observed for the free hydrocarbons (Fig. 2). The most abundant
295 products were C_{18} and C_{21} *n*-alkanes. *N*-C_{37} was also detected in unusually high abundance.
296 Phytane, present in the free aliphatic fraction was also identified in the sulfur-bound fraction.
297 The C_{31} 17β,21β-homohopane, detected in the free hydrocarbon fraction, was also present in
298 the sulfur-bound fraction in low abundance. Nevertheless, it was the most abundant hopanoid
299 in the *191* fragmentogram of layer 3 (Fig. 8). Layer 3 of the mat presented the highest
300 abundance of sulfur-bound hopanes (ranging from C_{27} to C_{31}) and a high relative abundance
301 of C_{31} 17β,21β-homohopane. Additional hopanes detected in trace amounts included C_{29} αβ
302 and βα hopanes and C_{30} αβ hopanes. Sulfur-bound steroids were also identified in the deep
303 layer 4. These included 20*R* 5α-cholestane and 5α-24-ethylcholestane, confirmed by MRM
304 GC-MS analysis (Fig. 9). The three top layers contained different sulfur-bound carotenoids
305 present at trace levels. These included β-renierapurpurane in layer 1, renieratane in layers 1
306 and 2 and isorenieratane in layer 3.
307
308 **Discussion**
309 *Biological signatures*
310 Cyanobacteria
311 Cyanobacterial signatures were detected in all mat layers (Fig. 2). The dominant *n*-C_{17}
312 was most likely from filamentous cyanobacteria [56, 57], although other sources are known
313 (e.g. eukaryotic algae [57]). Its detection in microbial mats from Shark Bay [25], as well as
314 previously from hot springs [58], freshwater [22], or hypersaline environments [59–61], have

315 all been attributed to a cyanobacterial source. In addition, PLFAs potentially deriving from
316 cyanobacteria (i.e., 16:1ω7, 17:1ω5, 18:4ω3, 18:2ω6 and 18:1ω9) [47, 48] were detected in
317 relatively high abundance in all layers (Table 1). Allen et al. [25] observed a similar
318 predominance of these PLFAs in a smooth mat from Hamelin Pool, Shark Bay.
319
320 Diatoms
321 Diatom communities were also detected in the mat. First, diatom PLFAs were
322 observed in all layers, although their relative abundance varied with depth (Fig. 5). These
323 PLFAs showed the most ^{13}C-enriched values (Fig. 6). Diatoms can be ^{13}C-rich compared to
324 other primary producers, as some species are capable of assimilating HCO^-_3 [62] and
325 bicarbonate is enriched by about 8 ‰ in comparison to CO_2(aq) [63].
326
327 In addition, C_{27}, C_{28} and C_{29} sterenes were detected in layer 4 (Fig. 2). Sterenes can be
328 derived from a variety of eukaryotic sources, including diatoms, green algae, other
329 phytoplankton, zooplankton, terrestrial higher plants and submerged macrophytes [64, 65].
330 Although specific steroid types can be derived from zooplankton (C_{27} sterenes),
331 phytoplankton (C_{28}), aquatic or terrestrial plants (C_{29}) or green algae (C_{29}; [64]), an even
332 distribution of C_{27}, C_{28} and C_{29} sterenes is consistent with a diatom source [64]. In addition,
333 C_{27}, C_{28} and C_{29} sterenes presented the most positive δ^{13}C values (Fig. 4) suggestive of a
334 specific ^{13}C-rich source, such as diatoms [62].
335
336 Furthermore, a C_{25} HBI alkene detected in layer 1 has previously been identified in
337 subtidal sediments, and both pustular and smooth microbial mats from Hamelin Pool [10, 25,
338 66]. It has also been observed in diatoms [67] and in diatomaceous ooze from Hamelin Pool
339 samples [68].

340 Purple sulfur bacteria

341 Purple sulfur bacteria (PSB) were present in all layers, based on *cyc*19:0 distributions
342 [69]. This PLFA was consistently the most ^{13}C depleted of all the PLFAs (Fig. 6). A similar
343 depletion in ^{13}C was observed for this PLFA in laminated microbial sediments from the
344 Wadden Sea and was attributed to photoautotrophic PSB [69]. Most PSB are
345 photoautotrophic, but some are also able of photoheterotrophy [70] or can use alternative
346 carbon fixation pathways such as the reversed tricarboxylic acid (TCA) cycle [71, 72].
347 Carbon fixation via the TCA cycle, however, usually leads to ^{13}C-enriched biolipids [73].

348

349 Sulfate-reducing bacteria

350 Methylhexadecanoic acid, a common SRB marker, was most prominent in the deepest
351 layer of the mat. However, other PLFAs generally associated with SRB (i.e. *iso*- and *anteiso*-
352 15:0 and 17:0, 17:1ω8 and 17:1ω5) were common to all layers (Table 1).

353

354 In addition, an even carbon-numbered preference, such as evident for the C_{20}-C_{26}
355 PLFAs, usually implies higher plant inputs [74] deriving from coastal macrophytes or
356 terrestrial plants brought to the mat which may be possible by aeolian transport [25]. These
357 PLFAs, however, were more ^{13}C-enriched (by on average of 7.7 ‰ in layer 4) than *n*-alkanes
358 of similar carbon number range (Nb. showing odd carbon number preference) (Fig. 4 and 6),
359 indicative of a separate source. Long chain PLFAs of comparable distribution with $\delta^{13}C$
360 values 4-5‰ enriched compared to co-occurring *n*-alkanes has also been reported for Shark
361 Bay ooids [75] where they were suggested to reflect a SRB *Firmicute* source [75] and a
362 similar source is possible for the studied mat.

363

364

365 Vascular plant input

366 An odd over even carbon-number preference for long-chain *n*-alkanes was evident
367 from all four layers (i.e., C_{25}-C_{33} carbon preference indexes were between 1.8 and 2.4) (Fig.
368 2) and is a distinctive characteristic of epicuticular leaf waxes [74, 76]. Higher plant material
369 supplied by aeolian transport has been observed to impact the microbial mats of Hamelin
370 Pool [18, 25]. Waxy *n*-alkanes with odd carbon-number predominance, however, may also be
371 derived from coastal seagrass beds [77, 78], which are highly abundant in Shark Bay [79] and
372 have been previously observed in Shark Bay mats [10]. In addition, the $\delta^{13}C$ values of the
373 long chain *n*-alkanes (C_{24}-C_{33}) from the four layers were notably more depleted in ^{13}C than
374 the short chain *n*-alkanes (C_{16}-C_{20}) (Fig. 4). Such a large isotopic difference is indicative of
375 different sources. The odd carbon preference (OCP) of the high molecular-weight *n*-alkanes
376 suggests a higher plant source [74]. Terrestrial C3 plants can produce long-chain *n*-alkanes
377 with $\delta^{13}C$ values ranging from -30 to -39 ‰, whilst coastal macrophytes can produce *n*-
378 alkanes with $\delta^{13}C$ values between -19 and -34 ‰ [80, 81].
379
380 *Biomarker and isotopic trends with depth*
381 Long-chain *n*-alkanes were in highest abundance in the top layer (Fig. 3), as observed
382 in other microbial mats [11], and probably due to higher plant material.
383
384 Diatom PLFAs were common to all layers but appeared to be most abundant in layers
385 1 and 4 (Fig. 5). $C_{25:1}$ HBI, a particularly diagnostic molecular biomarker of diatoms [67, 68],
386 was also detected in layer 1 (Fig. 3). Diatoms are usually found at the very top layer of
387 microbial mats where light is available for photosynthesis [82]. In association with
388 cyanobacteria, diatoms provide large quantities of photosynthate to anaerobic organisms [82].
389 A possible higher abundance of diatoms in the deepest layer, as suggested by the layer 4

390 PLFA data and also possibly by the high C_{27}-C_{29} sterene signal [64], is unusual. However,
391 diatoms are capable of using NO_3^- to perform respiration under dark anoxic conditions [83]
392 and have been detected in deep anoxic sediments [84]. High abundances of diatoms have also
393 been detected vertically deep in laminated microbial sediments from the Wadden Sea [69].
394
395 Based on *cyc*$C_{19:0}$ PLFA distribution, PSB appeared to be present in all layers, but
396 were most abundant in layer 2 (Fig. 5). This is in concordance with the distinctly purple
397 colour of layer 2, which distinguishes it from the green/brown colour of the overlying
398 cyanobacterial rich layer 1 (Fig. 1).
399
400 Terpenoid products including 22,29,30-trisnorhop-17(21)-ene, diploptene and C_{31}
401 17β,21β-homohopane were most abundant in the deepest layer (Fig. 2 and 3) suggesting they
402 likely derived from anaerobic organisms such as SRB which have been previously identified
403 as a source of these hopanoids compounds [45]. SRB indicative PLFAs were also detected in
404 highest abundance in the deepest layers (i.e., 10 % and 15 % of the total PLFA signal in layer
405 3 and 4, respectively; Fig. 5). In contrast, layer 1 contained a 6 % representation of SRB
406 PLFAs.
407
408 Previous studies highlighted a mosaic-like distribution of porewater solutes (i.e.
409 sulfide, iron(II), phosphate) within Shark bay microbial mats [11, 29]. Therefore,
410 environmental conditions suitable for most organisms have been observed throughout the
411 different layers of the mat with localised oxidised areas in the deeper parts of the mat and
412 anoxic sulfidic zones in the upper layers. For example, “hotspots” of sulfide with
413 concentrations up to 230 μM were measured within the upper predominantly oxic layer of
414 Shark Bay mats under daylight conditions [11]. Temporal variability also influences

community distributions with zones shifting from inhibitory to stimulatory conditions for certain groups of organisms over a diel cycle [11, 29]. Finally, metabolic versatility also allows organisms to be present in several mat layers or switch metabolism within a specific layer. PSB, for instance, can grow photoautotrophically, photoheterotrophically or as Fe and/or S-oxidising chemoautotrophs [9]. In addition, although SRB are commonly regarded as obligate anaerobic organisms, specific species can undertake limited aerobic respiration [85] and sulfate-reduction has been observed under fully oxic conditions [85, 86]. SRB have been detected in oxic waters and sediments [87–89], as well as oxygen supersaturated mat layers [90–93]. High rates of sulfate reduction have also been measured in the oxic layers of Bahamian [94] and Shark Bay [29] microbial mats. Therefore, due to this complex and highly heterogeneous porewater solute distribution and the metabolic versatility of some organisms, different groups of microorganisms can be detected within a same layer. However, as observed in this study, specific groups tend to dominate in specific layers because of the general trends in light and chemical gradient with depth.

Significance of sulfur in modern stromatolites

Sulfurisation during primary diagenesis, producing organic sulfur compounds (OSCs) with intramolecular or intermolecular linkages, is a key process in the sedimentary preservation of biolipids [95]. Sulfate-reducers have existed on Earth for ca. 3.5 billion years [96] and the activity of these bacteria has played a significant role in preservation of OM in the rock record, for instance in carbonate laminae of the ca. 2.72 Ga Tumbiana stromatolites where sulfur-rich globules representing microbial cells encapsulated in minerals have been identified [97]. SRB were also recently shown to play an important role in the microbially induced carbonate encapsulation of a Devonian crustacean under euxinic conditions, allowing

439 the preservation of intact Devonian sterols [98]. In contemporary sediments, the metabolism
440 of SRB generates H_2S providing a reducing agent for abiotic reduction of biolipids [40].
441
442 In the present study, OSCs were detected in the four mat layers. In layer 4, *n*-C_{37} was
443 present in unusually high abundance (Fig. 7) and might be attributed to haptophyte-derived
444 alkadienes [38]. A similarly prominent *n*-C_{37} has been reported in the sulfurised fraction from
445 a prymnesiophyte impacted sediments [36] and haptophytes have also been reported to occur
446 in other hypersaline and coastal marine microbial mats [99]. Phytane was also identified in
447 the sulfur-bound fraction. We recently detected a high abundance of phytane (and phytene) in
448 the Raney-nickel released sulfur-bound fraction of estuarine sediments associated with a high
449 concentration of monosulfidic black ooze (R. Lockhart, unpublished). Layer 3 presented the
450 highest abundance of sulfur-bound hopanes (C_{27}-C_{31}) and a high proportion of C_{31} 17β,21β-
451 homohopane (Fig. 8). A high relative abundance of ββ hopanes has been previously reported
452 in the sulfur-bound fractions of other sediments, including evaporitic sequences and lignites
453 [36, 100]. Additional hopanes included C_{29} αβ and βα hopanes and C_{30} αβ hopanes. The more
454 thermally stable αβ isomers are usually dominant in crude oils, but have also been reported in
455 recent lacustrine sediments [101] and Holocene peat deposits [102, 103]. Furthermore, a clear
456 predominance of αβ-hopanoids over ββ-hopanoids in a living microbial mat suggests that
457 "geologically" favoured isomers can also be biological metabolites [104]. C_{31} 17β,21β-
458 homohopane is usually attributed to cyanobacteria [44] but was recently detected in high
459 abundance in anoxic mono-sulfidic black ooze sediments (R. Lockhart, unpublished). These
460 results suggest that this hopane could be either derived from cyanobacteria that are dominant
461 microbial communities in this mat or from SRB [45] that are relatively abundant in anoxic
462 parts of the mat. Sulfur-bound steroids were also identified in the deep layer 4 (Fig. 9).

463 Sulfur-bound C_{27} - C_{29} steranes with a predominance of 5α over 5β configuration have been
464 seen in early diagenetic sulfurised sediments [105, 106].
465
466 Sulfur-bound carotenoids were detected at trace levels in the first three layers. The
467 natural product precursors of β-renierapurpurane (detected in layer 1) and renieratane
468 (observed in layers 1 and 2) derive from *Chromatiacea* [107]. Isorenieratane (present in layer
469 3) may originate from isorenieratene present in brown-pigmented *Chlorobi* [108] or
470 actinomycetes [109]. The presence of fully reduced carotenoids in the sulfur bound fraction
471 implies they have been hydrogenated *in-situ* or during the desulfurisation process [110]. Such
472 possible *in-situ* transformation of unstable oxygen-sensitive carotenoids into more stable
473 configurations typical of fossil biomarkers confirms the crucial role that sulfur-cycling
474 organisms play in the preservation of OM in modern stromatolites.
475
476 *Application to ancient microbial mats*
477 The distribution of microbial communities in a well-laminated modern microbial mat
478 from Shark Bay was found to be quite heterogeneous with different microbial groups present
479 within the same layers, although some general trends with depth were evident. Microbial
480 mats contain complex microbial communities that interact with each other and their
481 surrounding environment [111]. The study of layered microbial mats such as the ones
482 observed in Shark Bay may have important implications for the understanding of ancient
483 stromatolites. Whereas previously, only rare glimpses of the details of microbial processes in
484 the formation of ancient mats have been possible (e.g. [97]), the current study provides an
485 alternative means of understanding ancient mat biosignature preservation. Specifically, our
486 study suggests the critical role of the sulfur cycle and biological processes that support it, in
487 the preservation of biolipids. Aerobic heterotrophs present in upper mat layers can strongly

488 degrade biolipids. For instance, in a hypersaline mat from a solar saltern, the concentration of
489 organic carbon decreased by 80% within the first cm [112]. In addition, within the first 2 cm
490 of a hot-spring mat, the concentration of cyanobacterial *n*-C_{17} decreased by 97% while more
491 resistant molecules such as hopanoids decreased by 57% [24]. Therefore, SRB activity can
492 help protect biomolecules from aerobic degradation and promote their preservation at early
493 stages of diagenesis. Preservation of microbial mats can be further enhanced by carbonate
494 precipitation, subsequently leading to the formation of lithified structures commonly referred
495 to as microbialites [113–115]. Highest sulfate-reduction rates in lithifying hypersaline mats
496 are concomitant with areas of maximum carbonate precipitation [116], consistent with the
497 significant role of sulfur cycling organisms in the lithification of stromatolites [117, 118].
498 Further investigation of contemporaneous S-rich systems such as stromatolites and analogous
499 samples may help identify the preservation pathways of specifically functionalised organic
500 compounds.

501

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510

LEGENDS TO FIGURES

Fig. 1: Photography of the microbial smooth mat with indication of the four different layers.

Fig.2: Total ion chromatograms from GC-MS analysis of the aliphatic hydrocarbon fractions from smooth mat layers 1-4. The major steroid products were 5α(H),14α(H),17α(H) 20*R* isomers of cholest-2-ene (C_{27}), 24-methylcholest-2-ene (C_{28}) and 24-ethylcholest-2-ene (C_{29}). $C_{27}H$= is 22,29,30-trisnorhop-17(21)-ene (C_{27}); $C_{30}H$= is hop-22(29)-ene (C_{30}); and $C_{31}H$ is 17β,21β-homohopane (C_{31}).

Fig. 3: Relative abundance of major hydrocarbon groups: *n*-alkanes in blue, isoprenoids in pink, steroids in orange and hopanoids in green. C_{27}, C_{28} and C_{29} sterenes represented the 5α(H),14α(- H),17α(H) 20*R* isomers of cholest-2-ene (C_{27}), 24-methylcholest-2-ene (C_{28}) and 24-ethylcholest-2-ene (C_{29}). C_{27}, C_{30} hopenes and C_{31} hopane represented the 22,29,30-trisnorhop-17(21)-ene (C_{27}), hop-22(29)-ene (C_{30}) and 17β,21β-homohopane (C_{31}). The numbers indicate the percentages of the different compounds.

Fig. 4: $\delta^{13}C$ values of aliphatic hydrocarbons from the different mat layers (1-4) analysed by CSIA. The maximum standard deviation of the $\delta^{13}C$ values is ± 0.2 ‰. The triangle in the red circle represents the C_{25} HBI alkene, the squares in the blue circles indicate hopanoids and the dashes in the green circles the steroids. C_{27}, C_{28} and C_{29} sterenes represented the 5α(H),14α(- H),17α(H) 20*R* isomers of cholest-2-ene (C_{27}), 24-methylcholest-2-ene (C_{28}) and

24-ethylcholest-2-ene (C_{29}). C_{27}, C_{30} hopenes and C_{31} hopane represented the 22,29,30-trisnorhop-17(21)-ene (C_{27}), hop-22(29)-ene (C_{30}) and 17β,21β-homohopane (C_{31}).

Fig. 5: Relative proportion of PLFA groups with depth (diatoms: $C_{16:4w1}$; $C_{20:5w3}$; $C_{20:4w6}$; PSB: *cyc*$C_{19:0}$; SRB: *i*$C_{15:0}$; *ai*$C_{15:0}$; 10-me16:0; *i*$C_{17:0}$; *ai*$C_{17:0}$; $C_{17:1w8}$; $C_{17:1w5}$, others).

Fig. 6: $\delta^{13}C$ values of PLFA from the four mat layers (1-4). The maximum standard deviation of the $\delta^{13}C$ values was ± 0.3‰. The squares in blue circles represent the SRB markers, the triangles in red circles represent the diatom markers and the diamonds in purple circles represent the PSB marker.

Fig. 7: Total Ion Chromatogram from GC-MS analysis of the aliphatic fraction of the sulfur-bound hydrocarbons released from layer 4. The black dots indicate even carbon-numbered *n*-alkanes.

Fig. 8: Mass fragmentogram (*m/z* = 191) from GC-MS analysis of the aliphatic fraction of the sulfur-bound hydrocarbons released from layer 3.

Fig. 9: Partial GC-MRM chromatograms (*m/z 217 → 372; 217 → 400*). A: Steroid standard. B: aliphatic fraction of sulfur-bound hydrocarbons released from layer 4.

LEGENDS TO TABLE

Table 1: Relative abundance of (methyl ester) PLFAs detected in the four layers of the smooth mat. Abundances are expressed as a % of total PLFA signal; *iso: iso* branching; *anteiso*: *anteiso* branching; br: branched at unspecified position; *cyc*: cyclopropyl.

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