

1 **Unusual butane- and pentanetriol-based tetraether lipids in *Methanomassiliicoccus***  
2 ***luminyensis*, a representative of the seventh order of methanogens**

3 Running title: Lipid composition of *Methanomassiliicoccus luminyensis*

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21 **Abstract**

22 A new clade of archaea has recently been proposed to constitute the seventh methanogenic  
23 order, the *Methanomassiliicoccales*, which is related to the *Thermoplasmatales* and the  
24 uncultivated archaeal clades Deep-Sea Hydrothermal Vent *Euryarchaeota* Group 2 and  
25 Marine Group-II *Euryarchaeota*, but only distantly related to other methanogens. In this  
26 study, we investigated the membrane lipid composition of *Methanomassiliicoccus*  
27 *luminyensis*, the sole cultured representative of this seventh order. The lipid inventory of *M.*  
28 *luminyensis* comprises a unique assemblage of novel lipids as well as lipids otherwise typical  
29 for either thermophilic, methanogenic, or halophilic archaea. For instance, glycerol  
30 sesterpanyl-phytanyl diether core lipids mainly found in halophilic archaea were detected,  
31 and so were compounds bearing either heptose or methoxylated glycosidic head groups,  
32 both of which have so far not been reported for other archaea. The absence of quinones or  
33 methanophenazines is consistent with a different biochemistry of methanogenesis compared  
34 to the methanophenazine-containing methylotrophic methanogens. The most distinctive  
35 characteristic of the membrane lipid composition of *M. luminyensis*, however, is the presence  
36 of tetraether lipids in which one glycerol backbone is substituted by either butane- or  
37 pentanetriol, i.e., lipids recently discovered in marine sediments. Butanetriol dibiphytanyl  
38 glycerol tetraether (BDGT) constitutes the most abundant core lipid type (>50% relative  
39 abundance) in *M. luminyensis*. We have thus identified a source for these unusual orphan  
40 lipids. The complementary analysis of diverse marine sediment samples showed that BDGTs  
41 are widespread in anoxic layers, suggesting an environmental significance of  
42 *Methanomassiliicoccales* and/or related BDGT producers beyond gastrointestinal tracts.

43 **Importance**

44 Cellular membranes of members of all three domains of life, *Archaea*, *Bacteria*, and *Eukarya*,  
45 are largely formed by lipids in which glycerol serves as backbone for the hydrophobic alkyl  
46 chains. Recently, however, archaeal tetraether lipids with either butanetriol or pentanetriol as  
47 backbone were identified in marine sediments and attributed to uncultured sediment-dwelling

48 archaea. Here we show that the butanetriol-based dibiphytanyl tetraethers constitute the  
49 major lipids in *Methanomassiliicoccus luminyensis*, the currently only isolate of the novel  
50 seventh order of methanogens. Given the absence of these lipids in a large set of archaeal  
51 isolates, these compounds may be diagnostic for the *Methanomassiliicoccales* and/or closely  
52 related archaea.

53 **Keywords:** methanogens; archaea; *Methanomassiliicoccus luminyensis*; membrane lipids;  
54 butane- and pentanetriol-based tetraether lipids.

## 55 **Introduction**

56 Methane is a potent greenhouse gas and an important intermediate in the global carbon  
57 cycle (1–3). Biogenic methane is predominantly produced by archaea inhabiting diverse  
58 anoxic environments such as sediments, soils, wetlands, and the digestive tracts of termites  
59 and ruminants (2, 4). All cultured methanogens to date belong to the phylum *Euryarchaeota*,  
60 while metagenomic sequencing revealed a putative methanogenic metabolism for members  
61 of the uncultivated *Bathyarchaeota* (formerly known as Miscellaneous Crenarchaeotal Group,  
62 MCG) indicating that methanogenesis might not be restricted to the *Euryarchaeota* (5).

63 Methanogens are classified into seven orders (*Methanobacteriales*, *Methanococcales*,  
64 *Methanomicrobiales*, *Methanosarcinales*, *Methanocellales*, *Methanopyrales* and  
65 *Methanomassiliicoccales*) that generate methane from H<sub>2</sub>/CO<sub>2</sub>, acetate, formate, or  
66 methylated substrates (2, 6–8). Of these, the *Methanomassiliicoccales* have only recently  
67 been described, representing the seventh order of methanogens (6, 7, 9). These  
68 *Euryarchaeota* have been detected based on gene biomarker analyses in diverse  
69 environments such as lakes, soils, and marine sediments, but are particularly abundant in  
70 the digestive tracts of ruminants (10–15). A single pure culture, *Methanomassiliicoccus*  
71 *luminyensis*, as well as a few enrichment cultures have been obtained, all of which reduce  
72 methanol or methylamines with H<sub>2</sub> as electron donor (6, 16–20).

73 The *Methanomassiliicoccales* are only distantly related to other methanogens and form a  
74 distinct cluster within the *Thermoplasmata* with the non-methanogenic thermoacidophilic  
75 *Thermoplasmatales* and other related lineages such as the Deep-Sea Hydrothermal Vent  
76 *Euryarchaeota* Group 2 (DHVEG-2), and the uncultivated Terrestrial Miscellaneous  
77 *Euryarchaeota* Group (TMEG), Marine Benthic Group D (MBG-D), and Marine Group II  
78 *Euryarchaeota* (MG-II; Fig. 1; 7, 21). Especially the latter two groups are widely distributed in  
79 marine sediments and the surface ocean, respectively, but lack cultured representatives (22,  
80 23). Along with the *Methanomassiliicoccales*, MBG-D and other benthic *Euryarchaeota* are of  
81 particular interest in environmental microbiology and geosciences as they could be important  
82 contributors to microbial biomass and activity in the sedimentary biosphere (24–26). In  
83 samples where MBG-D and MG-II dominated 16S rRNA gene libraries, glycerol dibiphytanyl  
84 glycerol tetraethers (GDGTs) have frequently been detected as major archaeal lipids,  
85 indicating that these archaeal groups may be able to synthesize these lipids (27–29).  
86 Moreover, intact GDGTs, e.g., GDGTs attached to glycosidic polar head groups, are  
87 commonly used for quantifying archaeal abundance in the subseafloor biosphere (25, 30–  
88 33). Understanding the potential sources of GDGTs is of primary importance for reliable  
89 quantification of benthic archaeal biomass using lipid biomarkers (31, 32).

90 Here, we report the lipid composition of the sole isolated representative of the  
91 *Methanomassiliicoccales*, *M. luminyensis* strain B10(T). The lipid analyses were facilitated by  
92 recently developed HPLC-MS methods that allow the comprehensive, simultaneous analysis  
93 of archaeal core and intact polar glycerol-based membrane lipids as well as respiratory  
94 quinones, i.e., membrane-bound electron carriers (34, 35). We show that *M. luminyensis*  
95 strain B10(T) contains a diverse suite of unique tetraether lipids with either butanetriol or  
96 pentanetriol substituting a glycerol backbone moiety. Such lipids were recently found in  
97 marine and estuarine sediments, but have not previously been detected in cultured archaeal  
98 representatives (36–38). We further documented the distribution of butanetriol-based lipids in  
99 diverse marine sediments, which suggested the widespread presence of relatives of *M.*  
100 *luminyensis*.

101 **Material and Methods**

102 ***Phylogenetic analysis***

103 High quality 16S rRNA gene sequences of archaeal groups of interest (alignment quality >90,  
104 pintail 100, sequence quality >90) with a minimum length of 1400 nt were obtained from the  
105 SILVA Ref NR SSU r123 database (39). If more than ten sequences per group were  
106 downloaded, the sequences were clustered with 94.5% sequence identity using cd-hit-est of  
107 the CD-HIT Suite (Huang et al 2010) to obtain representative sequences of different genera  
108 (40). After aligning the sequences using the SINA online alignment tool (41) the alignment  
109 was improved by gap removal with Gblocks using the least stringent parameters to avoid  
110 losing phylogenetic information (42). The alignment was uploaded to the Model Selection tool  
111 of the IQ-TREE web server to select the best suited nucleotide substitution model. A  
112 maximum likelihood tree was calculated with IQ-TREE applying the GTR model (+F+I+G4)  
113 (43). Ultrafast bootstrap (1000 replicates) was used to verify branch support (44). FigTree  
114 (<http://tree.bio.ed.ac.uk/software/figtree/>) and Adobe Illustrator (Adobe Systems Inc., San  
115 Jose, CA) were used for visualizing the phylogenetic tree.

116 ***Cultivation and lipid extraction***

117 *M. luminyensis* was grown in an anaerobic medium based on the medium published by Lang  
118 et al. (20). Cultures (2 x 20 mL), inoculated with 10% of a previous culture grown under the  
119 same conditions, were grown in 120 mL serum flasks at 37 °C for 7 days under an  
120 atmosphere containing 80% H<sub>2</sub> and 20% CO<sub>2</sub>. Cells were harvested by centrifugation (20  
121 minutes; 13,000 g) and were subsequently lyophilized.

122 Lipids from *M. luminyensis* were ultrasonically extracted following a modified Bligh & Dyer  
123 protocol (45) using a monophasic mixture of methanol, dichloromethane, and aqueous buffer  
124 (2:1:0.8, v:v:v). A 50 mM phosphate buffer (pH 7.4) was used for the first two extractions  
125 while a 50 mM trichloroacetic acid buffer (pH 2) was used for two additional extractions. The  
126 total lipid extracts (TLE) were dried under a stream of N<sub>2</sub> and stored at -20 °C until

127 measurement. In addition to *M. luminyensis*, twelve marine sediment samples from a variety  
128 of depositional environments (Table 2) were analyzed and prepared as described in Liu et al.  
129 (31).

### 130 ***Intact polar and core lipid analysis***

131 Intact polar and core lipids were analyzed by injecting TLE aliquots dissolved in  
132 methanol:dichloromethane (9:1, v:v) on a Dionex Ultimate 3000 high performance liquid  
133 chromatography (HPLC) system connected to a Bruker maXis Ultra-High Resolution  
134 quadrupole time-of-flight tandem mass spectrometer equipped with an electrospray ion  
135 source operating in positive mode (Bruker Daltonik, Bremen, Germany). The mass  
136 spectrometer was set to a resolving power of 27,000 at  $m/z$  1,222 and every analysis was  
137 mass-calibrated by loop injections of a calibration standard and correction by lock mass,  
138 leading to a mass accuracy of better than 1-3 ppm. Ion source and other MS parameters  
139 were optimized by infusion of standards (acyclic GDGT (GDGT-0), monoglycosidic (1G-)  
140 GDGT-0, diglycosidic (2G-) GDGT-0) into the eluent flow from the LC system using a T-  
141 piece.

142 Analyte separation was achieved using reversed phase (RP) HPLC on an Acquity UPLC  
143 BEH C<sub>18</sub> column (1.7  $\mu$ m, 2.1 x 150 mm, Waters, Eschborn, Germany) maintained at 65 °C  
144 as described by Wörmer et al. (34). The injection volumes was 10  $\mu$ L and analytes were  
145 eluted at a flow rate of 0.4 mL min<sup>-1</sup> using linear gradients of methanol:water (85:15, v:v,  
146 eluent A) to methanol:isopropanol (50:50, v:v, eluent B) both with 0.04% formic acid and  
147 0.1% NH<sub>3</sub>. The initial condition was 100% A held for 2 min, followed by a gradient to 15% B  
148 in 0.1 min and a gradient to 85% B in 18 min. The column was then washed with 100% B for  
149 8 min.

150 To determine relative abundances of core lipids, 50% of the TLE was hydrolyzed with 1 M  
151 HCl in methanol for 3 h at 70 °C to yield core lipids (46). Additionally, biomass was  
152 hydrolyzed directly using 1 M HCl in methanol for 16 h at 70 °C; subsequently lipids were

153 ultrasonically extracted three times from hydrolyzed biomass using DCM:MeOH 5:1 (v:v).  
154 The hydrolyzed TLE and the extract obtained from hydrolyzed biomass were analyzed on the  
155 same HPLC-MS system using normal phase (NP) chromatography and an atmospheric  
156 pressure chemical ionization-II ion source operated in positive mode, as described by Becker  
157 et al. (47). Briefly, hydrolyzed TLE aliquots were dissolved in *n*-hexane:2-propanol (99.5:0.5,  
158 v:v) and injected onto two coupled Acquity BEH Amide columns (2.1 x 150 mm, 1.7  $\mu$ m  
159 particle size, Waters, Eschborn, Germany) maintained at 50 °C. The injection volume was 10  
160  $\mu$ L. Lipids were eluted using linear gradients of *n*-hexane (eluent A) to *n*-hexane:2-propanol  
161 (90:10, v:v; eluent B) at a flow rate of 0.5 mL min<sup>-1</sup>. The initial gradient was 3% B to 5% B in  
162 2 min, followed by increasing B to 10% in 8 min, to 20% in 10 min, to 50% in 15 min and  
163 100% in 10 min, followed by 6 min at 100% B to flush and 9 min at 3% B to re-equilibrate the  
164 columns.

165 Lipids were identified by retention time as well as accurate molecular mass and isotope  
166 pattern match of proposed sum formulas in full scan mode and MS<sup>2</sup> fragment spectra.  
167 Integration of peaks was performed on extracted ion chromatograms of  $\pm$ 10 mDa width and  
168 included the [M+H]<sup>+</sup> ions for NP-HPLC-MS and additionally [M+NH<sub>4</sub>]<sup>+</sup> and [M+Na]<sup>+</sup> ions for  
169 RP-HPLC-MS. Where applicable, doubly charged ions were included in the integration.

170 Lipid abundances were corrected for response factors of commercially available as well as  
171 purified standards. Purified standards were obtained from extracts of *Archaeoglobus fulgidus*  
172 as described in Elling et al. (46). The abundances of monoglycosidic (1G) glycerol  
173 dibiphytanyl glycerol tetraethers (GDGTs) and butanetriol dibiphytanyl glycerol tetraethers  
174 (BDGTs) were corrected for the response of purified acyclic 1G-GDGT standard, while  
175 monoheptose (1Hp)-1G-BDGT was corrected for the response of purified acyclic 2G-GDGT  
176 standard due to the structural similarity of the lipids (Fig.1). The abundances of  
177 phosphatidylglycerol (PG), 1G-PG-BDGTs and 1Hp-1G-PG-BDGT were corrected for the  
178 response of a commercially available 1G-PG-GDGT standard (Matreya LLC, Pleasant Gap,  
179 PA, USA). The abundances of 1G- and 2G-archaeols (ARs) were corrected for the response

180 of respective purified standard, while triglycosidic (3G-) ARs as well as Methoxy-1G  
181 (1MeOG) 1G and 1MeOG-2G-ARs were corrected for the response of 2G-AR. PG-AR  
182 abundances were corrected for the response of a commercial phosphatidylethanolamine  
183 archaeol standard (Avanti Polar Lipids Inc., Alabaster, AL, USA). Due to the lack of  
184 appropriate standards, polyprenols were not corrected for their relative response. The  
185 abundances of core GDGTs, BDGTs, pentanetriol dibiphytanyl glycerol tetraethers (PDGTs),  
186 glycerol dibiphytanol diethers (GDDs) and butanetriol dibiphytanol diethers (BDDs) were  
187 corrected for the response factors of purified GDGT-0, while the abundance of core AR was  
188 corrected for the response factors of the respective purified standard. The lower limit of  
189 detection for lipids was  $< 1 \text{ pg } \mu\text{L}^{-1}$ .

## 190 **Results**

### 191 ***Intact polar and core lipid composition***

192 Eighteen different intact polar lipids (IPLs) with either di- or tetraether core structure and nine  
193 different polar head groups were detected in *M. luminyensis*. Head groups include mono-, di  
194 and trihexose, methoxy hexose, phosphatidylglycerol, monoheptose and combinations of the  
195 different head group types (Fig. 2 and 3). Detected IPLs comprise AR (two  $\text{C}_{20}$  isoprenoid  
196 side chains), GDGT-0, extended (Ext) and diextended (diExt) AR, the latter containing  $\text{C}_{20-25}$   
197 and  $\text{C}_{25-25}$  isoprenoidal chains, respectively, as core lipid structures. Methoxy hexose and  
198 heptose-containing lipids have been tentatively identified by multiple stage mass  
199 spectrometry (Fig. 4, Table 1). Moreover, the dominant compounds were identified as IPLs  
200 possessing a butanetriol dibiphytanyl glycerol tetraether (BDGT) core (Fig. 5, Table 1).  
201 These unusual tetraether lipids are characterized by the replacement of one glycerol moiety  
202 with a butanetriol (37) and have not been found in any other cultured archaea to date. Free  
203 core lipids were relatively abundant and occurred as AR, GDGT, BDGT, as well as GDD and  
204 BDD. Neither GDD nor BDD lipids were detected as IPLs (see Fig. 3). Besides these IPLs  
205 and core lipids, we detected saturated and unsaturated  $\text{C}_{45}$  and  $\text{C}_{50}$  polyprenols, which



206 contained up to one double bond per isoprenoid unit. Methanophenazines and respiratory  
207 quinones were not detected.

208 Di- and tetraether based IPLs with glycosidic head groups account for 49% of the total lipids,  
209 Total phosphate-based lipids comprise 33%, while non-polar free core lipids and polyprenols  
210 contribute the remaining 18%. The most abundant single lipid in *M. luminyensis* is a PG-  
211 BDGT, contributing 20% to the total lipid pool (see Table 1). Phosphatidylglycerol is the  
212 dominant single head group representing 25% of total head groups (Fig. 6a) followed by 1G  
213 and 1Hp-1G with 14% and 16%, respectively, while other head groups are equally distributed  
214 with 8-9%, except for 3G, which showed the lowest relative abundance (3%). The dominant  
215 core structure in *M. luminyensis* in the total di- and tetraether lipid pool, including IPLs, is  
216 BDGT, accounting for more than 50% (Fig. 6b). The second most abundant core lipid is  
217 diExt-AR with 30%, while all other core lipids comprise <10% of total core lipids.

218 Since not all lipids might be solvent-extractable from cells (48, 49), we acid-hydrolyzed the  
219 biomass and compared the core lipid distribution with that obtained from the TLE as well as  
220 to that obtained from acid hydrolysis of the TLE (Fig. 6b). The relative abundance of BDGTs  
221 was substantially higher (up to 82%) in the extracts obtained after acid hydrolysis of the TLE  
222 and direct hydrolysis of the biomass compared to the TLE (Fig. 6b). Similarly, the relative  
223 abundance of GDGTs increased to almost 20%. Consequently, several lipids showed  
224 strongly reduced abundances in the hydrolyzed extracts or were not detectable anymore as  
225 in case of the diether lipids Ext- and diExt-AR. While AR showed a similar relative  
226 abundance in the hydrolyzed TLE compared to direct analysis of the TLE, its abundance was  
227 particularly low in the hydrolyzed biomass extract. As shown by Huguet et al. (49),  
228 concentrations of GDGTs were substantially higher (one to two orders of magnitude) in  
229 directly hydrolyzed biomass compared to regular lipid extraction protocols for *Nitrosopumilus*  
230 *maritimus* biomass. Thus, although we did not generate quantitative information, BDGT and  
231 GDGT concentration in the hydrolyzed biomass might be so high that they overwhelm the  
232 signal of the diether compounds during mass spectrometry. Interestingly, in the hydrolyzed

233 extracts, acyclic to dicyclic PDGT and mono- and bicyclic BDGTs were detected; both  
234 compound groups were not detectable in the TLE.

#### 235 ***Occurrence of BDGTs in the marine environment***

236 To further examine the environmental significance of the unusual BDGTs, we investigated  
237 their distribution in 12 marine sediments from diverse settings (Table 2). BDGTs were  
238 detected in two-thirds of the samples, including the Peru Margin, Hydrate Ridge,  
239 Mediterranean sapropels, Cascadia Margin and Namibia Margin. In these samples, BDGT  
240 core lipids accounted for 0.1 and 3.5% of archaeal core tetraethers (GDGTs + BDGTs; Table  
241 2). BDGTs were not detected in sediments from the Equatorial Pacific, Namibia Margin  
242 surface sediment and the deep subsurface of the Cascadia Margin.

#### 243 **Discussion**

##### 244 ***Lipid inventory of *M. luminyensis* compared to other archaea***

245 The most distinctive characteristics of the membrane lipid composition of *M. luminyensis* are  
246 BDGTs and PDGTs which are present as IPLs and free core lipids and have not been  
247 reported from other archaea. Thus, these unusual lipids may be diagnostic for members or  
248 close relatives of the *Methanomassiliicoccales*. Both, BDGT and PDGT lipids seem to be  
249 selectively bound in the acid-hydrolysable fraction, indicated by higher relative abundances  
250 in the hydrolyzed biomass compared to the Bligh and Dyer extract (Fig. 6). Potentially, these  
251 lipids are preferentially bound to proteins in the membranes and were released by the acid  
252 treatment. In bacteria as well as in archaea, membrane proteins have been shown to  
253 selectively bind lipids, such as fatty acids, isoprenoids, and different phospho- and  
254 glycolipids, influencing the structural and functional integrity of proteins (e.g., 50, 51–53).

255 However, the biological function as well as the biosynthetic pathway of BDGTs and PDGTs  
256 remain unknown. Since the biosynthesis of archaeal membrane lipids typically involves  
257 dihydroxyacetone phosphate (DHAP) as an intermediate that is converted to glycerol-1-

258 phosphate (G1P; 54), a different biosynthetic pathway might be required for butane- and  
259 pentanetriol-containing lipids (37). The genomes of *Methanomassiliicoccales* contain genes  
260 well known to be involved in archaeal ether lipid biosynthesis, including genes encoding  
261 homologues of G1P dehydrogenase, 3-O-geranylgeranyl-*sn*-glycerlyl-1-phosphate (GGGP)  
262 and digeranylgeranylglyceryl phosphate (DGGGP) synthases, and four enzymes responsible  
263 for the activation of the diglyceride, the addition of polar head groups to the glycerol moiety,  
264 and the final production of archaeol via the subsequent reduction of the unsaturated  
265 isoprenoid chains (20, 21). Only one gene for GGGP synthase, and no second homologue to  
266 this gene could be identified that might encode a hypothetical enzyme catalyzing the  
267 formation of a GG-butanetriyl-P or GG-pentanetriyl-P intermediate from butanetriol or  
268 pentanetriol, respectively, and geranylgeranyl diphosphate (GGPP). Nevertheless, future  
269 studies on *Methanomassiliicoccus* BDGT and PDGT biosynthesis might help to elucidate  
270 their unresolved biochemistry. Moreover, although the identification of BDGTs and PDGTs  
271 based on HPLC-MS as well as degradation experiments by Zhu et al. (37) seems conclusive,  
272 the exact structures of these unusual lipids, e.g., the stereochemistry of the butane- and  
273 pentanetriol backbone, remain to be fully resolved, e.g., by using NMR-spectroscopy.

274 *M. luminyensis* further possesses a unique membrane lipid composition of mixed di- and  
275 tetraethers with glycosidic and phosphatidylglycerol head groups, which differs distinctly from  
276 all other methanogens and archaea. While Ext-AR as major core lipid in *M. luminyensis* is  
277 widespread in halophilic archaea (55–57), it is only present in trace amounts in other  
278 methanogens including *Methanosarcina barkeri* (58) and *Methanothermobacter*  
279 *thermautotrophicus* (59). In environmental samples, the detection of Ext-AR has been  
280 frequently associated with methane oxidizing archaea (60–62). The diExt-AR that we  
281 detected in *M. luminyensis* has so far only been reported in halophiles (63) and as the  
282 dominant lipid in the thermophile *Aeropyrum pernix* (64), but not in methanogens. Moreover,  
283 heptose-based membrane lipids have not been reported in Archaea, while heptose is a  
284 common constituent of polysaccharides in Bacteria (e.g., 65, 66, 67).

285 Phosphate-bound polyprenols occur widespread in all domains of life and they mainly  
286 function as membrane-bound (poly-)saccharide carriers involved in cell wall assembly (68–  
287 72). We found abundant phosphate-free polyprenols in *M. luminyensis*, which have  
288 previously been detected in the thermophilic methanogen *M. thermautotrophicus* grown  
289 under hydrogen-limitation; it was suggested that they may play a role in membrane  
290 stabilization (73). Their high relative abundance (14% of total detected lipids, Table 1) in *M.*  
291 *luminyensis* implies an important role of free polyprenols also in this archaeon. However,  
292 their distribution among other archaea has not yet been studied and the function of  
293 phosphate-free polyprenols in archaeal cells remains elusive.

294 The unusual membrane lipid composition of *M. luminyensis* is consistent with its  
295 phylogenetically distant relationship to other orders of methanogens (9). Additionally, in  
296 contrast to the high diversity of respiratory quinones in related members of the  
297 *Thermoplasmatales* (35, 74, 75), no quinones were detected in *M. luminyensis*. Similarly, no  
298 methanophenazines, respiratory quinone-analogs found in *Methanosarcinales* (35, 76) and  
299 *Methanosaeta* (77), were detected in *M. luminyensis*. This finding supports studies of Lang et  
300 al. (20) and Söllinger et al. (15) who suggested that the biochemistry of methanogenesis in  
301 *Methanomassiliicoccales* may be fundamentally different from that of other,  
302 methanophenazine- and cytochrome-containing methylotrophic archaea.

303 We further validated the potential of BDGTs and PDGTs as biomarkers for *M. luminyensis* by  
304 analyzing 25 cultured archaea that we recently analyzed for their respiratory quinone  
305 composition (35). These species cover the phyla *Eury-*, *Cren-* and *Thaumarchaeota* and  
306 within the *Euryarchaeota* several methanogens as well as the *Methanomassiliicoccales*-  
307 related thermoacidophile *Thermoplasma acidophilum*. We did not detect BDGTs or PDGTs in  
308 any of these archaea. This indicates a high chemotaxonomic potential of these lipids for  
309 *Methanomassiliicoccales*, although we cannot exclude that other, uncultured archaeal  
310 lineages also synthesize these lipids. Thus, BDGT biosynthesis might represent another  
311 evolutionarily distinct feature of *Methanomassiliicoccales* similar to the unique pathways for

312 methanogenesis and energy conservation (9, 20, 78). Specific membrane lipid adaptation  
313 within the *Thermoplasmata* is supported by the fact that for example *Aciduliprofundum*  
314 *boonei* belonging to DSHVE-2 contains H-shaped GDGTs (79), while they have not been  
315 reported from *Thermoplasmatales* species (80) as well as were not detected in *M.*  
316 *luminyensis*. Analysis of the lipid inventory of other cultured representatives of the seventh  
317 order of methanogens are, however, required to provide detailed information about the  
318 phylogenetic patterns for the biosynthesis of butane- and pentanetriol-based lipids.

### 319 ***Implications for environmental studies***

320 Butane- and pentanetriol-based tetraether lipids have recently been identified in a number of  
321 environmental settings, such as deeply buried marine (37) and shallow estuarine sediments  
322 (36). Meador et al. (36) suggested a potential association of BDGTs with the Miscellaneous  
323 Crenarchaeotal Group (MCG) due to the positive correlation of BDGT lipids with the relative  
324 abundance of MCG 16S rRNA sequences in microbial communities of estuarine sediments  
325 from the White Oak River, NC, USA (hereafter WOR). The authors further interpreted the  
326 notable <sup>13</sup>C-depletion of BDGTs as evidence for BDGT-producers either being autotrophs or  
327 heterotrophs feeding on <sup>13</sup>C-depleted substrates. While being consistent with the isotopic  
328 lines of evidence, our results suggest that the source of BDGTs in WOR sediments might  
329 instead be members or relatives of the *Methanomassiliicoccales* (see Fig. 1). In fact,  
330 members of the “environmental clade” of *Methanomassiliicoccales* were previously assigned  
331 to TMEG (15). Although *Methanomassiliicoccales* were not specifically described in WOR  
332 sediments, which is likely due to the low coverage of commonly used primers for  
333 *Methanomassiliicoccales*, closely related clades such as MBG-D and TMEG were abundant  
334 in these samples (36, 81). Indeed, we evaluated commonly used primers against  
335 *Methanomassiliicoccales*, including the one used to sequence archaea from WOR sediments  
336 (23, 82) using TestPrime 1.0 and SILVA SSU r126 RefNR database (83), and the coverage  
337 was only between 0.5% and 56% for zero mismatches. Accordingly, the positive correlation  
338 of MCG with BDGTs in the WOR sediments (36) may instead result from the co-occurrence

339 of MCG and *Methanomassiliicoccales* and/or uncultivated *Thermoplasmata*, such as TMEG  
340 and MBG-D, which was observed by operational taxonomic unit network analysis in various  
341 marine sediments (84). However, in samples where MBG-D dominate 16S rRNA clone  
342 libraries, both BDGTs and PDGTs were not detected, while GDGTs were the most abundant  
343 lipids (27). This suggests that members of the MBG-D inhabiting the Pakistan margin  
344 sediments are not a major source for BDGT and PDGT lipids and that BDGT synthesis is  
345 limited to a subgroup within the *Thermoplasmata*.

346 The analysis of twelve globally distributed marine sediments from various environmental  
347 settings revealed the widespread occurrence of BDGT core lipids (Table 2), implying a large  
348 environmental distribution of *Methanomassiliicoccales* and/or relatives that may constitute  
349 additional sources. This supports recent metagenomic studies, which showed that besides  
350 gut and rumen (6, 13, 14) *Methanomassiliicoccales* also occur ubiquitously in marine and  
351 terrestrial anaerobic environments (10, 15, 84, 85). Similarly to WOR, 16S rRNA gene  
352 sequences of *Methanomassiliicoccales* were not reported for the investigated sites likely due  
353 to a mismatch of commonly used primers against hitherto undetected clades (23), while other  
354 uncultured *Thermoplasmatale* were detected (86). In some samples, for instance in the  
355 equatorial Pacific, BDGTs were not detected, which is probably related to low TOC  
356 concentrations (30) and sulfate reducing conditions (87) at this site. The relative abundance  
357 of BDGTs in the 12 samples analyzed in this study (Table 2) is much lower than in the  
358 estuarine WOR sediments, where BDGTs accounted for 15% of the total archaeal core lipid  
359 pool on average (36). While this data indicates that the conditions in the WOR sediments  
360 select for the BDGT-producers, the factors controlling the distribution of the seventh order of  
361 methanogens and how they compete with hydrogenotrophic and methylotrophic  
362 methanogens, as they require both H<sub>2</sub> and methanol, remains a target for future studies. If  
363 future studies confirm the specificity of BDGTs as biomarkers for the seventh order of  
364 methanogens, their detection will enrich strategies for investigating these aspects in  
365 environmental samples, cultivation experiments, and the gastro-intestinal tracts of humans  
366 and ruminant animals (e.g., 14, 17).

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672 **Table and Figure legends**

673 **Table 1.** IPLs and core lipids in the TLE of *M. luminyensis*. Molecular masses of [M+H]<sup>+</sup>,  
674 [M+NH<sub>4</sub>]<sup>+</sup> and [M+Na]<sup>+</sup> adducts in positive ion mode RP-HPLC-MS<sup>1</sup>, diagnostic fragment ions  
675 in MS<sup>2</sup> experiments and relative abundance of lipids are shown. For interpretations of mass  
676 spectra see main text. Abbreviations: AR, glycerol diphytanyl diether (archaeol); Ext-AR,  
677 glycerol sesterpanyl-phytanyl diether (extended archaeol); diExt-AR, glycerol disesterpanyl  
678 diether (diextended archaeol); GDGT, glycerol dibiphytanyl glycerol tetraether; GDD, glycerol  
679 dibiphytanol diether; BDGT, butanetriol dibiphytanyl glycerol tetraether; BDD, butanetriol  
680 dibiphytanol diether; 1G, monoglycosyl; 2G, diglycosyl; 3G, triglycosyl; 1Hp, monoheptose;  
681 PG, phosphatidylglycerol; 1MeOG, methoxyglycosyl.

682 **Table 2.** Percentage of core BDGTs relative to total isoprenoidal core tetraethers  
683 [BDGTs/(BDGTs + GDGTs) x 100] in selected sediment samples (n.d., not detected).  
684 Detailed information on sampling sites has been published in Liu et al. (31, 88).

685 **Fig. 1.** Phylogenetic tree of archaea, including methanogens and clades found in marine  
686 sediments, and the major core lipids described for cultivated and enriched representatives.  
687 Lipid data of *Methanomassiliicoccales* from this study, for other cultivated archaea from 27,  
688 79, 89–93 and for ANME enrichments from 94, 95. The maximum likelihood tree is derived  
689 from nearly full-length 16S rRNA gene sequences. Bootstrap values (1000 replicates) were  
690 calculated to verify branch support (● ≥ 95 %; ○ >80%). The scale bar indicates substitutions  
691 per site. Abbreviations: MG-II, Marine Group II, DHVEG-2, Deep-Sea Hydrothermal Vent  
692 *Euryarchaeota* Group 2; TMEG, Terrestrial Miscellaneous *Euryarchaeota* Group; MBG,  
693 Marine Benthic Group; MG, Marine Group; ANME, anaerobic methanotroph; MCG,  
694 Miscellaneous Crenarchaeotal Group; GDGT, glycerol dibiphytanyl glycerol tetraether;  
695 GTGT, glycerol trialkyl glycerol tetraether; GDD, glycerol dibiphytanyl diether; BDD,  
696 butanetriol dibiphytanyl diether; BDGT, butanetriol dibiphytanyl glycerol tetraether; PDGT,  
697 pentanetriol dibiphytanyl glycerol tetraether; Uns, unsaturated; Ext, extended, OH, hydroxy;  
698 M, macrocyclic; MeO, methoxy; Me, methylated; H, H-shaped.

699 **Fig. 2.** Molecular structures of all identified intact polar and core lipids in  
700 *Methanomassiliicoccus luminyensis*. Lipids include glycerol diphytanyl diether (archaeol),  
701 glycerol sesterpanyl-phytanyl diether (extended archaeol), glycerol disesterpanyl diether  
702 (diextended archaeol), glycerol dibiphytanyl glycerol tetraether (GDGT), glycerol dibiphytanol  
703 diether (GDD), butanetriol dibiphytanyl glycerol tetraether (BDGT), butanetriol dibiphytanol  
704 diether (BDD), pentanetriol dibiphytanyl glycerol tetraether (PDGT) core lipids and saturated  
705 and unsaturated C<sub>45</sub> and C<sub>50</sub> polyprenols with up to one double bond per isoprenoid unit.  
706 BDGT and PDGT core lipids with one and two cyclopentyl moieties are also shown. Intact  
707 polar lipids consist of di- and tetraether core lipids attached to a polar head group.

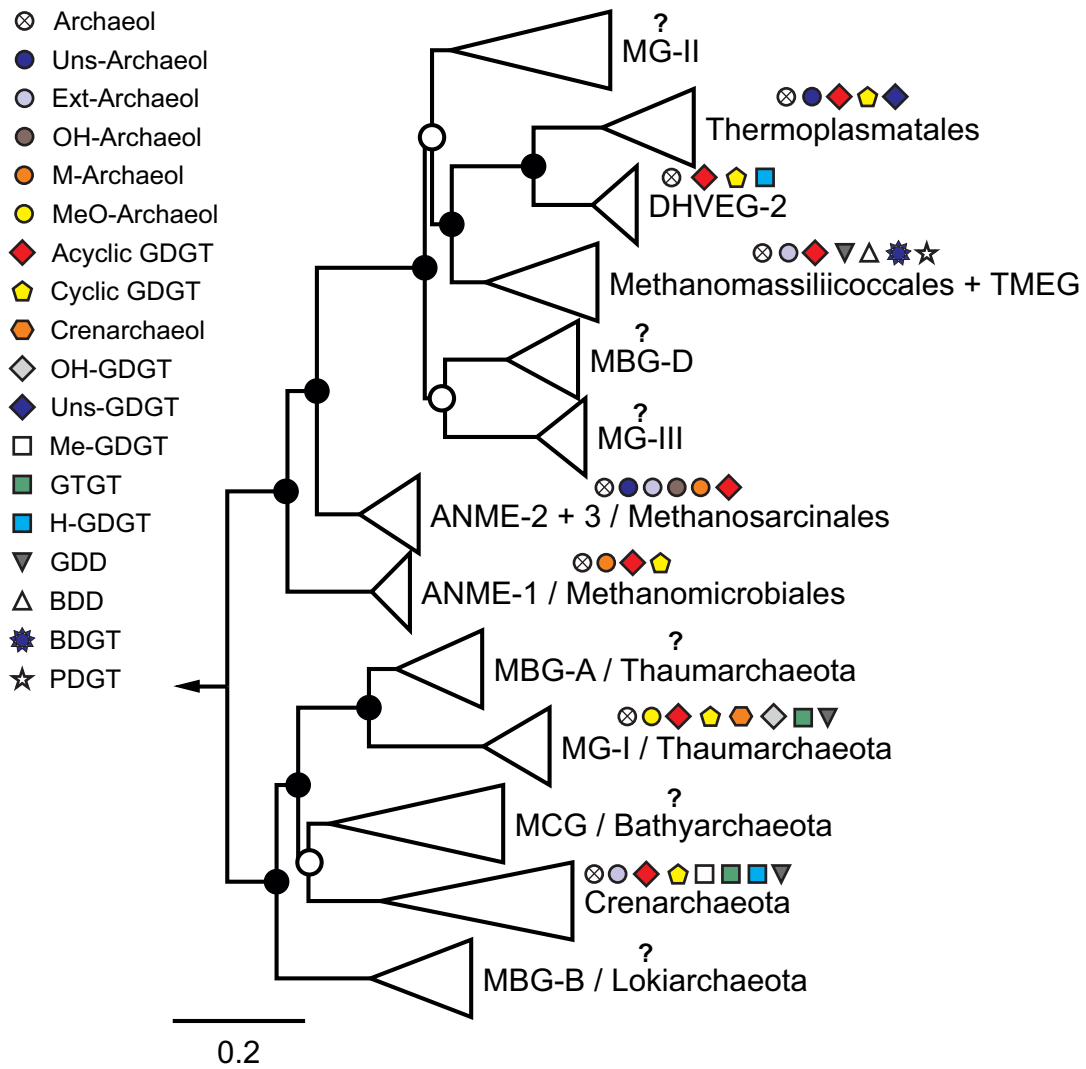
708 **Fig. 3.** Reversed phase HPLC-MS analyses of *M. luminyensis* TLE showing (a) extracted ion  
709 chromatogram of all identified lipids (including C<sub>46</sub>-GTGT injection standard) and (b) density  
710 map plot allowing three-dimensional view on chromatographic separation and mass-to-  
711 charge ratio (*m/z*) with intensity on the z-axis (from low intensities indicated by white colors to  
712 intermediate intensities indicated by blue color and high intensities indicated by red color).  
713 Lipid nomenclature designates combinations of core lipid types (AR, archaeol; Ext-AR,  
714 extended-AR; diExt-AR, diextended-AR; GDGT, glycerol dibiphytanyl glycerol tetraether;  
715 GDD, glycerol dialkanol diether; BDGT, butanetriol dibiphytanyl glycerol tetraether; BDD,  
716 butanetriol dibiphytanol diether) and head groups (PG, phosphatidylglycerol; 1G,  
717 monoglycosyl; 2G, diglycosyl; 3G, triglycosyl; PG, phosphatidyl glycerol; 1Hp-1G,  
718 monoheptose-1G; 1G-PG; 1Hp-1G-PG; 1MeOG-1G, methoxy-1G; 1MeOG-2G). For  
719 structures of lipids see Fig. 2.

720 **Fig. 4.** MS<sup>2</sup> spectra of ammoniated ([M+NH<sub>4</sub>]<sup>+</sup>) 1Hp-1G-BDGT (*m/z* 1687.5) and 1MeOG-1G-  
721 AR (*m/z* 1078.9), respectively. The chemical structures and the formation of major product  
722 ions are also drawn. The glycerol extension in the BDGT structure is either located at *sn*-1 or  
723 *sn*-3 positions of the glycerol. Both, 1MeOG and 1Hp head group structures have only been  
724 tentatively identified based on their exact mass in full scan and MS<sup>2</sup> experiments and their  
725 full characterization requires further structural elucidation. However, for example for the peak

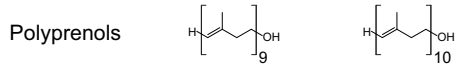
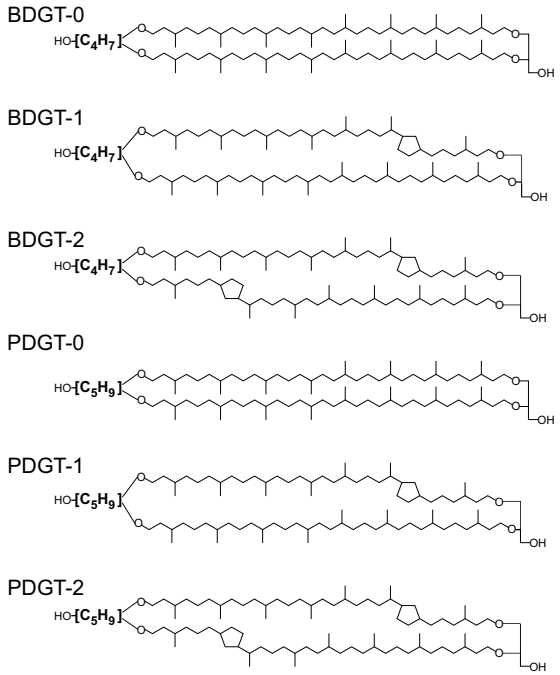
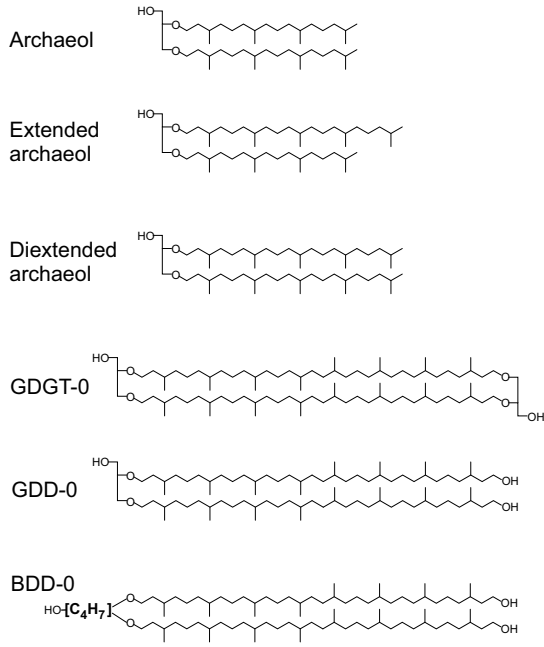
726 at  $m/z$  1078.9  $[M+NH_4]^+$  we observed a dominant fragment ion associated with core Ext-AR  
727 (62) in the MS<sup>2</sup> spectrum, resulting from a neutral loss of 1G + 176.1 Da + NH<sub>3</sub> and likely  
728 indicating a methylated dihexose head group (96). We interpreted the spectrum to represent  
729 a 1MeO-1G-Ext-AR. Similarly, we observed a loss of 2G + CH<sub>2</sub>O + NH<sub>3</sub> (354.1 Da) and  
730 BDGT core lipid fragment ions (37, 38) for the peak at  $m/z$  1687.3  $[M+NH_4]^+$  and tentatively  
731 identified this IPL as heptose-containing lipid, 1Hp-1G-BDGT. The polar head group is either  
732 located at the glycerol or butanetriol moiety.

733 **Fig. 5.** (a) Magnified section of density map plot in the tetraether area showing the major  
734 diagnostic ions of butanetriol and corresponding solely glycerol containing lipids in the TLE of  
735 *M. luminyensis*, analyzed by RP-HPLC-MS. (b) and (c) show MS<sup>2</sup> spectra of sodiated  
736 ( $[M+Na]^+$ ) core BDGT ( $m/z$  1338.3) and 1G-BDGT ( $m/z$  1500.4), respectively. BDGT spectra  
737 match those shown by Zhu et al. (37) and Knappy et al. (38). The glycerol extension in the  
738 BDGT structure is either located at *sn*-1 or *sn*-3 positions of the glycerol and the polar head  
739 group of intact BDGTs is either located at the glycerol or butanetriol moiety.

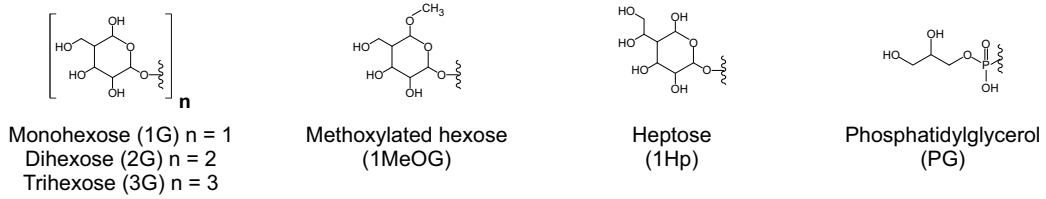
740 **Fig. 6.** (a) Relative abundance of different head groups in the TLE of *M. luminyensis*. (b)  
741 Relative abundance of core lipids in the TLE, acid hydrolyzed TLE and acid hydrolyzed  
742 biomass of *M. luminyensis*. For the TLE, free and head group-bound core lipids were  
743 considered. For chemical structures and abbreviations see Fig. 2.

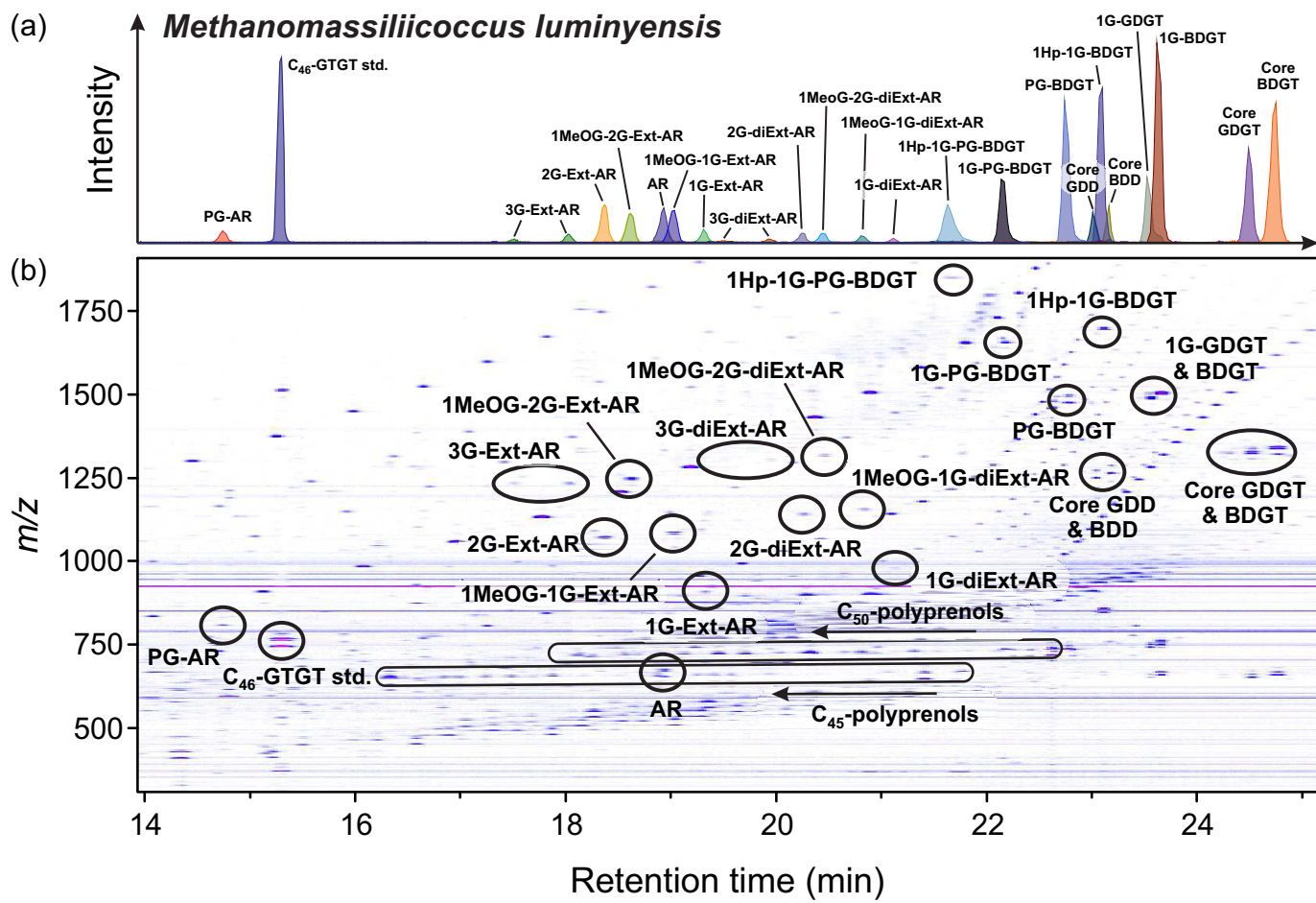


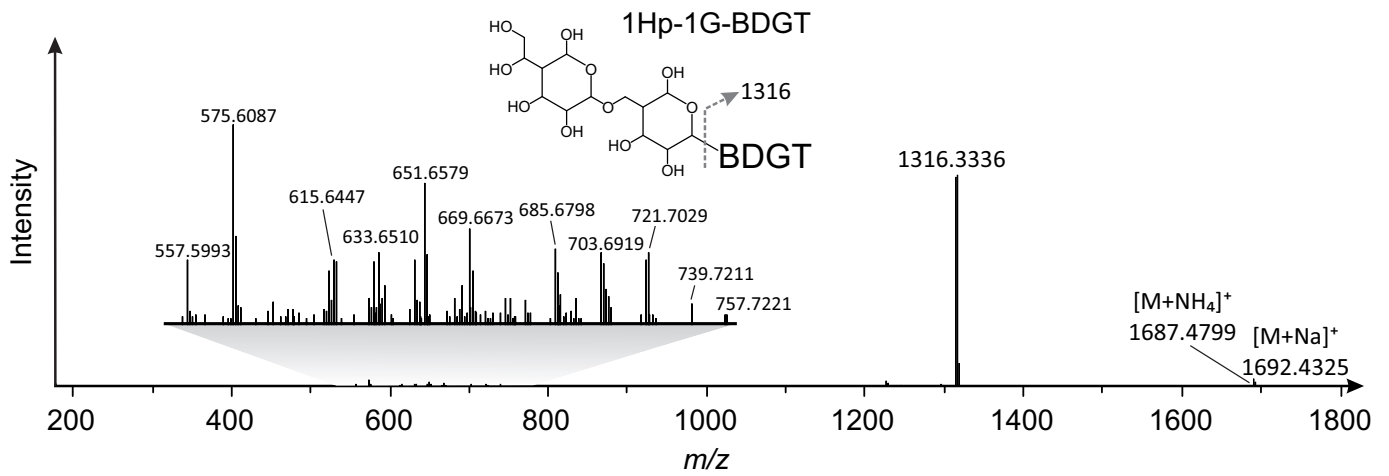
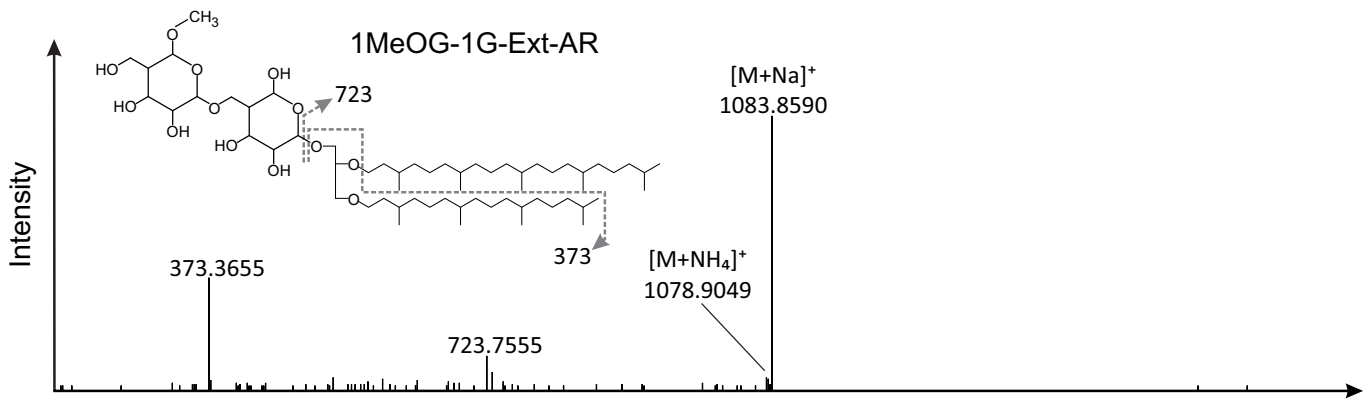
**Core lipids**



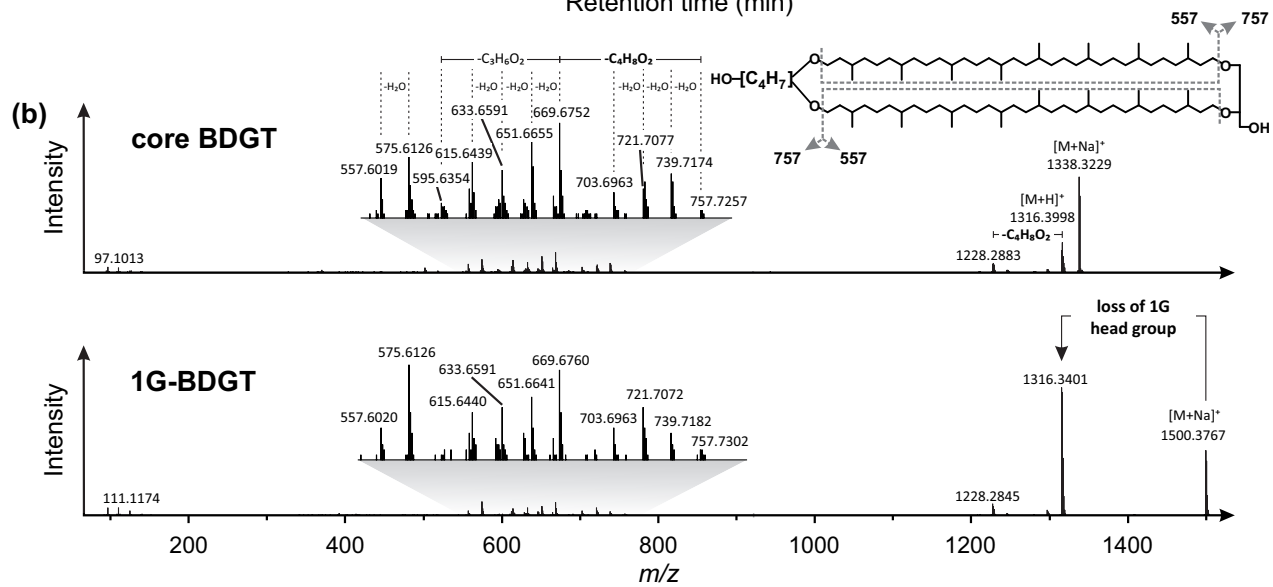
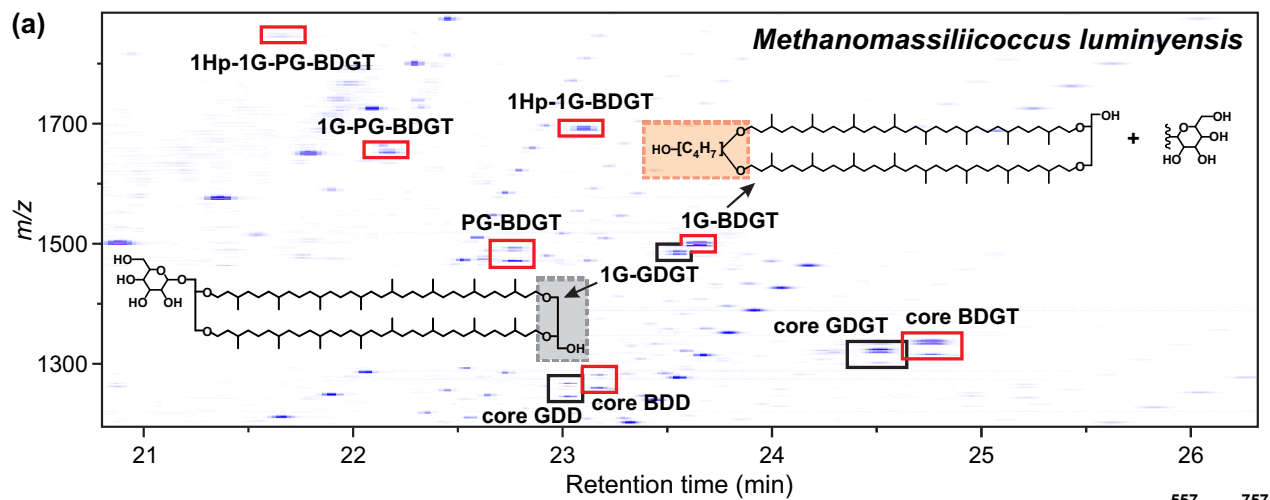
**Head groups**

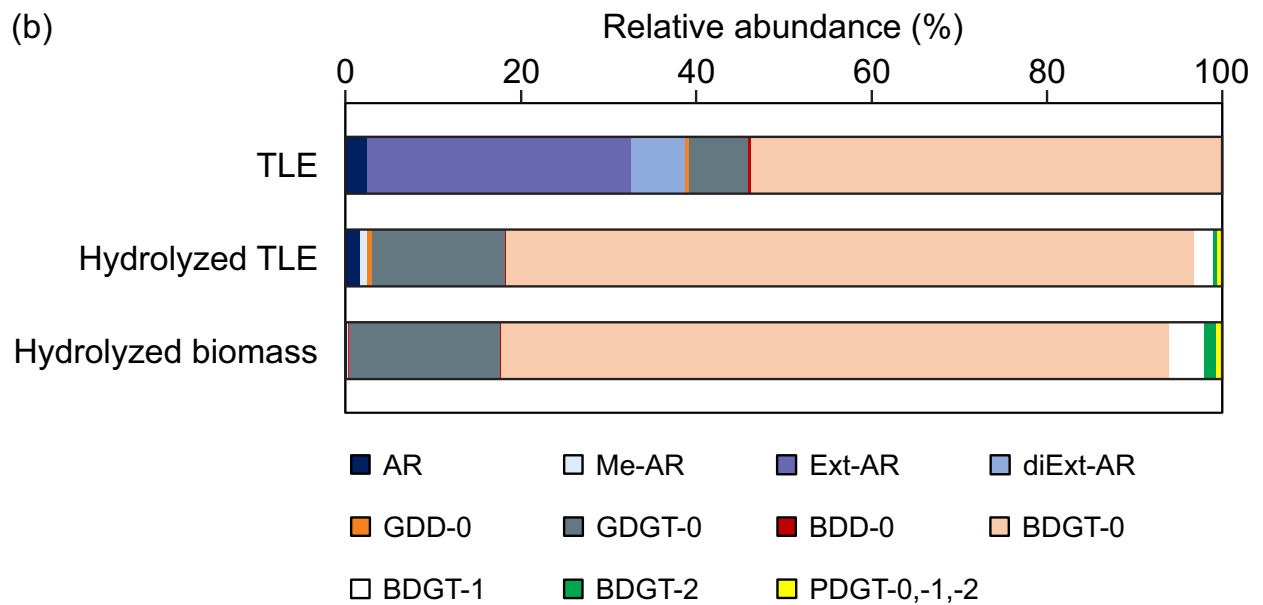
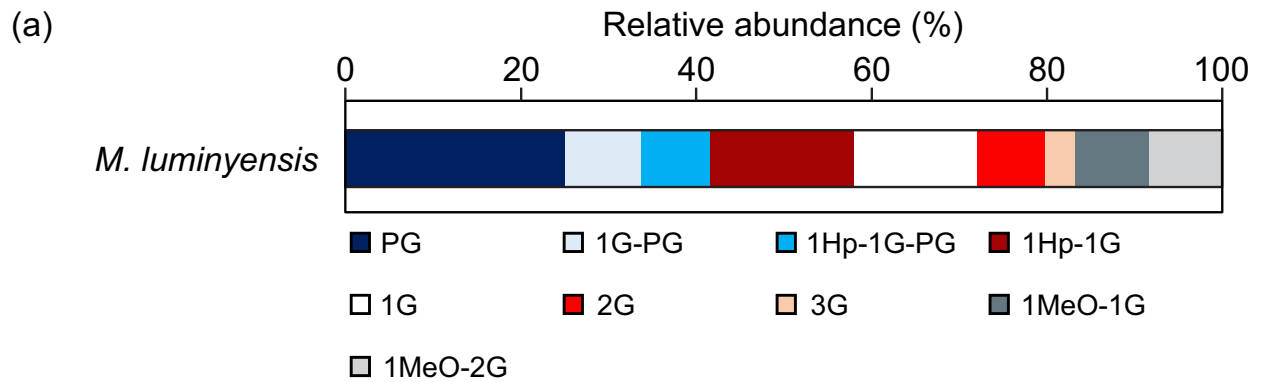












Compound	$m/z$ ( $[M+H]^+$ ; $[M+NH_4]^+$ ; $[M+Na]^+$ )	Characteristic fragment ions in MS <sup>2</sup>	Retention time (min)	Relative lipid abundance (%)
GDD	1246.2965; 1263.3230; 1268.2784	669.7	23.0	0.3
BDD	1260.3121; 1277.3387; 1282.2940	683.7	23.2	0.3
GDGT	1302.3227; 1319.3492; 1324.3046	743.7	24.5	1.3
BDGT	1316.3383; 1333.3649; 1338.3203	757.7	24.8	2.1
1G-GDGT	1464.3755; 1481.4020; 1486.3574	1302.3; 743.7	23.5	3.0
1G-BDGT	1478.3911; 1495.4177; 1500.3731	1316.3; 757.7	23.6	6.7
PG-BDGT	1470.3414; 1487.3680; 1492.3234	a)	22.8	19.0
1G-PG-BDGT	1632.3943; 1649.4208; 1654.3762	1470.3	22.2	6.8
1Hp-1G-BDGT	1670.4545; 1687.4811; 1692.4365	1316.3	23.1	13.1
1Hp-1G-PG-BDGT	1824.4576; 1841.4842; 1846.4396	b)	21.6	6.2
AR	653.6806; 670.7072; 675.6626	373.4	19.0	0.8
PG-AR	807.6837; 824.7103; 829.6657	733.6; 537.4	14.8	0.8
1G-Ext-AR	885.8117; 902.8382; 907.7936	373.4; 443.5; 723.8	19.3	1.1
1G-diExt-AR	955.8899; 972.9165; 977.8719	443.5; 793.8	21.1	0.4
1MeOG-1G-Ext-AR	1061.8802; 1078.9067; 1083.8621	373.4; 443.5; 723.8	19.0	5.5
2G-Ext-AR	1047.8645; 1064.8911; 1069.8465	373.4; 443.5; 723.8	18.4	6.1
1MeOG-1G-diExt-AR	1131.9584; 1148.9850; 1153.9404	443.5; 793.8	20.8	1.3
2G-diExt-AR	1117.9428; 1134.9693; 1139.9247	443.5; 793.8	20.3	1.7
1MeOG-2G-Ext-AR	1223.9330; 1240.9595; 1245.9149	373.4; 443.5; 723.8	18.6	4.9
3G-Ext-AR (a)	1209.9173; 1226.9439; 1231.8993	373.4; 443.5; 723.8	17.5	0.6
3G-Ext-AR (b)	1209.9173; 1226.9439; 1231.8993	373.4; 443.5; 723.8	18.0	1.4
1MeOG-2G-diExt-AR	1294.0112; 1311.0378; 1315.9932	443.5; 793.8	20.5	1.6
3G-diExt-AR (a)	1279.9956; 1297.0221; 1301.9775	443.5; 793.8	19.5	0.3
3G-diExt-AR (b)	1279.9956; 1297.0221; 1301.9775	443.5; 793.8	20.0	0.5
C <sub>50:1</sub> - C <sub>50:10</sub> polyprenols	699.6-717.8; 716.7-734.8; 721.6-739.8	loss of H <sub>2</sub> O (-18.0 Da)	18.1-22.6	8.5
C <sub>45:0</sub> - C <sub>45:9</sub> polyprenols	631.6-649.7; 648.6- 666.7; 653.6-671.7	loss of H <sub>2</sub> O (-18.0 Da)	16.4-21.7	5.6

a) fragmentation not fully resolved

b) no MS<sup>2</sup> data available

Cruise	Site and Core	Sediment depth (m)	Total organic carbon (wt%)	% BDGTs
M76/1	GeoB12806-2	0.1	8.9	n.d.
(Namibia Margin)	GeoB12807-2	3.1	7.4	0.21
ODP201	1229D 4H4	30.7	4.7	0.36
(Peru Margin)	1229A 22H1	185.9	0.47	3.5
ODP201	1226B 10H3	83.8	1.1	n.d.
(Equatorial Pacific)	1226E 20H3	320	0.28	n.d.
ODP204	1250D 6H5	43.5	0.96	1.1
(Hydrate Ridge)	1250D 12H5	100.3	1.3	0.12
IODP311	1237C 10H5	79.8	0.64	n.d.
(Cascadia Margin)	1237C 13C6	109.8	0.56	0.21
ODP 160	966C 5H02	40	5.7	0.81
(Mediterranean Sapropel)	966C 7H04	65	7.4	0.34