1 **Stable carbon isotopic compositions of intact polar lipids reveal complex carbon flow**

2 **patterns among hydrocarbon degrading microbial communities at the Chapopote**

3 **asphalt volcano**

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23 **Abstract**

24   Seepage of asphalt forms the basis of a cold seep system at 3000 m water depth at the

25 Chapopote Knoll in the southern Gulf of Mexico. Anaerobic microbial communities are

26 stimulated in the oil-impregnated sediments as evidenced by the presence of intact polar

27 membrane lipids (IPLs) derived from Archaea and Bacteria at depths up to 7 m below the

28 seafloor. Detailed investigation of stable carbon isotope composition (δ^{13}C) of alkyl and acyl

29 moieties derived from a range of IPL precursors with distinct polar head groups resolved the

30 complexity of carbon metabolisms and utilization of diverse carbon sources by uncultured

31 microbial communities. In surface sediments most of the polar lipid-derived fatty acids with

32 phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and disphosphatidylglycerol

33 (DPG) head groups could be tentatively assigned to autotrophic sulfate-reducing bacteria,

34 with a relatively small proportion involved in the anaerobic oxidation of methane.

35 Derivatives of phosphatidyl-(N)-methylethanolamine (PME) were abundant and could be

36 predominantly assigned to heterotrophic oil-degrading bacteria. Archaeal IPLs with

37 phosphate-based hydroxyarchaeols and diglycosidic glyceroldibiphytanylglyceroltetraethers

38 (GDGTs) were assigned to methanotrophic archaea of the ANME-2 and ANME-1 cluster,

39 respectively, whereas δ^{13}C values of phosphate-based archaeols and mixed phosphate-based

40 and diglycosidic GDGTs point to methanogenic archaea. At a 7 m deep sulfate-methane

41 transition zone that is linked to the upward movement of gas-laden petroleum, a distinct

42 increase in abundance of archaeal IPLs such as phosphate-based hydroxyarchaeols and

43 diglycosidic archaeol and GDGTs is observed; their δ^{13}C values are consistent with their

44 origin from both methanotrophic and methanogenic archaea. This study reveals previously

45 hidden, highly complex patterns in the carbon-flow of versatile microbial communities

46 involved in the degradation of heavy oil including hydrocarbon gases that would not have

47 been evident from classical compound-specific isotope analyses of either bulk IPL or apolar

48 lipid derivatives.

49

50   **1. INTRODUCTION**

51

52   The microbial remediation of pollutants and xenobiotics, such as spilled oil introduced

53 into the environment by human activities, is of great socioeconomic interest. In the marine

54 environment, sulfate-reducing bacteria (SRB) are the most important anaerobic degraders of

55 petroleum hydrocarbons (Widdel et al., 2003). The capability of SRB to degrade both

56 aliphatic and aromatic compounds present in crude oil has been observed both in cultures

57 (Rueter et al., 1994; Rabus et al., 1996; Musat et al., 2009) and in natural environments

58 (Connan, 1984; Lovley, 1997; Caldwell et al., 1998). Petroleum seeps in the Gulf of Mexico

59 (GoM) are an ideal location to study the natural processes of hydrocarbon degradation,

60 because there microbial life is stimulated by both oil and methane (Sassen, 1980; Sassen et

61 al., 1993; Aharon et al., 2000; Joye et al., 2004). An important process at petroleum seeps in

62 the GoM is the anaerobic oxidation of methane (AOM; Joye et al., 2004; Orcutt et al., 2005),

63 which is mediated by a consortium of methanotrophic archaea and SRB (Hinrichs et al., 1999;

64 Boetius et al., 2000). This process is of global relevance because it accounts for the

65 consumption of up to 90% of the greenhouse gas methane within marine sediments

66 (Reeburgh, 2007; Knittel and Boetius, 2009).

67   Specific types of bacterial polar lipid fatty acids (PLFAs) and archaeal ether lipids are

68 useful chemotaxonomic biomarkers for certain groups of microorganisms. PLFA analyses

69 have been successfully used to monitor bioremediation of petroleum hydrocarbons by specific

70 groups of SRB (e.g., Aries et al., 2001; Pelz et al., 2001). However, so far intact polar lipids

71 have not been targeted in these studies. While PLFAs can be diagnostic with regard to the

72   source organisms (e.g., Dowling et al., 1986; Kaneda, 1991), the valuable information on

73   head group-lipid relationships is lost during these analyses. In order to increase specificity, it

74   is recommended to analyze PLFAs in combination with head group type (Fang et al., 2000).

75   This statement can be extended to archaeal lipids, which also encode taxonomic information

76   in their polar head group (cf. Koga and Nakano, 2008; Rossel et al., 2008). Microbial

77   signatures in the northern GoM have been investigated by non-polar lipid biomarkers,

78   complemented with stable carbon isotope studies as well as with gene-based analyses (Zhang

79   et al., 2002, 2003; Orcutt et al., 2005; Pancost et al., 2005; Lloyd et al., 2006). These studies

80   have shown that at methane seeps in the GoM, as in similar environments worldwide, AOM is

81   an important process. However, due to the additional presence of oil, AOM and sulfate-

82   reduction are often decoupled and sulfate reduction rates exceed rates of AOM due to the

83   additional presence of oil-degrading SRB (Joye et al., 2004; Orcutt et al., 2005).

84   At the Chapopote Knoll, located in the northwest of the Campeche Knolls, in the

85   southern GoM, extensive asphalt deposits cover an area of over 1 km^2 in 3000 m water depth

86   (MacDonald et al. 2004; Brüning et al., 2010). Heavily biodegraded oil at this location is

87   apparently a result of intense microbial activity in the asphalts and sediments, as demonstrated

88   by the presence of intact polar lipids (IPLs; Schubotz et al., *in press*) and the formation of

89   authigenic carbonates (Nähr et al., 2009). The asphalt flows at the Chapopote Knolls therefore

90   pose a natural laboratory for the biodegradation of heavy oils.

91   In this study we will investigate the effect of petroleum and methane seepage on the

92   microbial community composition as evidenced by the distribution and isotopic composition

93   of microbial lipids and interpret the results in terms of metabolic function and sedimentary

94   carbon flow. We will compare surface sediments that were retrieved from a zone of high

95   fluid flow and active petroleum seepage with deeper sediments at a sulfate-methane transition

96   zone (SMTZ) between 6 and 8 meters below seafloor (mbsf), the latter showing the presence

97   of petroleum in the deeper parts of the core (Fig. 1, Schubotz et al., *in press*).   The

98   mechanistic insights gained from this study will be relevant to our understanding of the

99   benthic microbial response to anthropogenic inputs of petroleum such as the Deep Water

100 Horizon oil spill in the Northern Gulf of Mexico. The analytical approach extends previously

101 applied compound-specific stable carbon isotope analysis (CSIA) of bulk IPLs to CSIA of

102 individual IPLs that were separated according to head group polarity.   This extension

103 diminishes mixing of signals from various taxonomic groups and provides more and detailed

104 insights into the complexity of the microbial community structure.

105

106   **2. MATERIAL AND METHODS**

107

108 *2.1. Sampling*

109

110   Sediment samples were retrieved during Meteor expedition M67/2 in March to April

111 2006 (Bohrmann and Spiess et al., 2008). Sediment cores were recovered by gravity coring

112 and by ROV operated push coring. Push core GeoB10619 (21°54,333'N, 93°26,497'E) was

113 recovered in close vicinity of the main asphalt site (Brüning et al., 2010; Schubotz et al., *in*

114 *press*).   The deep core GeoB10610 (21°54,25'N, 93°25,88'E) was retrieved approximately

115 ~1 km northwest of the main asphalt site in the trough of the Chapopote Knoll 'crater', where

116 oil slicks were observed on the surface of the water.

117

118 *2.2. Lipid biomarkers*

119

120 *2.2.1. Extraction of IPLs*

121

122   IPLs were extracted from freeze-dried surface and deep sediments with a modified

123   Bligh and Dyer method according to the detailed description in Schubotz et al. (*in press*). To

124   have sufficient material for stable carbon isotope analyses of surface sediment samples were

125   combined from several depth intervals were combined resulting in two samples: 2.5-

126   10 centimeters below seafloor (cmbsf) and 10-15 cmbsf. In brief, 4 mL solvent mixture of

127   dichloromethane:methanol:buffer (DCM:MeOH:buffer, 1:2:0.8; v/v/v) was added to 1 g

128   sediment and ultrasonicated for 10 min in four steps. In the first two extraction steps a

129   phosphate buffer was used (pH 7.4) and the last two steps were done with a trichloroacetic

130   acid buffer (50 g/L, pH 2.0). After each ultrasonication step the mixture was centrifuged and

131   the supernatants collected in a separatory funnel. The solvent composition was adjusted to

132   achieve a final ratio of DCM/MeOH/buffer of 1:1:0.8 (v:v:v) by further adding DCM and

133   deionized (MilliQ) water. The organic phase was washed three times with MilliQ water,

134   carefully reduced to dryness under a stream of nitrogen at 37°C, and stored as total lipid

135   extract (TLE) at -20°C until analysis.

136

137   *2.2.2. Analysis of IPLs by liquid chromatography – mass spectrometry (LC-MS)*

138

139   The analysis of IPLs was previously described in Sturt et al. (2004), briefly, an aliquot

140   of the TLE was re-dissolved in DCM:MeOH (5:1) and injected on to a ThermoFinnigan

141   Surveyor HPLC System equipped with a LiChrosphere Diol-100 column (2.1 x 150 mm;

142   Alltech, Germany) coupled to a ThermoFinnigan LCQ Deca XP Plus ion trap mass

143   spectrometer using an electrospray ionization (ESI) interface.   Instrument settings were

144   previously described by Sturt et al. (2004). Identification of compounds was based on mass

145   spectral information including complementary fragmentation patterns in positive and negative

146   ionization mode and verification with standards as well as previously published data (Sturt et

147 al., 2004; Rossel et al., 2011; Schubotz et al. *in press*). Concentrations were calculated from

148 the relative response of the analyte compared to an injection standard (di-C$_{19}$-PC, see Table 1

149 for IPL abbreviation) and corrected by relative response factors of different IPL standards

150 relative to the injection standard (for details see Schubotz et al., *in press*). For those IPLs

151 lacking suitable reference standards, the mean response factor derived from the available IPL

152 standards (di-C$_{17}$-PG, di-C$_{16}$-PA, di-C$_{16}$-PE, di-C$_{16}$-PS, Gly-DAG, 2Gly-DAG, PC-AR,

153 Gly-GDGT-PG; Matreya, USA, Avanti Polar Lipids, USA) was used for correction. The

154 range of response factors of these IPL standards relative to the injection standard (di-C$_{19}$-PC)

155 varied between a factor of 0.5 and 1.2.

156

157 *2.2.3. Preparation of fractions enriched in IPL classes*

158

159 Preparation of IPL-enriched fractions and simultaneous removal of petroleum hydrocarbons

160 was achieved by preparative HPLC following the protocol by Biddle et al. (2006). A

161 preparative LiChrosphere Si60 column (250 x 10 mm, 5 um, Alltech, Germany) was

162 connected to a ThermoFinnigan Surveyor HPLC equipped with a Gilson FC204 fraction

163 collector. The flow rate was set to 1.5 mL min^{-1}, and the eluent gradient was: 100% A to

164 100% B in 120 min, hold at 100% B for 30 min, then 30 min column re-equilibration with

165 100% A, where eluent A was composed of *n*-hexane/2-propanol (79:20, v:v) and eluent B was

166 2-propanol/MilliQ water (90:10, v:v). In order to determine the fraction collection time

167 windows a micro-splitter was placed between column and fraction collector and part of the

168 flow was split to a ThermoFinnigan LCQ Deca XP Plus mass spectrometer after post-column

169 infusion of ammonium formate in methanol with a tee-piece and a syringe pump. A test

170 sample containing representative compounds (core GDGTs, 1Gly-GDGT, 2Gly-GDGT, PE-

171 DAG and PG-DAG) was then injected and fraction collection time windows were set

172   according to the test sample. In total 14 fraction were collected over 120 min (Table 1). All

173   sample fractions were reanalyzed on the analytical column to check the separation and purity

174   of IPL classes (Fig. 2).

175

176   *2.2.4. Preparation of polar lipid fatty acids (PLFAs) and alcohols*

177

178   To obtain the apolar derivatives of the IPLs, an aliquot of the TLE was saponified according

179   to the method described by Elvert et al. (2003). In brief, an aliquot of the polar fraction was

180   dissolved in 2 mL of methanolic KOH (6% KOH in MeOH, w/v) and the reaction took place

181   for 3 h at 80°C in an oven. The mixture was vortexed several times. After cooling to room

182   temperature, 2 mL of a 0.05M KCl solution were added and phospholipid-derived alcohols

183   were extracted by three times shaking with 2 mL *n*-hexane. The pH value was adjusted to

184   pH 1 with 25% HCl and fatty acids were extracted by three times shaking with 2 mL *n*-

185   hexane. After drying under a nitrogen stream, the reaction products were stored at -20°C until

186   derivatization and analysis.

187

188   *2.2.5.Preparation of hydrocarbon derivatives from IPLs*

189

190   Preparation of apolar derivatives of ether-bound IPLs was conducted following the

191   procedure by Jahnke et al. (2002). In brief, an aliquot of the intact GDGT-containing

192   fractions (Table 1) was dissolved in 200 μL of BBr_3 in DCM (Sigma-Aldrich, Germany). The

193   cleavage reaction took place under argon atmosphere at 60°C for 2 h. After cooling, the

194   mixture was carefully evaporated under an argon stream before 200 μL of a reaction mixture

195   of superhydride (lithium triethylborohydride) in THF (Sigma-Aldrich, Germany) was added

196   under argon atmosphere. The mixture was put in an oven for 2 h at 60°C before cooling

197 down to room temperature. 200 μL of de-ionized MilliQ water was added to quench the

198 reaction and hydrocarbons were extracted by washing three times with 500 μL hexane. After

199 evaporation under a nitrogen stream, the reaction mixture was separated on a silica column

200 (500 mg, Supelco, Germany) using 5 mL of hexane to prepare a clean hydrocarbon fraction.

201

202 *2.2.6. Gas chromatographic techniques*

203

204 The apolar derivatives of IPLs were analyzed by gas chromatography (GC) using three

205 different detectors: (*i*) GC-mass spectrometry (MS) for identification of compounds, (*ii*) GC-

206 flame ionization detection (FID) for quantification of compounds, and (*iii*) GC-isotope ratio

207 (ir)MS for determination of the stable carbon isotopic compositions.

208 Prior to analysis, an aliquot of the PFLAs and alcohols was derivatized with

209 bis-(trimethylsilyl)trifluoroacetamide (BSTFA, Merck, Germany) in pyridine at 70°C for 1 h

210 to synthesize trimethylsilyl(TMS)-derivatives. The derivatives were dissolved in hexane,

211 squalane was added as injection standard, and the mixture was analyzed on a ThermoFinnigan

212 Trace GC coupled to a ThermoFinnigan TraceMS for structural identification through mass

213 spectral information. The MS was operated in electron impact mode at 70 eV with a full scan

214 mass range of *m/z* 40-800. Determination of double bond positions of PLFAs and

215 monoalkylglycerolethers (MAGEs) was achieved via formation of dimethyldisulfide adducts

216 following the procedure by Elvert et al. (2003). Double bond determination was not possible

217 for some minor compounds, i.e. PLFAs $C_{14:1}$ and $C_{17:1}$ and MAGE $C_{17:1}$. For determination of

218 relative abundances, the GC was coupled to a FID. Compound-specific stable carbon isotopic

219 compositions were determined on a ThermoFinnigan GC coupled to a ThermoFinnigan

220 Deltaplus XP isotope ratio MS via GC-combustion interface, some replicates were also

221 measured on a MAT 252 mass spectrometer connected via a Finnigan Combustion Interface

222   III to a HP Series GC. The initial oven temperature was held at 60°C for 1 min, increased to

223   150°C with a rate of 10°C min^{-1}, then raised to a temperature of 310°C with a rate of 4°C min^{-}

224   ^{1} and held at 310°C for 35 min. The carrier gas was helium with a constant flow of 1.0 ml

225   min^{-1}. The isotopic compositions of the TMS-derivatives were corrected for the additional

226   methyl groups introduced during derivatization. The precision of replicate analysis (n=2) was

227   ≤1‰. All isotopic values are reported in the delta notation as $\delta^{13}C$ relative to the Vienna

228   PeeDee Belemnite (VPDB) Standard.

229

230   **3. RESULTS**

231

232   *3.2. PLFA distribution and $\delta^{13}C$ of individual bacterial IPLs*

233

234   In order to gain more insights into the biological source organisms of the polar lipids at

235   site GeoB10619 we separated the IPLs according to their head group by preparative HPLC

236   and analyzed the respective PLFA composition. Preparative LC yielded three different

237   fractions with phospholipids for each of the two depth intervals (Fig. 3, Appendix 1). The

238   first phospholipid fraction (F8) contains almost exclusively phosphatidylethanolamine

239   diacylglycerol (PE-DAG; >98%), the second fraction (F9) contains a mixture of PE (41 to

240   46%), diphosphatidylglycerol (DPG, cardiolipin; 24 to 29%), phosphatidylglycerol (PG; <1 to

241   13%), phosphatidic acid (PA; <1 to 5%) and an IPL with an unidentified head group (UK; 7

242   to 33%) with DAG structures. The last phospholipid fraction (F10) contains phosphatidyl-

243   (N)-methylethanolamine (PME, 30-98%), with small admixtures of phosphatidyl-(N,N)-

244   dimethylethanolamine (PDME) and in the depth interval 2.5-10 cmbsf also PA-DAG

245   (Table 2). Phosphatidylcholine (PC)-DAG, although previously detected during TLE analysis

246   of the total lipid extract (Fig. 1; Schubotz et al., *in press*), could not be recovered in any of the

247   fractions due to its retention on the preparative LC column.

248   Distinct differences in the fatty acid (FA) composition of the three fractions become

249   apparent. PLFAs of F8, mainly derived from PE, are dominated by $C_{16:0}$, *ai*-$C_{15:0}$ and $C_{14:0}$.

250   Other major FAs are *i*-$C_{15:0}$, *i*-$C_{16:0}$, $C_{15:0}$, $C_{16:1\omega7}$, and $C_{18:0}$ (Fig. 3, Appendix 1 and 2).

251   Notably, F8 also contains a diverse suite of methyl branched FAs in the carbon number range

252    $C_{14:0}$ to $C_{17:0}$ With depth the PLFA distribution remains similar, but $C_{18:1\omega7}$ and C_{17} FAs are

253   more abundant. There is some variety in the $\delta^{13}C$ of the PLFAs: saturated C_{16} and C_{18} FAs

254   have lowest $\delta^{13}C$ values ranging from -30 to -25‰, while highest ^{13}C-depletions are observed

255   for *ai*-$C_{17:0}$, $C_{17:1}$, $C_{16:1\omega5}$ and 10me$C_{16:0}$ ranging from -55 to -50‰ (Fig. 3, Appendix 1,). F9,

256   composed of a mixture of five IPLs with different head groups, has a very similar PLFA

257   distribution to F8, but $C_{16:0}$ is less abundant and monounsaturated C_{16} and C_{18} FA ($C_{16:1\omega7}$,

258    $C_{16:1\omega5}$, $C_{18:1\omega7}$, and $C_{18:1\omega9}$) become more dominant. Similar to F8, the relative abundance of

259    $C_{18:1\omega7}$ as well as $C_{16:1\omega7}$ and $C_{16:0}$ increases with depth. The distribution of $\delta^{13}C$ values is also

260   comparable to F8: higher $\delta^{13}C$ values are observed for the saturated C_{16} and C_{18} FAs, and

261    $C_{16:1\omega7}$ is comparably enriched in ^{13}C (-28 to -26‰) and minimal values are again found for

262   *ai*-$C_{17:0}$, 10me$C_{16:0}$ and $C_{16:1\omega5}$ (-55 to -53‰). F10 is distinct from the other two fractions as it

263   contains only minor amounts of methyl branched FAs and is almost solely dominated by $C_{16:0}$

264   (up to 31% of total FAs, Table 2). The FA *ai*-$C_{15:0}$ comprises only a minor part of PLFAs,

265   and monounsaturated C_{18} FAs are more abundant (Fig. 3, Appendix 2).   The PLFA

266   distribution of F10 does not change greatly with depth. The $\delta^{13}C$ values are also distinct from

267   F8 and F9: Fatty acids are generally enriched in ^{13}C, averaging at -29 and -26‰ (Table 2);

268   most enriched are saturated C_{16} and C_{18} and monounsaturated C_{18} FAs (-27 to -17‰). The

269   lowest isotopic values are observed for *ai*-$C_{15:0}$, *ai*-$C_{17:0}$ and 10me$C_{16:0}$ (Fig. 3, Appendix 1).

270

271   *3.3. Distribution of bacterial polar alkylglycerolethers*

272

273   The ether linked alcohols in MAGEs of the polar lipids collected in F9 resemble the

274   distribution of the FAs: *ai*-$C_{15:0}$ and $C_{16:0}$ and $C_{14:0}$ are the predominant MAGEs, followed by

275     $C_{16:1\omega7}$, $C_{16:1\omega5}$, 10Me-$C_{16:0}$ and $C_{14:0}$. The carbon isotopic compositions of all MAGEs are

276   depleted in ^{13}C throughout the core and range from -64‰ to -39‰ (Fig. 3, Appendix 1). The

277   most abundant dialkylglycerolether (DAGE) is composed of two *ai*-$C_{15:0}$ alkyl chains and its

278   δ^{13}C fluctuates from -46‰ (2.5 to 10 cmbsf) to -47‰ (10 to 17.5 cmbsf).

279

280   *3.4. Separation of archaeal diether and tetraether IPLs*

281

282   In the surface sediments at site GeoB10619 the most abundant archaeal diether lipid

283   was hydroxyarchaeol (OH-AR) with phosphate-based head groups PG, PA,

284   phosphatidylinositol (PI) and phosphatidylserine (PS), followed by extended hydroxyarchaeol

285   (OH-eAR) with PI as head group (Fig. 1, Schubotz et al., *in press*). After fractionation of IPL

286   classes with preparative LC, we were not able to separate all the OH-ARs according to their

287   head group. In total we obtained three fractions: F8 comprised of PG-OH-AR, F9, which

288   contains mixtures of PG- (29-49%), PA- (43-62%), and PI-OH-AR (8-10%), and a third

289   fraction (F10) with solely PS-OH-AR (Table 1). Additionally, after preparative sample clean

290   up we could detect intact archaeols (ARs) with PG and PE head groups in the prepared

291   fractions (Fig. 2), that were not detected previously due to a high limit of detection during

292   TLE analysis. Intact polar (IP)-ARs were separated into PG-AR in one fraction (F8) and a

293   mixture of PG (0-17%) and PE-ARs (83-100%) in the other fraction (F9). Carbon isotopic

294   compositions for OH-ARs and ARs with different head groups were very similar (Table 2);

295   for OH-eARs we could not obtain isotope values due to their low concentration. The δ^{13}C

296   values for OH-ARs ranged from -106 to -104‰. In comparison, all P-ARs were enriched in

297   ^{13}C compared to the P-OH-ARs with values around -73 to -64‰.

298   The main GDGTs in the surface sediments (2.5 to 17.5 cm) were 2Gly-GDGT, 2Gly-

299   GDGT-PG, PG-GDGT-PG and PG-GDGT. We were able to separate 2Gly-GDGT from

300   PG-GDGT and the remaining IP-GDGTs by preparative LC. Ether cleavage of GDGTs

301   obtained from the individual head group fractions revealed very low isotopic compositions for

302   GDGT-derived biphytanes (bp). The most negative δ^{13}C values were observed for

303   2Gly-GDGT, where isotopic values of bp with 0 to 3 cyclopentane rings (bp0 to bp3) ranged

304   from -94‰ to -82‰. PG-GDGT was more enriched in ^{13}C with values ranging from -82 to

305   -62‰. The remaining IP-GDGTs contained bp0 to bp2 with values spanning from -84 to

306   -63‰. For all IP-GDGTs, bp0 was 6 to 17‰ more enriched in ^{13}C compared to bp1 and bp2.

307   The most abundant archaeal diether IPLs in the deep sediments (600 to 800 cmbsf) from

308   core GeoB10610 were PE-OH-AR and 2Gly-AR (Fig. 1). Since concentrations of these

309   compounds were low, saponification and acid hydrolysis of the corresponding preparative

310   fractions (F7 and F9) did not result in sufficient yields for isotopic analysis. Therefore, we

311   analyzed the corresponding phytane after ether cleavage of the 2Gly-AR containing fraction

312   (F7). For PE-OH-AR, not enough material was left after saponification to conduct ether

313   cleavage. Therefore, we used the core lipid fraction (F3) in which the presence of OH-AR

314   and AR was confirmed by HPLC-APCI-MS analysis (cf. Turich et al., 2007). The phytane of

315   the ether-cleaved F3 fraction represented a mixture of AR and OH-AR, derived most likely

316   from the degradation of 2Gly-AR (<12%) and PE-OH-AR (>88%; Fig. 1). Since the δ^{13}C

317   values for phytane from F3 and F7 were very similar we assume that also the δ^{13}C values for

318   2Gly-AR and PE-OH-AR are similar (Table 3).

319   The most abundant intact tetraether lipid in the deep SMTZ was 2Gly-GDGT. After

320   preparative LC we also detected small amounts of 1Gly- and 3Gly-GDGT at 680 and 710

321   cmbsf (<1% of total IPLs). 1Gly-, 2Gly-, and 3Gly-GDGT could be separated according to

322   their head groups prior to δ^{13}C analysis (Table 1), however, no carbon isotope values could be

323   determined for 3Gly-GDGT due to its low amounts in all samples. δ^{13}C values for bp0 and

324   bp1 were most negative for both 1Gly and 2Gly-GDGTs (-96‰ to -74‰), highest δ^{13}C values

325   were observed for bp3-cren (-18‰ to -21‰), i.e., the biphytane with two cyclopentane rings

326   and one cyclohexane ring derived from crenarchaeol (Sinninghe-Damsté et al., 2002). Core

327   GDGTs were found in F3 with less ^{13}C-depleted carbon isotopic compositions for bp0 and

328   bp1 that ranged from -64 to -50‰, highest δ^{13}C values were observed for bp2 and bp3-cren,

329   spanning from -25 to -18‰ (Table 3).

330

331   **4. DISCUSSION**

332

333   *4.1. Distribution and carbon sources of bacteria in oily surface sediments*

334

335   Bacterial IPLs in the oil-impregnated surface sediments of site GeoB10619 are

336   dominated by DAG phospholipids with PE, PME, PG, DPG, PA, and PC head groups (Fig.

337   1). Some of these phospholipids probably derive from SRB based on IPL and PLFA

338   inventories found in cultures (e.g., Makula and Finnerty, 1974; Taylor and Parkes, 1983) and

339   on sulfate being the most plausible electron acceptor during hydrocarbon degradation in the

340   studied sediment cores (Fig. 1; Schubotz et al., *in press*). However, head group specific δ^{13}C

341   analysis testifies to a variety of different types of carbon metabolisms among members of the

342   bacterial community, and suggests the presence of additional bacterial groups that are

343   involved in the degradation of the oil.

344

345   *4.1.1. Autotrophic bacteria*

346

347   A distinct feature of the PLFA from F8 and F9 (mainly containing PE, PG, DPG, PA

348   head groups and an unknown head group at 10-17.5 cm) is the unusual variety of branched

349    $C_{14:0}$ to $C_{17:0}$ fatty acids (Fig. 3, Appendix 2). Branched and odd-carbon-numbered fatty acids

350   are typical components in cultures of SRB (Boon et al., 1977; Taylor and Parkes, 1983;

351   Dowling et al., 1986). The only other bacteria known to produce branched fatty acids, apart

352   from SRB are gram-positive Bacillus and Actinomycetes (Kaneda 1991; Wallace et al., 1995).

353   However, we can exclude the latter as potential source organism in these sediments since

354   many Bacillus and Actinomycetes species contain additional unique IPLs in their cell

355   membrane, such as surfactin (cf. Hue et al., 2001) and glycolipids (e.g., Shaw, 1970) that

356   were not detected in this study. The known fractionation between dissolved CO_2 and

357   dissolved inorganic carbon (DIC) of ca. -10‰ (Mook et al., 1974) at the *in situ* temperature (4

358   to 10°C; Bohrmann and Spiess et al., 2008; Klapp et al., 2010) and *in situ* pH between 7.3 to

359   7.6 (Bohrmann and Spiess et al., 2008) is applied to estimate the $\delta^{13}C$ of CO_2 from the

360   measured DIC (Fig. 1). The $\Delta\delta^{13}C$ values of branched $C_{15:0}$ and $C_{17:0}$ relative to CO_2 range

361   from -14 to -26‰. This wide range in isotopic values suggests a mixed signal of heterotrophic

362   and autotrophic growth, with the largest difference of around -26‰ in branched PLFA being

363   consistent with a predominantly autotrophic metabolism of their putative source organisms,

364   i.e. SRB (Preuß et al., 1989; Londry and Des Marais, 2003; Londry et al., 2004).

365   Another distinct feature in the PLFA distribution of F8 and F9, which contain the

366   common SRB phospholipids PE, PG and DPG (Makula and Finnerty, 1974), is the high

367   amount of *ai*-$C_{15:0}$ and $C_{16:1\omega5}$. Both FAs have been used as markers for SRB associated with

368   anaerobic methanotrophs of the ANME-1 and ANME-2 clusters, respectively (cf. Niemann

369   and Elvert, 2008), and were also observed as prominent FAs in *Desulfosarcina variabilis*

370   (Rütters et al., 2001), a cultured representative of the *Desulfosarcina/Desulfoccocus* (DSS)

371   group involved in AOM. The close association of these PLFAs to the process of AOM is

372   underlined by lower $\delta^{13}C$ values compared to all other FAs, with average $\delta^{13}C$ values of

373   -45±5‰ (Appendix 1). Nevertheless, the resulting $\Delta\delta^{13}C$ relative to CO_2 of -17±4‰ is not in

374   the range of the typically observed $\Delta\delta^{13}C$ of -40 to -50‰ for other AOM-dominated systems

375   (Hinrichs and Boetius, 2002), including the northern GoM (Zhang et al., 2002; Orcutt et al.,

376   2005). Even the most ^{13}C-depleted PLFAs found among the C_{17} cluster ($10meC_{16:0}$, $C_{17:1}$ and

377   ai-$C_{17:0}$ with PE, PG, DPG, PA, and unidentified head groups) still show a $\Delta\delta^{13}C$ relative to

378   CO_2 of less than -23±3‰, which is in the range of autotrophic growth using either the

379   acetyl-CoA-carbon monoxide dehydrogenase pathway or the reversed TCA cycle for carbon

380   assimilation (Londry et al., 2004). Sources for the above mentioned monounsaturated and

381   branched C_{17} fatty acids could also be SRBs that are able degrade petroleum hydrocarbons,

382   such as *Desulfobacter*, *Desulfobacterales* and *Desulfovibrio* (e.g., Kohring et al., 1994).

383   Taking this into consideration, we consequently assign the higher ^{13}C-enrichment of the

384   PLFAs typically associated with AOM to a mixed signal of autotrophic SRB directly involved

385   in AOM (Wegener et al., 2008) and other non AOM-related SRBs being either autotrophs as

386   well or heterotrophs (Fig.4; Londry et al., 2004). We note that other, non-specified bacterial

387   sources could have likely contributed to this group of PLFAs as well.

388   Decoupling of sulfate reduction from AOM is common at hydrocarbon seep sites in the

389   northern GoM due to the additional degradation of petroleum by SRB (e.g., Joye et al. 2004;

390   Formolo et al., 2004; Orcutt et al., 2005; Lloyd et al., 2006). The relatively less negative

391   carbon isotope values for the typical AOM-related PLFAs fortify the importance of petroleum

392   hydrocarbon degradation in the oil-impregnated sediments at the Chapopote asphalt volcano.

393   Solely some of the MAGEs ($C_{14:0}$, $C_{16:1\omega5}$, and $C_{17:1}$) derived from intact

394   acyl/alkyletherglycerols (AEG) show higher $\Delta\delta^{13}C$ values of up to -34‰ relative to CO_2 (Fig.

395   4). Therefore it can be assumed that these compounds are mainly derived from autotrophic

396   SRB, partly involved in AOM, which matches previous findings (Hinrichs et al., 2000;

397   Pancost et al., 2001; Niemann and Elvert, 2008). In SRB cultures MAGEs have been detected

398   in *Desulfosarcina variabilis* and *Desulforhabdus amnigenus* (Rütters et al., 2001), therefore

399   SRB are indeed likely precursor organisms for these compounds. It is noteworthy that lowest

400   carbon isotope values in PLFAs are always associated with PE, PG, DPG, PA and an

401   unidentified head group (Fig. 3, 4), indicating that autotrophic SRB, partly involved in AOM,

402   are responsible for their production.

403

404   *4.1.2. Heterotrophic bacteria*

405

406   Within the PLFAs of F8, F9 and F10 there is considerable variation in δ^{13}C; FAs with

407   δ^{13}C values close to those of total organic carbon (TOC) are observed in all three

408   phospholipid fractions and are mainly comprised of saturated C_{16} to C_{18} and monounsaturated

409   C_{18} fatty acids (Fig. 3, Appendix 1). Although these FAs are considered as generic, they are

410   often the most abundant FAs in oil-degrading bacteria (e.g., Kohring et al., 1994, Aries et al.,

411   2001). Based on the δ^{13}C values, we therefore assign these PLFAs to mainly heterotrophic

412   oil-degrading bacteria, in accordance with observed isotopic relationships of heterotrophic

413   SRB in laboratory studies (Londry et al., 2004) and heterotrophic bacteria in general (Blair et

414   al., 1985; Hayes, 2001). The presence of $10MeC_{16:0}$, $cyC_{17:0}$, *ai*-$C_{15:0}$ and $C_{16:1\omega5}$ points to

415   *Desulfobacterium sp.* or *Desulfobacter sp.* as potential SRB source candidates (Taylor and

416   Parkes, 1983; Dowling et al., 1986; Kohring et al., 1994; Londry et al., 2004). Members of

417   the Desulfobacteraceae (e.g., *Desulfobacterium*, *Desulfobacter* or *Desulfobacula*), which are

418   known degraders of aromatic hydrocarbons (Rabus et al., 2003; Musat et al., 2009), are

419   indeed present in these sediments, as indicated by gene-based analyses (Orcutt et al., 2010;

420   Santillano et al., 2010). For some of the FAs, particularly for saturated and monounsaturated

421   C_{18} FAs, even an enrichment in ^{13}C of up to 10‰ relative to TOC is found (Fig. 4). Such

422   enrichments relative to the substrate have been observed for SRB under substrate limitation

423   during heterotrophic growth, however, until now only in SRB of the gram-positive

424   Firmicutes, which utilize the carbon monoxide dehydrogenase (CODH) pathway during

425   carbon assimilation (Londry et al., 2004).

426   Comparison of the compound-specific $\delta^{13}C$ values of the different IPL fractions suggest

427   that heterotrophic and autotrophic bacteria produce different head groups: mean $\delta^{13}C$ values

428   of PLFAs from F8 and F9, containing PE, PG, DPG, PA, and the UK head groups, are

429   consistently ^{13}C-depleted relative to CO_2 by -8‰ to -15‰ (Figs. 3 and 4, Appendix 1),

430   reflecting autotrophy as primary carbon metabolism (Londry et al., 2004). By contrast,

431   PLFAs from F10, containing mainly PME, PDME and PA head groups, have a comparably

432   lower mean $\Delta\delta^{13}C$ relative to CO_2 of 4 to -7‰ with $\delta^{13}C$ values more closely resembling

433   those of TOC (Figs. 3 and 4, Appendix 1). Therefore we conclude that oil-degrading bacteria

434   mainly synthesize PLFAs with PME and PDME head groups containing predominantly

435   saturated and monounsaturated C_{16} and C_{18} PLFAs, while PE, PG and DPG head groups with

436   branched and odd-carbon numbered PLFAs and alkylglycerolethers are mainly synthesized by

437   autotrophic (AOM-associated) SRBs (cf. Fig. 4). Since SRB are known to predominantly

438   synthesize PE, PG and DPG head groups (Makula and Finnerty, 1974; Rütters et al., 2001;

439   Sturt et al., 2004; Seidel, 2009), we suggest that other oil-degrading bacteria, could be

440   potential sources for PME- and PDME-DAGs. These could include nitrifying bacteria, or

441   members of the Alpha- or Gammaproteobacteria, Bacteroides-Flavobacteria, and also some

442   gram-positive bacteria (Sohlenkamp et al., 2003).

443

444   *4.2. Distribution of methanotrophic and methanogenic archaea in oily surface sediments*

445

446   Abundant archaeal IPLs with OH-AR and GDGT core structures in surface sediments at

447   site GeoB10619 were assigned to both methanotrophic and methanogenic archaea (Schubotz

448   et al., *in press*). Since methanotrophic and methanogenic archaea are likely to produce very

449   similar lipids (cf. Koga and Nakano, 2008; Rossel et al., 2008), lipid $\delta^{13}C$ values are required

450   for assessing the relative importance of these two microbial groups in marine sediments.

451

452   *4.2.1. Archaeol- and hydroxyarchaeol-based IPLs*

453

454   Almost all archaeal IPLs in surface sediments are more depleted in ^{13}C than methane,

455   indicating a major contribution of methane-derived carbon to archaeal biomass in these depth

456   intervals. However, there are strong variations in $\delta^{13}C$ of different archaeal diethers and

457   tetraethers and also between lipids of different polar head group classes. Compounds that are

458   most depleted in ^{13}C relative to methane by -40 to -42‰ are OH-ARs of F8 and F9 (Fig. 4),

459   with only little variations between different phospho-based head groups (Table 2). This strong

460   depletion relative to methane is consistent with field observations where lipids from

461   methanotrophic archaea are typically depleted in ^{13}C relative to methane by 40 to 50‰

462   (Hinrichs et al., 2000; Niemann and Elvert, 2008). ARs with PG and PE head groups have

463   substantially more positive $\delta^{13}C$ values than OH-AR and are only 1 to 8‰ enriched in ^{13}C

464   relative to methane, which is similar to observations in gas hydrate-rich sediments from

465   Hydrate Ridge (Elvert et al., 2005). Therefore an exclusive methanotrophic source for these

466   lipids is unlikely. $\Delta\delta^{13}C$ values of IP-AR relative to CO_2 are around -33 to -50‰ (Fig. 4).

467   Methanogenic archaea are known to have fractionation factors of -23 to -45‰ between

468   biomass and CO_2 during autotrophic methanogenesis (House et al., 2003), with an additional

469   observed fractionation factor from lipid to biomass of up to -18‰ (Hayes, 2001). Recent

470   studies confirmed that fractionation between lipid and CO_2 can be as high as -47‰ under H_2

471 limitation, and around -14‰ with abundant H_2 present (Londry et al., 2008). Consequently,

472 these large isotopic differences are consistent with isotopic fractionation and synthesis of

473 P-AR by autotrophic methanogens under H_2 limitation. In addition, admixtures from

474 acetoclastic methanogens are conceivable. However, since the fractionation between substrate

475 and archaeol can be as little as -5‰ in *M. barkeri* (Londry et al., 2008), strongly ^{13}C-depleted

476 acetate as found at some methane seeps (Heuer et al., 2006) would be required. In summary,

477 P-OH-ARs in our samples are derived from methanotrophic archaea, probably ANME-2,

478 while P-ARs predominantly have a methanogenic source, possibly of a mixture of autotrophic

479 and acetoclastic methanogens.

480

481 *4.2.2. GDGT-based IPLs*

482

483 In the surface sediments (0 to 17.5 cm), IP-GDGTs were observed with both phospho-

484 based and diglycosidic polar head groups or mixtures of both (Fig. 1a). The analysis of

485 biphytanes derived from IP-GDGTs with different head groups revealed distinct patterns in

486 $\delta^{13}C$ values. Biphytanes of 2Gly-GDGT showed maximum ^{13}C-depletion with $\delta^{13}C$ ranging

487 from -94 to -82‰, i.e., 20‰ ^{13}C-enriched relative to the IP-OH-AR and up to 30‰ [13]C-

488 depleted compared to the IP-AR. Consequently the $\delta^{13}C$ values of biphytanes from

489 2Gly-GDGT most likely represent a mixture of methanotrophic and methanogenic archaeal

490 sources. This suggestion is supported by the observed shift to more negative isotopic values

491 with increasing sediment depth, mirroring the increasing ^{13}C-depletion of CO_2 with constant

492 $\Delta\delta^{13}C$ values of the biphytanes relative to CO_2 between -57 to -67‰ (Fig. 4), strongly

493 suggesting CO_2 as carbon source utilized by the 2Gly-GDGT-producing archaea. Rossel et al.

494 (2011) proposed that at seep environments 2Gly-GDGTs were primarily derived from

495 methanotrophic archaea of the ANME-1 cluster. The presence of ANME-1 archaea could

496   also explain the lower fractionation of biphytanes in ^{13}C compared for the OH-ARs: δ^{13}C

497   analyses on whole cells and cell aggregates have have shown that ANME-1 archaea tend to be

498   enriched in ^{13}C relative to ANME-2 archaea (Orphan et al., 2002). The variation in δ^{13}C of

499   the IP-GDGT-derived biphytanes of one IPL class within one depth interval is minimal.

500   Nevertheless, bp0 can be up to 10‰ enriched in ^{13}C relative to bp1 and bp2 (Table 3), which

501   suggests a predominant origin of 2Gly-GDGT-0 from methanogens, while 2Gly-GDGT with

502   1 to 3 cyclopentane rings are derived mainly from ANME-1 archaea. This observation is

503   consistent with analyses of methanogenic archaea mainly synthesizing GDGT-0 (Koga et al.,

504   1998) as core lipids, while ANME-1 archaea were shown to contain a suite of biphytanes with

505   up to two cyclopentane rings, deriving from GDGT-1, GDGT-2 and GDGT-3 (cf.

506   Blumenberg et al., 2004; Rossel et al., 2008; 2011).

507   The biphytanes of phospho-GDGTs and mixed phospho-glyco-GDGTs are more

508   enriched in ^{13}C compared to 2Gly-GDGT. Mean $\Delta\delta^{13}$C values of the resulting biphytanes

509   relative to CO_2 are -45 to -48‰ (Fig. 4), which is in a similar range as those of IP-ARs and

510   can be thus partly assigned to methanogenic archaea (Hayes, 2001; House et al., 2003; Londry

511   et al., 2008). In contrast to the ARs, the $\Delta\delta^{13}$C values of the biphytanes relative to CO_2 (-45

512   to -48‰) do not change with depth, suggesting a metabolically uniform source of these lipids.

513   With increasing numbers of cyclopentane rings biphytanes become increasingly ^{13}C-depleted,

514   indicating an increased contribution by methanotrophic archaea. The observation that

515   methanotrophic and methanogenic archaea produce different head groups in the environment

516   is novel, but is generally consistent with published data showing that 2Gly-GDGT is the main

517   IPL in ANME-1 methanotrophic archaea (Rossel et al., 2008; 2011) and that phospho- and

518   mixed phospho/glyco-GDGTs, e.g., PG-GDGT, PG-GDGT-PG, and 2Gly-GDGT-PG, are

519   abundant IPLs in methanogenic archaea (Koga et al., 1998; Strąpoć et al., 2008).

520

4.3. Shift from ANME-2 to ANME-1 dominated communities in surface sediments

The mixture of ^{13}C-depleted phospho-OH-ARs and slightly less depleted 2Gly-GDGTs in the oil-impregnated surface sediments of GeoB10619 points to the presence of both ANME-1 and ANME-2 archaea (cf. Rossel et al., 2008; 2011). Although subtle, we observe an increase in 2Gly-GDGT with depth, indicating that ANME-1 archaea become more important in the deeper layers. The phospho-GDGTs, PG-GDGT-PG and 2Gly-GDGT-PG, increase in a similar manner, but these IPLs can be predominantly assigned to a methanogenic source, based on their δ^{13}C analysis (see 4.2.2). Similar to the apparent presence of both ANME-1 and ANME-2 communities in the oil-impregnated surface sediments, the bacterial PLFA profiles show both markers for ANME-1 and ANME-2 associated SRB: ai-C$_{15:0}$, and C$_{16:1\omega5}$, respectively (cf. Niemann and Elvert, 2008), although these PLFAs might also have non-AOM associated bacterial sources as indicated by their δ^{13}C values (Fig. 4). A slight dominance of ANME-1 associated communities with depth is nevertheless supported by a relative increase in ai-C$_{15:0}$ over i-C$_{15:0}$ (Fig. 3), together with a relative increase of phospho-DEGs with depth (Fig. 1; Blumenberg et al., 2004; Niemann and Elvert 2008). Finally, the increase in ANME-1 archaea with depth is accompanied by a decrease in sulfate concentration. This matches previous observations where ANME-1 groups were more abundant at locations with lower sulfate levels (Elvert et al., 2005; Knittel et al., 2005; Harrison et al., 2009; Rossel et al., 2011).

4.4. Methanogenic, methanotrophic and heterotrophic archaea in a deep oil-influenced SMTZ

545　　　　The ratio of archaeal to bacterial IPLs increases with sediment depth. This trend is

546　　evident in the surface sediments of site GeoB10619 but even more apparent in the subsurface

547　　sediments at the SMTZ at site GeoB10610 where more than 80% of IPLs are derived from

548　　Archaea (Fig. 1). Typically, this trend is expressed in an increase of GDGTs with

549　　diglycosidic head groups (Lipp et al., 2008; Lipp and Hinrichs, 2009), and associated with

550　　$\delta^{13}C$ values of biphytanes that reflect a heterotrophic metabolism, even in deeply buried

551　　SMTZ (10 to 100 mbsf) with high methane fluxes (cf. Biddle et al., 2006). However, at

552　　Chapopote abundant phospho-OH-ARs were also observed in the deeply buried SMTZ and

553　　$\delta^{13}C$ values of most of the archaeal IPLs are highly ^{13}C-depleted (<-70‰), even in the deeper

554　　sediment layers at the bottom of the SMTZ (Table 3; Fig. 4). Here, the concentration profile

555　　(Fig. 1) is strongly suggestive of *in situ* production of these archaeal IPLs in the SMTZ.

556　　　　In contrast to surface sediments, IP-ARs and IP-GDGTs at the SMTZ contain mainly

557　　glycosidic-based head groups, and IP-OH-AR contains PE as head group, which was not

558　　observed in the surface sediments of site GeoB10619. This is an indication that the archaeal

559　　community differs from that in surface sediments. Phytane derived from PE-OH-AR

560　　(determined through F3, see methods) and 2Gly-AR are very similar in its isotopic

561　　compositions, being both ca. 20‰ more ^{13}C-depleted at the bottom of the SMTZ (710 cmbsf)

562　　than 30 cm above. At the top of the SMTZ, methane becomes increasingly depleted in ^{13}C,

563　　suggesting the presence of co-occurring methanogenic communities that utilize the ^{13}C-

564　　depleted pool of CO_2 fueled by AOM. Since no isotopic data for DIC is available below 620

565　　cmbsf (Fig. 1a), isotopic relationships between lipids and CO_2 could not be determined for

566　　these depths (cf. Figs. 1 and 4).

567　　The $\delta^{13}C$ values of the IP-GDGT-derived biphytanes from different head groups are quite

568　　variable and likely reflect different carbon sources, source organisms, and/or mixing ratios of

569　　precursors with different metabolisms. In contrast to IP-GDGTs from the surface sediments,

570 the IP-GDGT from the SMTZ also contain crenarchaeol as GDGT core lipid. Crenarchaeol is

571 considered to be a characteristic marker for crenarchaea (Sinninghe Damsté et al., 2002) and

572 its IPL-derivatives in subsurface sediments have been assigned to indigenous benthic

573 crenarchaea (Biddle et al., 2006; Lipp and Hinrichs, 2009). In contrast to euryarchaea,

574 including methanotrophic and methanogenic archaea, benthic crenarchaea are not known to

575 metabolize methane, instead they have been linked to heterotrophy in subseafloor sediments

576 (Biddle et al., 2006). A mixture of methane-oxidizing and heterotrophic archaea as potential

577 source organisms is indeed reflected in δ^{13}C of the GDGT-derived biphytanes of 1Gly- and

578 2Gly-GDGT. Whereas δ^{13}C values of bp0 and bp1 from 1Gly- and 2Gly-GDGT always

579 ranged from -95 to -74‰, the corresponding bp3-cren has δ^{13}C values between -21 and -18‰

580 and bp2 shows mixed δ^{13}C values ranging from -71 to -40‰. The lower δ^{13}C values for bp0

581 and bp1 resemble those of 2Gly-AR and PE-OH-AR and indicate methanotrophy or mixed

582 methanotrophy and methanogenesis as carbon metabolism for the source organisms of these

583 lipids. The highest δ^{13}C values observed for bp3-cren are best explained by a source of

584 heterotrophic benthic archaea (Fig. 4; Biddle et al., 2006) and/or reutilization of fossil

585 tetraether core lipids by benthic archaea (Table 3; Takano et al., 2010; cf. Liu et al., 2011).

586

587   **5. SUMMARY AND CONCLUSIONS**

588

589   This study provides detailed insight into the structure and function of the complex

590 microbial communities involved in hydrocarbon and methane turnover at an asphalt seep in

591 the southern GoM. Pronounced contrast of δ^{13}C in different polar lipid derivatives were

592 observed. We were therefore able to obtain a highly differentiated view of the carbon

593 metabolism of bacteria and archaea that would have been intractable by conventional bulk-

594 IPL CSIA.

595   Seepage of heavy oil stimulates strong production of microbial biomass as evidenced

596   the presence of substantial concentrations of microbial lipids; combined chemotaxonomic and

597   isotopic evidence suggests that these communities are comprised by multiple functional

598   groups including anaerobic methanotrophc archaea, methanogenic archaea, hetero- and

599   autotrophic SRB, and other bacteria involved in petroleum degradation and fermentative

600   break-down of complex organic matter. The study increases our mechanistic understanding

601   of a microbial response to a relatively recent input of strongly biodegraded petroleum to the

602   seafloor in an oligotrophic ocean.

603

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851

852 **TABLES**

853 **Table 1.** Fractions of IPLs recovered by preparative LC, separated according to head group

854 polarity. See methods for compound separation and refer to text for abbreviations.

	Fraction	Abbreviation	Full name
	F1	No IPLs detected	
	F2	hydrocarbons, fatty acids	
	F3	Core GDGTs, archaeol, hydroxyarchaeol	
	F4	No IPLs detected	
	F5	No IPLs detected	
	F11	No IPLs detected	
	F12	No IPLs detected	
	F13	No IPLs detected	
Archaeal IPLs	F6	1Gly-GDGT	Monoglycosyl-glyceroldibiphytanylglyceroltetraether
	F7	2Gly-GDGT	Diglycosyl-glyceroldibiphytanylglyceroltetraether
		2Gly-AR	Diglycosyl-archaeol
	F8	3Gly-GDGT	Triglycosyl-glyceroldibiphytanylglyceroltetraether
		PG-GDGT	Phosphatidylglycerol-glyceroldibiphytanylglyceroltetraether
		PE-AR	Phosphatidylethanolamine-archaeol
		PG-AR	Phosphatidylglycerol-archaeol
		PG-OH-AR	Phosphatidylglycerol-hydroxyarchaeol
	F9	PG-AR	Phosphatidylglycerol-archaeol
		PA-OH-AR	Phosphatidic acid-hydroxyarchaeol
		PG-OH-AR	Phosphatidylglycerol-hydroxyarchaeol
		PE-OH-AR	Phosphatidylethanolamine-hydroxyarchaeol
		PI-OH-AR	Phosphatidylinositol-hydroxyarchaeol
		PI-OHeAR	Phosphatidylinositol-extended hydroxyarchaeol
	F10	2Gly-GDGT-PG	Diglycosyl-glyceroldibiphytanylglyceroltetraether-phosphatidylglycerol
		PG-GDGT-PG	Phosphatidylglycerol-glyceroldibiphytanylglyceroltetraether-phosphatidylglycerol
		PG-GDGT-PE	Phosphatidylglycerol-glyceroldibiphytanylglyceroltetraether-phosphatidylethanolamine
		PS-OH-AR	Phosphatidylserine-hydroxyarchaeol
Bacterial IPLs	F8	PE-DAG	Phosphatidylethanolamine-diacylglycerol
	F9	PE-DAG	Phosphatidylethanolamine-diacylglycerol
		PE-DEG,	Phosphatidylethanolamine-dietherglycerol
		DPG	Diphosphatidylglycerol
		PG-DAG	Phosphatidylglycerol-diacylglycerol
		PA-DAG	Phosphatidic acid-diacylglycerol
		UK-DAG	Unknown head group-diacylglycerol
	F10	PME-DAG	Phosphatidyl-(N)-methylethanolamine-diacylglycerol
		PDME-DAG	Phosphatidyl-(N,N)-dimethylethanolamine-diacylglycerol
		PA-DAG	Phosphatidic acid-diacylglycerol
	nr[a]	PC-DAG	Phosphatidylcholine-diacylglycerol
		BL	Betaine lipids

855 (a) nr – not recovered in any fraction due to retention on the column

856

Table 2. Carbon isotopic composition of archaeal diether and tetraether IPL-cleavage products in surface sediments of GeoB10619

Compound	2.5-10 cmbsf					10-17.5 cmbsf				
	2Gly	PG-PG (47), 2Gly-PG(52)	PG	PE (83), PG (17)	PG (29), PA (62), PI (10),	2Gly	PG-PG (66), 2Gly-PG(34)	PG	PE	PG (49), PA (43), PI (8)
	δ^{13}C in ‰ (relative abundance in % in fraction)					δ^{13}C in ‰ (relative abundance in %)				
Archaeol	nd	nd	-71	-73	nd	nd	nd	-71	-64	nd
Hydroxyarchaeol	nd	nd	nd	nd	-106	nd	nd	-105	nd	-104
Biphytane 0	-82 (40)	-63 (51)	-62 (68)	nd	nd	-88 (37)	-67 (43)	-68 (56)	nd	nd
Biphytane 1	-89 (41)	-74 (49)	-73 (73)	nd	nd	-94 (43)	-84 (35)	-82 (44)	nd	nd
Biphytane 2	-87 (19)	nd	nd	nd	nd	-93 (19)	-83 (23)	nd	nd	nd
Total biphytanes[a]	**-86**	**-68**	**-66**			**-91**	**-76**	**-74**		

nd – not detected

(a) Summed carbon isotope values are depicted as the weighted mean of all compounds from which isotope values were available

861 **Table 3.** Carbon isotopic composition of archaeal diether and tetraether IPL-cleavage

862 products in the subsurface SMTZ at site GeoB10610.

	680 cmbsf			*710 cmbsf*		
	1Gly	2Gly	Core	1Gly	2Gly	Core
Compound	δ^{13}C in ‰ (relative abundance in % in fraction)			δ^{13}C in ‰ (relative abundance in % in fraction)		
Phytane (AR-derived)[a]	nd	-76	-75	nd	-96	-93
Biphytane 0	-78 (53)	-89 (59)	-64 (36)	-74 (45)	-95 (57)	-50 (30)
Biphytane 1	-79 (32)	-87 (34)	-62 (21)	-78 (27)	-96 (30)	-63 (19)
Biphytane 2	-38 (15)	-71 (7)	-25 (21)	-40 (13)	-68 (9)	-22 (28)
Biphytane 3 cren	nd	nd	-18 (22)	-18 (16)	-21 (4)	-19 (23)
Total biphytanes[b]	-72	-87	-45	-62	-89	-38

nd – not detected of below detection

(a) Phytane is a mixture of AR and OH-AR, derived from 2Gly-AR and PE-OH-AR, respectively, in the core lipid fraction.

(b) Summed carbon isotope values are depicted as the weighted mean of all compounds for which isotope values were available

863

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866 **FIGURES**

867 **Figure captions**

868 **Figure 1:** Geochemical profiles of sulfate and methane concentrations, stable carbon isotopic

869 composition ($\delta^{13}C$) of methane (CH_4), dissolved inorganic carbon (DIC) and total organic

870 carbon (TOC), and absolute and relative abundances of intact polar lipids (IPLs) at sites (a)

871 GeoB10619 and (b) GeoB10610. CO_2*: $\delta^{13}C$ of CO_2 was calculated based on $\delta^{13}C$ of DIC

872 according to Mook et al. (1974). For details and IPL abbreviations refer to the text. *At some

873 depths also PDME was observed. **At site GeoB10610 also PME was observed. Plots

874 modified after Schubotz et al. (*in press*).

875 **Figure 2:** a) Density map and base peak chromatogram of an analytical run of the total lipid

876 extract of sample GeoB10619 10-17.5 cmbsf before separation by preparative HPLC (b)

877 Density maps of fractions 7 to 9 of sample GeoB10619 10-17.5 cmbsf after separation by

878 preparative HPLC. Note that the purification of the TLE resulted in the detection of

879 compounds previously masked in the TLE (e.g., PE-AR and PG-AR in F8). * contamination.

880 **Figure 3:** Distribution of polar lipid-derived fatty acids of different head groups from

881 preparative LC-fractions 8, 9 and 10 at site GeoB10619. *PE is the exclusive head group for

882 the DAGE. **PME also contains unquantifiable admixtures of PDME as head group.

883 **Figure 4:** Variations in $\delta^{13}C$ of archaeal and selected bacterial IPL-derived apolar derivatives

884 (rectangles and diamonds, respectively) relative to TOC, CO_2 and methane (vertical bars) in

885 two surface and two subsurface sediments. Standard deviation in $\delta^{13}C$ measurements ($\leq\pm1‰$)

886 defines the width of the rectangles and diamonds. Bottom of each panel: isotopic relationships

887 of representative lipid biomarkers relative to the inferred carbon source ($\Delta\delta^{13}C$) for four

888 biogeochemical processes. The reported range of $\Delta\delta^{13}C$ values represent the overlap of the

889   observed $\Delta\delta^{13}C$ of individual lipids (see footnotes 1-15) and the $\Delta\delta^{13}C$ values of the

890   biogeochemical process reported in the literature (see footnotes a-e). The $\delta^{13}C$ value for PE-

891   OH-AR at site GeoB10610 was determined indirectly by analysis of the F3 core lipid fraction

892   (see main text). $\delta^{13}C$ values for CO_2 were calculated based on the isotopic composition of the

893   DIC according to Mook et al. (1974). * Unquantifiable amounts of PDME are present. For

894   details and IPL abbreviations refer to the text.

895

896
897 **APPENDIX**

898

899 **Appendix 1.** Carbon isotopic composition and relative distribution (in parentheses) of fatty

900 acids, MAGE and DAGE derived from bacterial IPLs.

	2.5-10 cmbsf						10-17.5 cmbsf					
	PE (>98)		PE (46), PG (13), PA (5), DPG (29), UK (7) [a]		PA (70), PME (30)		PE (>98)		PE (41), PG (<1), PA (<1), DPG (24), UK (33) [a]		PME (98) [b]	
	δ ^{13}C in ‰ (relative abundance in % of total FA or total MAGE in respective fraction)											
Fatty acids (FA)												
C14:0	nd	(9)	-38	(7)	-36	(9)	-43	(7)	-42	(7)	-31	(6)
meC14:0	nd	(3)	nd	(3)	nd	(<1)	-49	(2)	nd	(2)	nd	(<1)
meC14:0	nd	(1)	-43	(4)	nd	(<1)	-38	(3)	-37	(2)	nd	(<1)
i-C15:0	nd	(4)	-43	(6)	nd	(2)	-44	(5)	-45	(3)	nd	(1)
ai-C15:0	-43	(14)	-41	(20)	-40	(6)	-47	(24)	-46	(20)	-46	(6)
C15:0	nd	(4)	-37	(5)	nd	(5)	-43	(<1)	-42	(4)	-28	(3)
i-C16:0	-38	(7)	-35	(4)	nd	nd	-38	(4)	-39	(3)	nd	(2)
ai-C16:0	nd	(2)	nd	(3)	nd	(3)	-36	(1)	nd	(1)	nd	(1)
meC15:0	nd	(4)	nd	(2)	nd	(6)	-36	(2)	nd	(1)	nd	(3)
C16:1ω7c	nd	(6)	-32	(9)	-29	(9)	-47	(9)	-28	(11)	-24	(10)
C16:1ω5c	nd	(3)	-41	(6)	nd	(4)	-50	(6)	-53	(7)	-38	(3)
C16:0	-25	(17)	-30	(11)	-27	(28)	-38	(12)	-26	(15)	-22	(31)
10meC16:0	nd	(3)	-48	(2)	nd	(<1)	-54	(3)	-55	(2)	nd	(<1)
ai-C17:0	nd	(1)	-47	(2)	nd	(<1)	-51	(2)	-54	(2)	-47	(1)
C17:1	nd	(<1)	nd	(1)	nd	(<1)	-55	(3)	nd	(2)	-35	(1)
cyC17:0	nd	(<1)	nd	(1)	nd	(<1)	nd	(1)	nd	(1)	nd	(<1)
C17:0	nd	(6)	nd	(<1)	nd	(<1)	-41	(<1)	nd	(<1)	-31	(1)
C18:1ω9c	nd	(4)	nd	(2)	-24	(6)	nd	(2)	nd	(1)	-20	(7)
C18:1ω7c	nd	(3)	-28	(5)	-27	(5)	-48	(7)	-29	(8)	-17	(6)
C18:1	nd	(1)		(4)		(7)		(5)	-26	(3)		(2)
C18:0	-28	(6)	-28	(2)	-25	(8)	-29	(2)	-28	(4)	-24	(12)
Total FA [c]	**-33**		**-37**		**-29**		**-42**		**-38**		**-26**	
Monoalkylglycerolether (MAGE)												
C14:0	nd	nd	-53	(9)	nd	nd	nd	nd	-43	(7)	nd	nd
i-C15:0	nd	nd	-46	(7)	nd	nd	nd	nd	-45	(6)	nd	nd
ai-C15:0	nd	nd	-44	(17)	nd	nd	nd	nd	-45	(21)	nd	nd
15:0	nd	nd	-44	(8)	nd	nd	nd	nd	-46	(7)	nd	nd
meC15:0	nd	nd	nd	nd	nd	nd	nd	nd	-40	(3)	nd	nd
i-C16:0	nd	nd	nd	(4)	nd	nd	nd	nd	-39	(6)	nd	nd
C16:1ω7c	nd	nd	nd	(6)	nd	nd	nd	nd	-62	(8)	nd	nd
C16:1ω5c	nd	nd	nd	(3)	nd	nd	nd	nd	-44	(3)	nd	nd
C16:0	nd	nd	-49	(21)	nd	nd	nd	nd	-42	(22)	nd	nd

10meC16:0	nd	nd	-41	(11)	nd	nd	nd	nd	-50	(9)	nd	nd
C17:1	nd	nd	nd	(14)	nd	nd	nd	nd	-64	(8)	nd	nd
Total MAGE[c]			**-46**						**-47**			
Dialkylglycerolether (DAGE)												
ai-C15:0/*ai*-C15:0	nd		-47		nd		-51		-50		nd	

Nd – not detected or below detection

(a) PE is the exclusive head group for DAGE

(b) PME with unquantifiable admixtures of PDME as head groups

(c) Summed carbon isotope values are depicted as the weighted mean of all compounds from which isotope values were available

901

902

903   **Appendix 2.** Chromatograms of PLFAs of different IPLs from preparative LC-fractions 8, 9

904   and 10 in the sediment horizon from 10 to 15 cm at site GeoB10619.