

1 **Bacterial precursors and unsaturated long-chain fatty acids are**
2 **biomarkers of North-Atlantic demosponges**

3
4 Short title: Fatty acid profiles of deep-sea demosponges

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6 Anna de Kluijver^{1*}, Klaas G.J. Nierop^{1*}, Teresa M. Morganti², Martijn C. Bart³, Beate M.
7 Slaby⁴, Ulrike Hanz⁵, Jasper M. de Goeij³, Furu Mienis⁵, Jack J. Middelburg¹

8
9 ¹Department of Earth Sciences, Faculty of Geosciences, Utrecht University, Utrecht,
10 Netherlands

11 ²Max Planck Institute for Marine Microbiology, Bremen, Germany

12 ³Department of Freshwater and Marine Ecology, Institute for Biodiversity and Ecosystem
13 Dynamics, University of Amsterdam, Amsterdam, Netherlands

14 ⁴GEOMAR Helmholtz Centre for Ocean Research Kiel, Kiel, Germany

15 ⁵NIOZ-Royal Netherlands Institute for Sea Research and Utrecht University, Den Burg,
16 Texel, Netherlands.

17
18 *corresponding authors

19 Email: a.dekluijver@uu.nl; k.g.j.nierop@uu.nl

20 **Abstract**

21 Sponges produce distinct fatty acids (FAs) that (potentially) can be used as
22 chemotaxonomic and ecological biomarkers to study endosymbiont-host interactions and the
23 functional ecology of sponges. Here, we present FA profiles of five common habitat-building
24 deep-sea sponges (class Demospongiae, order Tetractinellida), which are classified as high
25 microbial abundance (HMA) species. *Geodia hentscheli*, *G. parva*, *G. atlantica*, *G. barretti*,
26 and *Stelletta raphidiophora* were collected from boreal and Arctic sponge grounds in the
27 North-Atlantic Ocean. Bacterial FAs dominated in all five species and particularly isomeric
28 mixtures of mid-chain branched FAs (MBFAs, 8- and 9-Me-C_{16:0} and 10 and 11-Me-C_{18:0})
29 were found in high abundance (together $\geq 20\%$ of total FAs) aside more common bacterial
30 markers. In addition, the sponges produced long-chain linear, mid- and *a(i)*-branched
31 unsaturated FAs (LCFAs) with a chain length of 24–28 C atoms and had predominantly the
32 typical $\Delta^{5,9}$ unsaturation, although also $\Delta^{9,19}$ and (yet undescribed) $\Delta^{11,21}$ unsaturations were
33 identified. *G. parva* and *S. raphidiophora* each produced distinct LCFAs, while *G. atlantica*,
34 *G. barretti*, and *G. hentscheli* produced similar LCFAs, but in different ratios. The different
35 bacterial precursors varied in carbon isotopic composition ($\delta^{13}\text{C}$), with MBFAs being more
36 enriched compared to other bacterial (linear and *a(i)*-branched) FAs. We propose biosynthetic
37 pathways for different LCFAs from their bacterial precursors, that are consistent with small
38 isotopic differences found in LCFAs. Indeed, FA profiles of deep-sea sponges can serve as
39 chemotaxonomic markers and support the conception that sponges acquire building blocks
40 from their endosymbiotic bacteria.

41 Introduction

42 Sponges are abundant inhabitants of nearly all aquatic ecosystems including the deep-
43 sea (1). They are sessile filter feeders with unique features, such as their enormous filtration
44 capacity and their symbiosis with dense and diverse communities of (sponge-specific)
45 microbes (algae, bacteria, archaea) (2,3) that contribute to their ability to thrive at nearly all
46 depths and latitudes. The endosymbionts, which can occupy >50 % of sponge volume (4),
47 serve as energy source for sponges and provide a diverse pallet of metabolites and metabolic
48 pathways that are beneficial to the sponge (reviewed in (2)). A prominent class of metabolites
49 produced by the sponge and its endosymbionts are lipids. Lipid analysis of sponges started in
50 the 1970s (5,6) and was sparked by the diversity and unique structures of fatty acids (FAs), of
51 which extensive reviews exist (7–9). Characteristic of sponges is the presence of unusual
52 poly-unsaturated, long chain (≥ 24 carbons(C)) FAs (LCFAs), with a typical $\Delta^{5,9}$ unsaturation
53 (named “demospongiic acids”, because of their first discovery in demosponges (5,10)). These
54 LCFAs constitute a major part of sponge membrane phospholipids (PLs) and probably serve
55 a structural and functional role (11). Sponges, because of their endosymbionts, are rich in
56 bacterial FAs with high diversity, including not only the common *iso* (*i*) and *anteiso* (*a*)-
57 branched FAs, but also more unusual ones. Typical of demosponges are a high abundance of
58 mid-chain branched FAs (MBFAs), that are thought to be produced by sponge-specific
59 eubacteria (12), and a presence of branched LCFAs (12,13). As branching is assumed to be
60 introduced by microbes and not by the sponge host, the presence of branched LCFAs
61 provides information on biosynthetic interactions between endosymbionts and host (12,14).
62 Monoenic FA, e.g. C_{16:1} ω 7, abundant in bacteria (15), have been identified as precursors for
63 LCFAs with ω 7 configuration (16). Accordingly, the position of unsaturation also provides
64 insight in bacteria-host biosynthetic interactions.

65 In addition, sponge FA composition may have taxonomic value, at least on a higher
66 classification level (e.g. class level), since Demospongiae, Hexactinellida ('glass' sponges),
67 Calcarea, and Homoscleromorpha have distinct FA profiles (17). However, the
68 chemotaxonomic value on a lower classification level is disputable, since composition may
69 alter with environmental conditions (18). The FA composition of sponges, especially
70 combined with (natural abundance) stable isotope analysis, has been shown a valuable tool to
71 infer dietary information on sponges, such as feeding on coral mucus (19), phytoplankton
72 (20) and methane-fixing endosymbionts (21).

73 The North-Atlantic Ocean is home to extensive sponge grounds, that are widespread
74 along the continental shelves, seamounts, and on the abyssal plains (22,23). *Geodiidae* and
75 other sponge species of order Tetractinellida (class Demospongiae) are major constituents of
76 these sponge grounds, representing >99 % of sponge ground benthic biomass (23–25).
77 *Geodiidae* spp. are high microbial abundance (HMA) sponges that harbor rich, diverse and
78 specific microbial communities (bacteria and archaea) involved in several biogeochemical
79 processes, as observed in *G. barretti* (26). This is reflected in the FA composition of *G.*
80 *barretti* that is dominated by bacterial FAs (12), including the distinct MBFAs that represent
81 28% of total FAs (12). However, the FA profiles of other *Geodiidae* are not described in the
82 literature yet.

83 In this study we analyzed the FA profiles of five common deep-sea Tetractinellids,
84 from different assemblages distinguished by temperature in the North Atlantic: the Arctic
85 sponge ground assemblages accommodate *G. parva*, *G. hentscheli*, and *Stelletta* spp. (e.g. *S.*
86 *rhapsodiophora*) dwelling at temperatures below 3–4 °C, and the boreal assemblages
87 accommodate *G. barretti* and *G. atlantica* amongst others, which are typically found at
88 temperatures above 3 °C (23,27). Based on the chemical configuration and the presence of
89 branching in LCFAs, we propose biosynthetic pathways and show that these are consistent

90 with the C isotope ($\delta^{13}\text{C}$) signatures of LCFAs and bacterial precursors. The high abundance
91 of endosymbiont markers that are precursors of LCFAs, indicate that these deep-sea sponges
92 use their endosymbionts as metabolic source.

93

94 **Methods**

95 **Sponge collection**

96 Common habitat-building sponges of class Demospongiae, order Tetractinellida, were
97 collected in the North-Atlantic Ocean by remotely operated vehicle (ROV) and box cores
98 during different scientific expeditions. *G. atlantica* ($n = 2$) specimens were collected on the
99 Sula Reef between 266–295 m depth during an expedition in August 2017 with the
100 Norwegian research vessel G.O. Sars (64°42'N 7°59'E). *G. barretti* ($n = 6$) individuals were
101 obtained from the Barents Sea (70°47'N 18°03'E) around 300 m water depth on a subsequent
102 G.O. Sars expedition in August 2018 (28). During the same expedition, *G. hentscheli*, *G.*
103 *parva*, *Stelletta rhapsodiophora* (all $n = 1$) were collected at 550–600 m depth on the summit
104 of Schulz Bank (73°50' N, 7°34' E) (29). *G. hentscheli* ($n = 3$), *G. parva* ($n = 3$), and *S.*
105 *rhapsodiophora* ($n = 2$) specimens were retrieved on an Arctic expedition with the German
106 research vessel Polarstern (AWI Expedition PS101) in September–October 2016 at 690–1000
107 m depth from Langseth Ridge, located in the permanently ice-covered Central Arctic
108 (86°N, 61°E). Sponges collected during the G.O. Sars expeditions were immediately dissected
109 on board and sponges collected from Langseth Ridge were frozen at -20°C and dissected
110 (frozen) in the lab. Subsamples ($n = 3$) from random parts of individual sponges were freeze-
111 dried, grinded to obtain a fine powder. The powdered subsamples of sponges from Schulz
112 Bank and Barents Sea were mixed to obtain a species representative sample, while a
113 subsample of the interior of sponges was analyzed in case of Langseth Ridge specimens.

114

115 **Lipid extraction and FAME preparation**

116 Approximately 100 mg of sponge powder of each individual sponge was used per
117 extraction. Sponge lipids were extracted with a modified Bligh and Dyer protocol (30), which
118 was developed at NIOZ Yerseke (31–33). We adjusted this protocol by replacing chloroform
119 with dichloromethane (DCM), because of lower toxicity. The whole protocol is available
120 online: [dx.doi.org/10.17504/protocols.io.bhnpj5dn](https://doi.org/10.17504/protocols.io.bhnpj5dn). In short, sponge tissue samples were
121 extracted in a solvent mixture (15 mL methanol, 7.5 mL DCM and 6 mL phosphate (P)-
122 buffer (pH 7-8)) on a roller table for at least 3 hours. Layer separation was achieved by
123 adding 7.5 mL DCM and 7.5 mL P-buffer. The DCM layer was collected, and the remaining
124 solution was washed a second time with DCM. The combined DCM fraction was evaporated
125 to obtain the total lipid extract (TLE), which was subsequently weighed. An aliquot of the
126 TLE was separated into different polarity classes over an activated silica column. The TLE
127 was first eluted with 7 mL DCM (neutral lipids), followed by 7 mL acetone (glycolipids) and
128 15 mL methanol (phospholipids). The phospholipid (PL) fraction, which was used for further
129 analysis, was converted into fatty acid methyl esters (FAMES) using alkaline methylation
130 (using sodium methoxide in methanol with known $\delta^{13}\text{C}$). Alkaline methylation is
131 recommended for complex lipid mixtures (34). After methylation, FAMES were collected in
132 hexane and concentrated to ~100 μL hexane for gas chromatography (GC) analysis.

133 For this study, two individual sponge samples per species were selected for detailed
134 analysis. Aliquots of the FAME samples were used for double bond identification using
135 dimethyl disulfide (DMDS) derivatization (35). Samples reacted overnight at 40°C in 50 μL
136 hexane, 50 μL DMDS and 10 μL 60 mg/mL I_2 . The reaction was stopped by adding 200 μL
137 hexane and 200 μL $\text{Na}_2\text{S}_2\text{O}_3$. The hexane layer was collected, and the aqueous phase was
138 washed twice with hexane. The combined hexane fraction was dried, subsequently eluted

139 over a small Na₂SO₄ column using in DCM: methanol (9:1) to remove any water and re-
140 dissolved in hexane in a GC-vial for GC-analysis. Another aliquot of FAME sample was used
141 for methyl-branching identification using catalytic hydrogenation with Adams catalyst (PtO₂)
142 and hydrogen. Each FAME sample, dissolved in ~3 mL ethyl acetate with 10-30 mg PtO₂ and
143 a drop of acetic acid, was bubbled with hydrogen gas for at least 1 h, after which the reaction
144 vial was closed, and stirred overnight at room temperature. Subsequently, each sample was
145 purified over a small column consisting of MgSO₄ (bottom) and Na₂CO₃ (top) using DCM
146 and analyzed after re-dissolving it in ethyl acetate.

147

148 **FAME analysis**

149 FAMES were analyzed on a gas chromatograph (GC) with flame ionization detector
150 (FID) (HP 6890 series) for concentrations and GC-mass spectrometry (MS) (Finnigan Trace
151 GC Ultra) for identification on a non-polar analytical column (Agilent, CP-Sil5 CB; 25 m x
152 0.32 mm x 0.12 μm). The GC oven was programmed from 70–130 °C at 20 °C/min and
153 subsequently at 4 °C/min to 320 °C, at which it was hold for 20 min. The GC–FID was
154 operated at a constant pressure of 100 kPa, whereas the GC–MS was operated at a constant
155 flow of 2.0 mL min⁻¹. The MS was operated in Full Data Acquisition mode, scanning ions
156 from *m/z* 50–800. The ¹³C/¹²C isotope ratios of individual FAMES were determined by
157 analyzing samples in duplicate on a GC-combustion-isotope ratio mass spectrometer (IRMS)
158 consisting of a HP 6890N GC (Hewlett-Packard) connected to a Delta-Plus XP IRMS via a
159 type-III combustion interface (Thermo Finnigan), using identical GC column and settings as
160 for GC-MS.

161 Retention times were converted to equivalent chain length (ecl) based on the retention
162 times of C_{12:0}, C_{16:0}, and C_{19:0} FAMES. The δ¹³C values obtained by GC-C-IRMS were

163 corrected for the added C atom of the methylation agent. The data were analyzed and plotted
164 in R (36) with R-package RLims (37).

165

166 **Results**

167 The lipid yield of *G. barretti*, *G. hentscheli*, *G. parva*, and *S. raphidiophora* was
168 similar, around 2–3 % of dry weight (DW). Only *G. atlantica* had a lower lipid yield, about
169 1.6 % of DW. The FA profiles of PL resembled those of TLE (Table S1). However,
170 identification was more difficult using TLEs, because LCFAs co-eluted with sterols, hence
171 PL chromatography was used for identification and composition analysis.

172

173 **Identification**

174 Chemical structures of individual FAs were identified by retention times (ecl),
175 interpretation of their mass spectra and/or by identification using a NIST library. The
176 assignments were verified with reference mixtures (bacterial and general FA mixtures from
177 Sigma Aldrich) and by literature comparison (e.g. the reference ecl lists from NIOZ Yerseke
178 (31)).

179 FAs are presented in both ω and Δ (IUPAC) annotation to avoid unambiguity and in a
180 hybrid form, which is typical of sponge LCFA annotation (17,38) (Table 1). Unsaturation is
181 described as $C_{x,y}$, where x is the number of C atoms and y is the number of double bonds,
182 which is followed by Δ and all double bond positions from the carboxylic acid end in Δ
183 notation, and the position of the first double bond from the methyl (terminal) end in ω
184 notation (Table 1, Fig. 1). Methyl branching according to IUPAC notation is described as y-
185 Me- C_x , where y is the position of the branching from the carboxylic acid end and x is the
186 number of C atoms at the backbone, excluding the branching (Fig. 1). The ω notation follows

187 the terminology of IUPAC for MBFAs, but deviates for terminally branched FAs. The
 188 penultimate ($\omega 2$) and pen-penultimate methyl branching ($\omega 3$) are described with ω notation
 189 as *iso* ($i-C_x$) and *anteiso* ($a-C_x$) where x is the total number of C atoms, including the
 190 branching (Table 1, Fig. 1).

191

192 **Table 1:** Fatty acid (FA) composition in % of total PLFA of deep-sea demosponge species
 193 (order Tetractinellida): *Geodia atlantica* (*Ga*), *G. barretti* (*Gb*), *G. hentscheli* (*Gh*), *G. parva*
 194 (*Gp*) and *Stelletta raphidiophora* (*Sr*). FA names are given in ω and Δ notation and a hybrid
 195 form, with corresponding total C atoms (C) and equivalent chain length (ecl). FA categories
 196 match with those of Fig. 2. Only FAs with abundance ≥ 1 % (in at least one species) are
 197 shown. Numbers in bold are ≥ 10 % of total FAs.

				Species	<i>Ga</i>	<i>Gb</i>	<i>Gh</i>	<i>Gp</i>	<i>Sr</i>
FA notation				<i>n</i>	2	6	4	4	3
Ecl	FA (ω)	FA (Δ)	C	Category	FA composition (%)				
13.68	C _{14:0}	C _{14:0}	14	Other	0.9	1.0	1.0	0.8	1.5
14.17	Me-C _{14:0}	Me-C _{14:0}	15	Bacteria	0.7	1.4	1.4	1.3	1.8
14.38	<i>i</i> -C _{15:0}	13-Me-C _{14:0}	15		3.0	3.5	3.1	2.5	4.0
14.46	<i>a</i> -C _{15:0}	12-Me-C _{14:0}	15		2.6	2.6	2.2	2.0	4.3
15.35	Me-C _{15:0}	Me-C _{15:0}	16		0.6	0.9	1.6	1.8	1.6
15.59	C _{16:1} $\omega 9$	C _{16:1} Δ^7	16		1.6	0.5	2.6	3.3	2.5
15.68	C _{16:1} $\omega 7$	C _{16:1} Δ^9	16		6.3	8.7	7.8	6.3	8.2
15.78	C _{16:1} $\omega 5$	C _{16:1} Δ^{11}	16		1.5	2.1	1.9	1.6	2.7
16	C _{16:0}	C _{16:0}	16		Other	5.7	3.7	3.5	3.2
16.31	<i>i</i> -C _{17:1} $\omega 7$	15-Me-C _{16:1} Δ^9	17	Bacteria	4.2	5.4	1.4	1.0	1.4
16.45	8- and 9-Me-C _{16:0}	8- and 9-Me-C _{16:0}	17		8.3	10	14	11	13
16.62	<i>i</i> -C _{17:0}	15-Me-C _{16:0}	17		1.1	1.3	1.2	1.3	2.2
16.68	<i>a</i> -C _{17:0}	14-Me-C _{16:0}	17		1.4	1.3	0.9	0.8	1.2
17.40	Me-C _{17:0}	Me-C _{17:0}	18		3.1	2.8	1.7	1.9	2.0
17.65	C _{18:1} $\omega 9$	C _{18:1} Δ^9	18		1.2	0.2	1.5	1.4	1.5
17.72	C _{18:1} $\omega 7$	C _{18:1} Δ^{11}	18		3.1	4.2	3.8	3.9	3.7
18	C _{18:0}	C _{18:0}	18		Other	4.6	3.7	3.0	3.0
18.11	Me-C _{18:1} $\omega 12$ or $\omega 14$	Me-C _{18:1} Δ^6 or Δ^4	19	Bacteria	2.0	2.9	4.0	4.4	4.9
18.46	10- and 11-Me-C _{18:0}	10- and 11-Me-C _{18:0}	19		12	17	20	23	19
18.78	<i>cy</i> -C _{19:0}	<i>cy</i> -C _{19:0}	19		1.0	1.2	1.2	0.8	1.3
20.85	C _{22:6} $\omega 3$	C _{22:6} $\Delta^{4,7,10,13,16,19}$	22	Other	1.8	1.3	0.2	0.6	
23.17	C _{24:2} $\Delta^{5,9}$ ($\omega 15$)	C _{24:2} $\Delta^{5,9}$	24	Sponge				1.2	
23.67	<i>i</i> -C _{25:2} $\Delta^{5,9}$ ($\omega 15$)	23-Me-C _{24:2} $\Delta^{5,9}$	25					11	

23.74	$a\text{-C}_{25:2}\Delta^{5,9}$ ($\omega 15$)	22-Me- $\text{C}_{24:2}\Delta^{5,9}$	25					4.2	
23.84	$i\text{-C}_{25:1}\omega 7$	23-Me- $\text{C}_{24:1}\Delta^{17}$	25		2.4	1.8			
24.73	$\text{C}_{26:2}\Delta^{5,9}$ ($\omega 17$)	$\text{C}_{26:2}\Delta^{5,9}$	26		2.4	2.4	5.4	0.4	0.4
24.81	$\text{C}_{26:2}\Delta^{9,19}$ ($\omega 7$)	$\text{C}_{26:2}\Delta^{9,19}$	26		4.3	8.4	8.5	0.6	
25.11	Me- $\text{C}_{26:2}\Delta^{5,9}$ ($\omega 17$)	Me- $\text{C}_{26:2}\Delta^{5,9}$	27		9.4	4.5	1.3		1.2
25.28	$(a)i\text{-C}_{27:2}\Delta^{5,9}$ ($\omega 7$) or $\Delta^{9,19}$ ($\omega 17$)	24 or 25-Me- $\text{C}_{26:2}\Delta^{5,9}$ or $\Delta^{9,19}$	27		4.9	2.9	0.1		0.7
25.96	$\text{C}_{28:2}\Delta^{5,9}$ ($\omega 21$)	$\text{C}_{28:2}\Delta^{5,9}$	28						1.5
26.14	$\text{C}_{28:2}\Delta^{11,21}$ ($\omega 7$)	$\text{C}_{28:2}\Delta^{11,21}$	28		1.9	1.8		3.6	
26.71	Me- $\text{C}_{28:2}\Delta^{5,9}$ ($\omega 21$)	Me- $\text{C}_{28:2}\Delta^{5,9}$	28						5.5

198

199 **Fig. 1: Illustration of ω and Δ annotation for the chemical structure of $i\text{-C}_{17:1}\omega 7 / 15\text{-Me-C}_{16:1}\Delta^9$.** The $a(i)$ -notation for methyl branching describes the total number of C, while
 200 the Me-notation describes the number of C in the backbone. For sponge LCFAs, a mixture of
 201 the Me-notation describes the number of C in the backbone. For sponge LCFAs, a mixture of
 202 both nomenclatures is, however, commonly used.

203

204 The elution order on an apolar column consists of FAMES with methyl-branching
 205 close to the functional group to elute first, followed by the terminally (penultimate) branched
 206 *iso* (i , $\omega 1$) and pen-penultimate *anteiso* (a , $\omega 2$) FAMES, and finally the unsaturated FAMES,
 207 for which unsaturation closest to the functional group elutes first. Branched unsaturated
 208 FAMES elute before branched straight FAMES and straight FAMES with the same C number
 209 elute last (Fig. 2, Table 1).

210

211 **Figure 2: GC trace of the FAME fraction extracted from demosponge *G. hentscheli***
 212 **from Langseth Ridge (Central Arctic).** LCFA isomers often co-eluted or were at least not
 213 well separated as shown in this PLFA profile $\text{C}_{26:2}\Delta^{5,9}$ and $\text{C}_{26:2}\Delta^{9,19}$.

214

215 The position of branching was also verified with MS spectra, as *i*-branching was
 216 characterized by a more intense $[M^+-43]$ fragment ion and *a*-branching was characterized by
 217 an elevated fragment ion at $[M^+-57]$. The position of methyl branching in saturated MBFAs

218 was identified via diagnostic mass fragments similar to (12). The relative intensity of m/z
219 171, 185, 199 and [185+213] was used to identify the relative contributions of 8, 9, 10, and
220 11-Me branching, respectively (Table S1). Because 11-Me-branching produces equal
221 fragments of m/z 185 and 213, the excess of m/z 185 ($213 - 185$) was produced by 9-Me
222 branching (Table S1) (12). The branching within unsaturated MBFAs was performed in
223 hydrogenated samples, using similar diagnostic fragments and ecl of saturated FAMES (Table
224 S1).

225 Identification of unsaturation positions was conducted after treatment with DMDS,
226 which is straight-forward with mono-unsaturated FAMES. However, for poly-unsaturated
227 FAMES, identification with DMDS becomes complicated, because of multiple possibilities
228 for S(-Me) adducts. The $\Delta^{5,9}$ unsaturation, typical of sponge LCFAs, forms a cyclic thioether
229 at the C_6 and C_9 position along with methylthio groups at C_5 and C_{10} positions upon
230 derivatization with DMDS. In addition, products are formed with either methylthio groups at
231 C_5 and C_6 and a (unreacted) double bond at C_9 and C_{10} , and vice versa (39). This has been
232 useful for identifying the typical $\Delta^{5,9}$ configuration in sponges (40). When unsaturation is far
233 apart, i.e. positions $\Delta^{9,19}$ and $\Delta^{11,21}$, both double bonds are converted to dimethyl disulfide
234 adducts (S1 Figure for their mass spectra). Based on ecl and a combination of DMDS and
235 hydrogenation, we identified branched-monoenic and dienic FAs.

236

237

238 **Fatty acid composition**

239 The Arctic species (*G. hentscheli*, *G. parva*, *S. raphidiophora*) from Schulz Bank
240 and Langseth Ridge had a similar FA profile (Table S1), so we pooled the compositional data
241 from the two locations (Table 1). The data are standardized to % of total FAs to facilitate
242 comparison, but actual concentrations ($\mu\text{g g}^{-1}$ DW) are available in Table S1.

243

244 **Bacterial fatty acids**

245 Bacterial FAs, comprising branched and monoenic FAs with chain length $< C_{20}$,
246 constituted the majority of total FAs in all five deep-sea demosponge species ($67 \pm 6 \%$
247 mean \pm SD of total FAs, used throughout text, $n = 19$, across all species) (Table 1, Fig. 3) and
248 can represent up to $79 \pm 2 \%$ (in *S. raphidiophora*).

249

250 **Fig. 3: Average contribution of bacterial FAs (blue), sponge LCFAs (orange) and other**
251 **FAs (green) to the total PLFAs of each species (abbreviated as in Table 1).**

252

253 MBFAs dominated the FA profiles of all deep-sea demosponge species (Table 1, Fig.
254 2), among them the most abundant were Me- $C_{18:0}$ (12–23 %, Table 1), with branching at 9,
255 10, 11 with a predominance at position 11 (m/z [213+185]; 49 % on average), followed by
256 position 10 (m/z 199; 37 % on average). The second most abundant FAs were Me- $C_{16:0}$ (8–14
257 % of total FAs, Table 1), with branching at 8, 9, 10, 11 and a predominance of position 9 (m/z
258 185; 36 % on average) and 10 (m/z 199; 33 % on average). Also, Me- $C_{14:0}$, Me- $C_{15:0}$, and Me-
259 $C_{17:0}$ were present, but in much lower abundance ($\leq 3 \%$ of total FAs for each, Table 1). Other
260 branched (saturated) FAs found in all demosponges but less abundant, included *i*- $C_{15:0}$ (13-
261 Me- $C_{14:0}$) and *a*- $C_{15:0}$ (12-Me- $C_{14:0}$) comprising 2–5 % of total FAs for each, and *i*- $C_{17:0}$ (15-
262 Me- $C_{16:0}$) and *a*- $C_{17:0}$ (14-Me- $C_{16:0}$) ranging from 1 to 2 % of total FAs for each (Table 1).

263 Multiple monoenic FAs were found in the deep-sea demosponges. The most abundant
264 were $C_{16:1}$ (ranging from 9 % in *G. atlantica* to 14 % in *G. parva* and *S. raphidiophora*),
265 consisting of different isomers with the double bond at $\omega 5$, $\omega 7$, $\omega 9$, and $\omega 11$ positions.
266 Isomers of $C_{18:1}$ with double bonds at $\omega 7$, $\omega 8$, $\omega 9$, $\omega 11$, $\omega 12$, $\omega 13$, $\omega 14$, and $\omega 15$ positions
267 constituted 4–7 % of total FAs. The $\omega 7$ unsaturation dominated in both $C_{16:1}$ and $C_{18:1}$ FAs. In
268 addition, rare $C_{16:1}$ and $C_{18:1}$ FAs with methyl-branching were found in demosponges. The

269 dominating unsaturation in C_{16:1} was ω7 and the hydrogenated FAME sample indicated *i*-
270 branching; *i*-C_{17:1}ω7 (15-Me-_{16:1}Δ⁹) represented 4–5 % in boreal species (*G. barretti*, *G.*
271 *atlantica*) and < 2 % in Arctic species (*G. hentscheli*, *G. parva*, *S. raphidiophora*) (Fig. 1,
272 Table 1). The most abundant unsaturation in C_{18:1} FAs was ω12 (Δ⁶) for *G. parva*, *G.*
273 *hentscheli* and *S. raphidiophora* and ω14 (Δ⁴) for *G. barretti* and *G. atlantica*, and the Me
274 group was in the middle of the chain, since no increased peaks for *i*- and *a*-C_{19:0} were found
275 in the corresponding hydrogenated fractions. The mid-Me branched C_{18:1} isomers (Me-
276 C_{18:1}ω4 and Me-C_{18:1}ω12) were found in all demosponge species and ranged between 2–5 %
277 (Table 1). Also, low amounts (< 1 %) of C_{15:1} and non-branched C_{17:1} were found.

278

279 **Other fatty acids**

280 Linear FAs were predominantly C_{14:0}, C_{16:0} and C_{18:0} in all species (Table 1). Sponges
281 contained only low amounts of FAs with a chain length between C₂₀ and C₂₄, such as C_{20:5}ω3
282 (< 1 % in all species) and C_{22:6}ω3 (1.4 ± 0.9 %, *n* = 8) in boreal species *G. atlantica* and *G.*
283 *barretti* and < 1% in Arctic species *G. hentscheli*, *G. parva* and *S. raphidiophora*.

284

285 **Sponge fatty acids**

286 LCFAs (≥ C₂₄), typical of sponges, differed per species and consisted of 24–29 C atoms
287 (Table 1). LCFAs represented 21 ± 6 % (*n* = 19, across all species), with the highest
288 contribution (29 %) in *G. atlantica* (Fig. 3). The most common unsaturation in demosponges
289 was Δ^{5,9}, but also unsaturation at Δ^{9,19} and Δ^{11,21} was observed.

290 - C₂₅: The dominant LCFA in *G. parva* was 23-Me-C_{24:2}Δ^{5,9} (*i*-C_{25:2}Δ^{5,9}), followed by
291 22-Me-C_{24:2}Δ^{5,9} (*a*-C_{25:2}Δ^{5,9}), making up 15 ± 1 % of total FAs. Isomers 23-Me-
292 C_{24:1}Δ¹⁷ (*i*-C_{25:1}ω7) and (mid-)Me-C_{24:1}Δ¹⁷ (Me-C_{24:1}ω7) were present in boreal
293 species (*G. atlantica* and *G. barretti*) representing together 2 ± 0.6 % (Table 1).

- 294 - **C₂₆**: The dominant LCFA in *G. hentscheli* was C_{26:2}Δ^{9,19}, followed by C_{26:2}Δ^{5,9},
295 together they represented 14 ± 6 % of total FAs in that species. *G. barretti* and *G.*
296 *atlantica* also synthesized C_{26:2}Δ^{5,9} and C_{26:2}Δ^{9,19} in comparable amounts, representing
297 together 11 ± 1% in *G. barretti* and 7 % in *G. atlantica*. Trace amounts (<1 %) of
298 C_{26:2}Δ^{5,9} were present in *G. parva* and *S. raphidiophora*. Similarly, trace amount of
299 C_{26:2}Δ^{11,21} (<1 %) was found in *G. parva*.
- 300 - **C₂₇**: (mid-)Me-C_{26:2}Δ^{5,9} were abundant in boreal species (*G. barretti*: 4 ± 3 %; *G.*
301 *atlantica*: 9 %). Also 25-Me-C_{26:2}Δ^{5,9} (*i*-C_{27:2}Δ^{5,9}), and 25-Me-C_{26:2}Δ^{9,19} (*i*-C_{27:2}Δ^{9,19})
302 were produced by boreal species, representing together 3 ± 2 % of total FAs in *G.*
303 *barretti* and 5 % in *G. atlantica*. Because these peaks co-eluted, the individual
304 concentrations might represent isomeric mixtures. *G. hentscheli* possessed low
305 amounts of (mid-)Me-C_{26:2}Δ^{5,9} and 25-Me-C_{26:2}Δ^{9,19} (*i*-C_{27:2}Δ^{9,19}) (< 2 %). Similarly,
306 *S. raphidiophora* had low amounts of (mid-)Me- C_{26:2}Δ^{5,9} and (*a*)*i*-C_{27:2}Δ^{5,9} (together
307 2 %, Table 1).
- 308 - **C₂₈**: *G. atlantica*, *G. barretti* and *G. parva* contained C_{28:2} with Δ^{11,21} configuration,
309 comprising 1.8 ± 1 % of total FAs in *G. barretti*, 1.9 % in *G. atlantica* and 3.6 ± 1.7
310 % in *G. parva*. *S. raphidiophora* contained a low amount of C_{28:2}Δ^{5,9} (1.5 ± 0.4 %)
311 (Table 1).
- 312 - **C₂₉**: The dominant LCFA in *S. raphidiophora* was (mid-)Me-C_{28:2}Δ^{5,9} with a
313 contribution of 5.5 ± 0.6 % to total FAs (Table 1).

314

315 **Stable C isotope values (δ¹³C)**

316 Stable C isotope values (δ¹³C) of dominant FAs ranged between -18 ‰ (95 percentile)
317 and -26 ‰ (5 percentile) and showed similar patterns across all demosponges (Fig. 4, Table
318 2). The δ¹³C values of the dominant MBFAs, Me-C_{16:0}, Me-C_{18:0}, and also Me-C_{18:1}ω12 (and

319 $\omega 14$) were enriched in ^{13}C compared to other bacterial fatty acids, (*a(i)*- $\text{C}_{15:0}$, $\text{C}_{16:1\omega 7}$,
 320 $\text{C}_{18:1\omega 7}$) (Fig. 4, Table 2). The most depleted FA was *i*- $\text{C}_{17:1\omega 7}$ ($-25.7 \pm 1.3 \text{‰ } \delta^{13}\text{C}$). The
 321 different LCFA isomers were analyzed as one, because isomers co-eluted or were at least not
 322 well separated on GC (Fig. 2). However, we could assign separate isotope values for (*a*)*i*-
 323 $\text{C}_{27:2}$ and Me- $\text{C}_{26:2}$ (Fig. 4). The LCFAs showed less isotopic variation compared to bacterial
 324 FAs, but still ranged between -25 and -19 ‰ (5–95 percentile) (Fig. 4, Table 2). Me- $\text{C}_{26:2}$
 325 and (*a*)*i*- $\text{C}_{27:2}$ had relatively similar $\delta^{13}\text{C}$ values, -20 and -21 ‰, in *G. barretti* and *G.*
 326 *atlantica*, but a more prominent difference of -21 and -24 ‰ was observed in the
 327 hydrogenated samples ($n = 1$ per species), indicating that peak overlap blurred the isotopic
 328 values.

329

330 **Table 2:** $\delta^{13}\text{C}$ values (mean \pm SD) of (bacterial) FA precursors and dominant LCFAs of all
 331 species combined.

Category	Fatty acid biomarker	Average $\delta^{13}\text{C}$ (‰) \pm SD
<i>a(i)</i> -branched FA	<i>(a)</i> <i>i</i> - $\text{C}_{15:0}$	-22.8 ± 0.6
	<i>(a)</i> <i>i</i> - $\text{C}_{25:2}$	-22.0 ± 0.6
	<i>i</i> - $\text{C}_{17:1\omega 7}$	-25.7 ± 1.3
	<i>i</i> - $\text{C}_{25:1\omega 7}$	-23.9 ± 0.7
	<i>(a)</i> <i>i</i> - $\text{C}_{27:2}$	-21.0 ± 1.2
Linear FA	$\text{C}_{16:0}$	-21.6 ± 1.1
	$\text{C}_{18:0}$	-20.2 ± 1.2
	$\text{C}_{16:1\omega 7}$	-23.0 ± 2.0
	$\text{C}_{18:1\omega 7}$	-23.6 ± 1.5
	$\text{C}_{26:2}$	-21.8 ± 0.9
	$\text{C}_{28:2}$	-23.2 ± 1.4
Mid-branched FA	Me- $\text{C}_{16:0}$	-19.3 ± 1.6
	Me- $\text{C}_{18:0}$	-18.4 ± 1.1
	Me- $\text{C}_{18:1}$	-17.4 ± 1.3
	Me- $\text{C}_{26:2}$	-20.2 ± 0.6
	Me- $\text{C}_{28:2}$	-19.4 ± 0.2

332

333 **Fig. 4:** $\delta^{13}\text{C}$ composition of precursors and dominant LCFAs in analyzed demosponges.

334 Sponge species were pooled together, and the median is indicated in the box plot as black

335 line. The numbers depict the sample size (individual FAME samples). The colors are used to
336 match bacterial precursor FAs with sponge-produced LCFAs. Pink is used for (mid-)Me-
337 branched FAs, grey is used for linear FAs, blue and yellow indicate (*a*)*i*-branched FAs with
338 distinct $\delta^{13}\text{C}$ that may end up in an isomeric mixture, indicated by green (see Fig. 5 for
339 biosynthetic pathways).

340

341 **Discussion**

342 **Bacterial FAs**

343 High concentrations of isomeric mixtures of MBFAs were found in all five sponge
344 species analyzed, independent of species and location (Table 1). A predominance of MBFAs
345 is considered to be a typical feature of Demospongiae, because it is not observed in any other
346 organism, sediment or water (12,17,41). Typical position of branching is between $\omega 5$ and $\omega 9$
347 (12), resulting in predominance of 8- and 9-Me- $\text{C}_{16:0}$ and 10- and 11-Me- $\text{C}_{18:0}$ in this study, in
348 agreement with previously reported MBFAs (42,43). MBFAs are typically produced by
349 bacteria, so they are presumably made by distinctive and sponge-specific eubacterial
350 symbionts. It has been hypothesized that these bacteria were widespread in the geological
351 past and were inherited in the protective environment of distinctive sponge hosts in modern
352 marine environments (8,12). This hypothesis has been further supported by genomic analysis
353 on *Geodia* sp. revealing similar microbial communities between species with little
354 geographical variation (44).

355 A proposed candidate phylum for MBFAs is Poribacteria, a unique and abundant
356 phylum in HMA sponges (45), since a positive relation between MBFA concentration and
357 Poribacteria abundance was found across several sponge species (43). Metagenome analyses
358 showed that Poribacteria are a prominent phylum in *G. barretti* (46,47), but are rare or even

359 absent in *G. hentscheli* (48), which shows a dominance of Acidobacteria, Chloroflexi, and
360 Proteobacteria, phyla that are also abundant in *G. barretti* (46,47). This suggests that either
361 the MBFAs belong to one of the above-mentioned phyla, or that the MBFAs are shared
362 among microbial phyla, as their chemotaxonomic resolution is lower compared to genomic
363 analysis. In the environment, MBFAs are primarily found in nitrogen and sulfur reducers
364 (chemoheterotrophs) and oxidizers (chemoautotrophs) that are mostly members of the (large)
365 proteobacteria family (49–52). Nitrogen and sulfur reduction and oxidation processes are
366 conducted in deep-sea sponges such as *G. barretti* (26,53,54), and oxidation processes are
367 coupled to CO₂ fixation, although associated CO₂ fixation is likely to contribute < 10 % of
368 the carbon demand of deep-sea sponges (55). The poribacteria in sponges were also
369 characterized as mixotrophic bacteria, able to fix CO₂ using the ancient Wood–Ljungdahl
370 (reversed acetyl-CoA) pathway (56). The isotopic enrichment in MBFAs (Fig. 4, Table 2),
371 agrees with earlier observations for *G. barretti* (57), and might thus be linked to nitrogen and
372 sulfur transforming processes and potentially CO₂ fixation. It will be interesting to perform
373 an isotope-tracer study (55) with ¹³C-CO₂ to assess CO₂ incorporation in the abundant
374 MBFAs, perhaps combined with nitrification (or sulfur oxidation) inhibitors, similar to
375 Veuger et al. (58).

376 The most depleted FA (*i*-C_{17:1}ω7, Fig. 4, Table 2) is considered a chemotaxonomic
377 marker for the sulfur reducing bacteria *Desulfovibrio* sp. (59). The isotopic difference
378 between *i*-C_{17:1}ω7 and MBFAs suggest that these markers are not from the same microbial
379 consortium. The more general bacterial markers (e.g. (*a*)*i*-C_{15:0}, typical of gram-positive
380 bacteria and C_{16:1}ω7 and C_{18:1}ω7, typical of general gram-negative bacteria (15)) had
381 intermediate δ¹³C values (Fig. 4, Table 2). Such values can be the result of isotopic averages
382 from different pathways, since they are more general bacterial markers, or they might

383 represent general heterotrophy on organic matter with $\delta^{13}\text{C}$ value from -24 to -22 ‰ in the
384 western Arctic (60).

385 The low contribution of FAs with a chain length of C_{20} to C_{24} typical of
386 phytoplankton and zooplankton (e.g. $\text{C}_{20:5\omega3}$ and $\text{C}_{22:6\omega3}$) indicates that sinking zoo- and
387 phytoplankton are not contributing much to sponge diet, at least not directly. These findings
388 support increasing evidence that *G. barretti* (and other North-Atlantic deep-sea sponges)
389 primarily feed on dissolved organic matter and pelagic and associated bacteria (61,62). A
390 higher contribution of phytoplankton markers in boreal *Geodia* spp. (*G. atlantica* and *G.*
391 *barretti*) compared to Arctic species (Table 1) might be linked to water depth, as boreal
392 species were sampled from ~300 m and Arctic species from ~600 m, while also
393 environmental factors, such as permanent ice coverage (Langseth Ridge) and a generally
394 lower primary production in the Arctic compared to the boreal North-Atlantic ocean (63)
395 might play a major role.

396 The overall high abundance of bacterial FAs (56–79 % of total FAs across all five
397 analyzed deep-sea demosponge species, Fig. 3) fits with their classification as HMA sponges
398 and supports the idea that microbial endosymbionts play a pivotal role in sponge metabolism
399 (2,3). It is important to notice that the contribution of endosymbionts is likely even higher,
400 since archaea are not detected with (PL)FA analysis (64), while they were also found to be
401 abundant in *G. barretti* (46,47).

402

403 **Sponge LCFAs**

404 Although bacterial FA profiles were very similar among the studied Tetractinellid
405 species, the sponge-specific LCFA composition was more species-specific. The dominant
406 unsaturation in LCFAs, was double unsaturation at $\Delta^{5,9}$ position in all species analyzed,
407 which is typical feature of demosponges (5,10). Similarly, the linear $\text{C}_{26:2}\Delta^{5,9}$, (*a*)*i*- $\text{C}_{25:2}\Delta^{5,9}$

408 and/or $i\text{-C}_{27:2}\Delta^{5,9}$, present in all species analyzed (Fig. 5, Table 1), are common LCFAs of
409 demosponges, (e.g. (17,38,65), for an overview see (8)). We found (mid-)Me-branched $\Delta^{5,9}$
410 LCFAs in all species, except *G. parva* (Fig. 5, Table 1). Also, Thiel et al. (17) found them in
411 *G. barretti* and some other Demospongiae (*Haliclona* sp., *Petrosia* sp.) but not in all analyzed
412 Demospongiae. We also identified novel LCFAs: $i\text{-C}_{27:2}\Delta^{9,19}$ (in *G. atlantica*, *G. barretti*, and
413 *G. hentscheli*) and $\Delta^{11,21}$ in $\text{C}_{26:2}$ and $\text{C}_{28:2}$ (*G. barretti* and *G. parva*). The presence of Δ^{11}
414 unsaturation ($\text{C}_{26:2}\Delta^{11,21}$ and $\text{C}_{28:2}\Delta^{11,21}$), identified via DMDS derivatization, is uncommon in
415 sponge LCFAs. Barnathan et al. (66) found Δ^{11} unsaturation in a series of monoenic FAs,
416 including $\text{C}_{28:1}$, in a tropical demosponge species (order Axinellida), but no dienic LCFAs
417 with Δ^{11} unsaturation have been described so far. The configuration indicates that Δ^{11}
418 desaturase might be active in these species; however, the activity of this enzyme in sponges
419 has not been reported.

420 *G. atlantica* and *G. barretti* had almost identical FA profiles (Table 1), suggesting that
421 these species might be closely related, as was earlier suggested based on their sterol and
422 amino acid composition (67), but deviates from molecular phylogeny that places them further
423 apart (27). The FA profile of *G. hentscheli* resembled those of *G. barretti* and *G. atlantica*
424 and based on molecular phenology, *G. hentscheli* is a sister species of *G. barretti*. On the
425 other hand, *G. parva* produced distinct LCFAs compared to the other *Geodia* spp., the i - and
426 $\alpha\text{-C}_{25:2}\Delta^{5,9}$ and this species is also phylogenetically apart from the other *Geodia* spp. (27). A
427 dominance of (α) i -branched $\text{C}_{25:2}$ has been found in another Geodiidae family (*Geodinella*)
428 (68). Finally, also *S. raphidiophora* produced a distinct LCFA, $\text{Me-C}_{28:2}\Delta^{5,9}$, a LCFA that
429 has been described for demosponges of the family *Aplysinidae* (13,42).

430 Each of the three dominant Tetractinellids of Arctic sponge grounds (*G. hentscheli*, *G.*
431 *parva* and *S. raphidiophora*) produced distinct LCFAs (Table 1) that can serve as
432 chemotaxonomic markers. The morphology of these sponges is very similar, so LCFA

433 analysis provides an additional method to identify each species. Furthermore, the distinct
434 LCFAs could be useful as trophic markers to study the ecological role of deep-sea sponges in
435 the environment. No geographical differences in LCFA composition of Arctic Tetractinellids
436 were found (Table S1) suggesting that the environment has a limited influence on the LCFA
437 composition, which is a prerequisite for using LCFA as chemotaxonomic markers.

438

439 **Biosynthetic pathways of prominent sponge fatty acids**

440 The identification of branching in LCFAs allows identification of its short chain
441 precursors and biosynthetic pathways. As demonstrated by various *in vivo* incorporation
442 studies with radioactive substrates (16,38,65,69), sponges elongate FA precursors by adding
443 2 C atoms at the carboxylic acid end and desaturate at Δ^5 and Δ^9 (visualized in (10)),
444 revealing $C_{16:0}$ as precursor for the common $C_{26:2}\Delta^{5,9}$, while $C_{16:1\omega7}$ was identified as
445 precursors for $C_{26:2}\Delta^{9,19}$ (Fig. 5). There is no evidence for branching to be introduced by
446 sponges, so *i*- and *a*- $C_{15:0}$ were identified as precursors of *i*- and *a*- $C_{27:2}\Delta^{5,9}$ (Fig. 5) (38),
447 while Me- $C_{16:0}$ has been identified as precursor for Me- $C_{26:2}\Delta^{5,9}$ and Me- $C_{28:2}\Delta^{5,9}$ (Fig. 5)
448 (13,16,70). Finally, we hypothesize that *i*- $C_{17:1\omega7}$ is the precursor for *i*- $C_{25:1\omega7}$ and *i*-
449 $C_{27:2}\Delta^{9,19}$ found in *G. atlantica*, *G. barretti*, and *G. hentscheli* (Fig. 5).

450 Application to the present study showed that most LCFAs could be linked to
451 precursors via established pathways, with hypothetical intermediates since hardly any were
452 found in detectable abundance (Fig. 5). The C isotopic differences in bacterial precursors
453 were (partially) reflected in C isotopic composition of LCFAs (Fig. 4, Table 2), although the
454 differences were not as prominent in LCFAs compared to their precursors. One explanation is
455 that a mixture of C sources is used by the host to elongate precursors to LCFAs, while also
456 methodological aspects might contribute. A (much) longer analytical column might help
457 improving separation of LCFAs.

458

459 **Fig. 5: Proposed biosynthetic pathways of (microbial) precursors to LCFA in examined**

460 **Tetractinellid species.** FAs detected in the studied sponges are shown in a black rectangle,

461 solid arrows indicate elongation, dashed arrows indicate $\Delta^{5,9}$ desaturation. The species

462 encompassing each LCFA are indicated with abbreviated names as in Table 1 and names in

463 bold means that the LCFA is dominant in that specific species.

464

465 The schematization of Fig. 5 shows the benefit of using both ω and Δ (and mixed)

466 nomenclatures in sponge lipid research. Annotations from the terminal end (ω and $a(i)$

467 notation) (Fig. 1) are convenient to show biosynthetic pathways as these positions do not

468 change with elongation (Fig. 5). However, the typical $\Delta^{5,9}$ unsaturation is more convenient to

469 show with Δ annotation, as an ω notation would alter with varying C chain length (Fig. 5).

470 Ambiguity arises in ω notation of methyl-branching, because $a(i)$ notation is used for

471 terminally branched FAs and describes total C atoms (including the methyl group(s)), while

472 Me notation is used for MBFAs and describes the C number of the backbone (excluding

473 methyl group (s)) and counts the position of the branching from the carboxylic acid end (and

474 not the terminal (ω) end, Fig. 1). This might lead to confusion about the total C number,

475 which is needed to correct measured isotope values for the extra methyl group, and about the

476 ω position of unsaturation (start counting from the end of the backbone, excluding the

477 methyl-group) and the conversion from ω to Δ notation (Fig. 1). We added this discussion to

478 create awareness and would like to recommend including a description of the notation in the

479 methods and presenting both nomenclature when a mixture of notation styles is used.

480

481 **Conclusions**

482 In this study we identified FAs of prominent habitat-building demosponges (order
483 Tetractinellida) from the boreal-Arctic Atlantic Ocean. All five species investigated
484 contained predominantly bacterial FAs, in particular isomeric mixtures of MBFAs (Me-C_{16:0}
485 and Me-C_{18:0}) (together >20% of total FAs). The MBFAs were isotopically enriched
486 compared to linear and (*ante*)*iso*-branched FAs. The sponge-produced LCFAs with chain
487 lengths of C₂₄-C₂₈ were linear, mid- and *a(i)*-branched and had predominantly the typical $\Delta^{5,9}$
488 saturation. They also produced (yet undescribed) branched and linear LCFAs with $\Delta^{9,19}$ and
489 $\Delta^{11,21}$ unsaturation, namely *i*-C_{27:2} $\Delta^{9,19}$, C_{26:2} $\Delta^{11,21}$, and C_{28:2} $\Delta^{11,21}$. *G. parva* and *S.*
490 *rhaphidiophora* each produced distinct LCFAs, while *G. atlantica*, *G. barretti*, and *G.*
491 *hentscheli* had a similar LCFA profile, although each species had different predominant ones.
492 The typical FA profiles of North-Atlantic deep-sea demosponges can be used as
493 chemotaxonomic and trophic markers. We proposed biosynthetic pathways for dominant
494 LCFAs from their bacterial precursors, which were supported by small isotopic differences in
495 LCFAs that support the idea that sponges acquire building blocks from their endosymbiotic
496 bacteria.

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509

510 **References**

- 511 1. van Soest RWM, Boury-Esnault N, Vacelet J, Dohrmann M, Erpenbeck D, de Voogd
512 NJ, et al. Global diversity of sponges (Porifera). PLoS One. 2012;7(4).
- 513 2. Taylor MW, Radax R, Steger D, Wagner M. Sponge-Associated microorganisms:
514 evolution, ecology, and biotechnological potential. Microbiol Mol Biol Rev.
515 2007;71(2):295–347.
- 516 3. Pita L, Rix L, Slaby BM, Franke A, Hentschel U. The sponge holobiont in a changing
517 ocean: from microbes to ecosystems. Microbiome. 2018;6(1):46.
- 518 4. Ribes M, Jiménez E, Yahel G, López-Sendino P, Diez B, Massana R, et al. Functional
519 convergence of microbes associated with temperate marine sponges. Environ
520 Microbiol. 2012;14(5):1224–39.
- 521 5. Litchfield C, Greenberg AJ, Noto G, Morales RW. Unusually high levels of C24-C30
522 fatty acids in sponges of the class demospongiae. Lipids. 1976;11(7):567–70.
- 523 6. Morales RW, Litchfield C. Unusual C24, C25, C26 and C27 polyunsaturated fatty
524 acids of the marine sponge *Microciona prolifera*. Biochim Biophys Acta, Lipids Lipid
525 Metab. 1976;431:206–16.
- 526 7. Rod’kina SA. Fatty acids and other lipids of marine sponges. Russ J Mar Biol.
527 2005;31:S49–60.
- 528 8. Rezanka T, Sigler K. Odd-numbered very-long-chain fatty acids from the microbial ,
529 animal and plant kingdoms. Prog Lipid Res J. 2009;48(3-4):206–38.

- 530 9. Mishra PM, Sree A, Panda P. Fatty acids of Marine sponges. In: Kim SK (eds),
531 Springer handbook of marine biotechnology. Springer Berlin Heidelberg; 2015. p.
532 851–68.
- 533 10. Kornprobst JM, Barnathan G. Demospongiac acids revisited. *Mar Drugs*.
534 2010;8(10):2569–77.
- 535 11. Lawson MP, Thompson JE, Djerassi C. Cell membrane localization of long chain C24-
536 C30 fatty acids in two marine demosponges. *Lipids*. 1988;23(8):741–9.
- 537 12. Thiel V, Jenisch A, Wörheide G, Löwenberg A, Reitner J, Michaelis W. Mid-chain
538 branched alkanolic acids from “living fossil” demosponges: A link to ancient
539 sedimentary lipids? *Org Geochem*. 1999;30(1):1–14.
- 540 13. Raederstorff D, Shu AYL, Thompson JE, Djerassi C. Biosynthetic studies of marine
541 lipids. 11.1 Synthesis, biosynthesis, and absolute configuration of the internally
542 branched demospongiac acid, 22-Methyl-5,9-octacosadienoic acid. *J Org Chem*.
543 1987;52(12):2337–46.
- 544 14. Bergé J-P, Barnathan G. Fatty acids from lipids of marine organisms: molecular
545 biodiversity, roles as biomarkers, biologically active compounds, and economical
546 aspects. *Mar Biotechnol*. 2005;1:51–111.
- 547 15. Zelles L. Phospholipid fatty acid profiles in selected members of soil microbial
548 communities. *Chemosphere*. 1997;35(1–2):275–94.
- 549 16. Djerassi C, Lam WK. Sponge Phospholipids. *Acc Chem Res*. 1991;24(3):69–75.
- 550 17. Thiel V, Blumenberg M, Hefter J, Pape T, Pomponi S, Reed J, et al. A chemical view
551 of the most ancient metazoa - Biomarker chemotaxonomy of hexactinellid sponges.
552 *Naturwissenschaften*. 2002;89(2):60–6.
- 553 18. Lawson MP, Bergquist PR, Cambie RC, Lawson MP, Lavis A, Cambie RC. Fatty acid
554 composition and the classification of the Porifera. *Biochem Syst Ecol*. 1984;12(1):63–

- 555 84.
- 556 19. van Duyl FC, Moodley L, Nieuwland G, van IJzerloo L, van Soest RWM, Houtekamer
557 M, et al. Coral cavity sponges depend on reef-derived food resources: Stable isotope
558 and fatty acid constraints. *Mar Biol.* 2011;158(7):1653–66.
- 559 20. Thurber AR. Diets of Antarctic sponges: Links between the pelagic microbial loop and
560 benthic metazoan food web. *Mar Ecol Prog Ser.* 2007;351:77–89.
- 561 21. Rubin-Blum M, Antony CP, Sayavedra L, Martínez-Pérez C, Birgel D, Peckmann J, et
562 al. Fueled by methane: Deep-sea sponges from asphalt seeps gain their nutrition from
563 methane-oxidizing symbionts. *ISME.* 2019;13:1209–1225.
- 564 22. Knudby A, Kenchington E, Murillo FJ. Modeling the distribution of *Geodia* sponges
565 and sponge grounds in the Northwest Atlantic. *PLoS One.* 2013;8(12):1–20.
- 566 23. Klitgaard AB, Tendal OS. Distribution and species composition of mass occurrences
567 of large-sized sponges in the northeast Atlantic. *Prog Oceanogr.* 2004;61(1):57–98.
- 568 24. Kutti T, Bannister RJ, Fosså JH. Community structure and ecological function of deep-
569 water sponge grounds in the Traenadypet MPA-Northern Norwegian continental shelf.
570 *Cont Shelf Res.* 2013;69:21–30.
- 571 25. Murillo FJ, Muñoz PD, Cristobo J, Ríos P, González C, Kenchington E, et al. Deep-
572 sea sponge grounds of the Flemish Cap, Flemish Pass and the Grand Banks of
573 Newfoundland (Northwest Atlantic Ocean): Distribution and species composition. *Mar*
574 *Biol Res.* 2012;8(9):842–54.
- 575 26. Hoffmann F, Larsen O, Thiel V, Rapp HT, Pape T, Michaelis W, et al. An anaerobic
576 world in sponges. *Geomicrobiol J.* 2005;22:1–10.
- 577 27. Cardenas P, Rapp HT, Klitgaard AB, Best M, Tholleson M, Secher Tendal O.
578 Taxonomy, biogeography and DNA barcodes of *Geodia* species (Porifera,
579 Demospongiae, Tetractinellida) in the Atlantic boreo-arctic region. *Zool J Linn Soc.*

- 580 2013;169(2):312–61.
- 581 28. Bart MC, Kluijver A De, Hoetjes S, Absalah S, Mueller B, Rapp HT, et al. Differential
582 processing of dissolved and particulate organic matter by deep-sea sponges and their
583 microbial symbionts. *Sci Rep*. Forthcoming.
- 584 29. Roberts EM, Mienis F, Rapp HT, Hanz U, Meyer HK, Davies AJ. Oceanographic
585 setting and short-timescale environmental variability at an Arctic seamount sponge
586 ground. *Deep Res Part I Oceanogr Res Pap*. 2018;138:98–113.
- 587 30. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J*
588 *Biochem Physiol*. 1959;37(8):911–17.
- 589 31. Koopmans M, Rijswijk P Van, Martens D, Wijffels RH. Seasonal variation of fatty
590 acids and stable carbon isotopes in sponges as indicators for nutrition : biomarkers in
591 sponges identified. *Mar biotechnol*. 2015;17(1):43–54.
- 592 32. Rix L, de Goeij JM, Mueller CE, Struck U, Middelburg JJ, van Duyl FC, et al. Coral
593 mucus fuels the sponge loop in warm- and cold-water coral reef ecosystems. *Sci Rep*.
594 2016;6:18715.
- 595 33. Boschker HTS. Linking microbial community structure and functioning: stable isotope
596 (^{13}C) labeling in combination with PLFA analysis. In: Kowalchuk GA, de Bruijn FJ et
597 al. (eds), *Molecular microbial ecology manual*. 2nd edition. Kluwer-Academic
598 publishers; 2004. p. 1673–88.
- 599 34. Christie WW. Preparation of Ester Derivatives of Fatty Acids for Chromatographic
600 Analysis. *Adv Lipid Methodol*. 1993;69–111.
- 601 35. Nichols PD, Guckert JB, White DC. Determination of monosaturated fatty acid
602 double-bond position and geometry for microbial monocultures and complex consortia
603 by capillary GC-MS of their dimethyl disulphide adducts. *J Microbiol Methods*.
604 1986;5(1):49–55.

- 605 36. Team RC. R: A language and environment for statistical computing. R Foundation for
606 Statistical Computing. 2019.
- 607 37. Soetaert K, Provoost P, Van Rijswijk P. RLims: R functions for Lab Analysis using
608 GC-FID and GC-c-IRMS. 2015.
- 609 38. Carballeira N, Thompson JE, Ayanoglu E, Djerassi C. Biosynthetic Studies of Marine
610 Lipids. 5. The biosynthesis of long-chain branched fatty acids in marine sponges. *J Org*
611 *Chem.* 1986;51(14):2751–6.
- 612 39. Vincenti M, Guglielmetti G, Cassani G, Tonini C. Determination of double bond
613 position in diunsaturated compounds by mass spectrometry of dimethyl disulfide
614 derivatives. *Anal Chem.* 1987;59(5):694–9.
- 615 40. Carballeira NM, Shalabi F. Unusual lipids in the Caribbean sponges *Amphimedon*
616 *viridis* and *Desmapsamma anchorata*. *J Nat Prod.* 1994;57(8):1152–9.
- 617 41. Gillan FT, Stoilov IL, Thompson JE, Hogg RW, Wilkinson CR, Djerassi C. Fatty acids
618 as biological markers for bacterial symbionts in sponges. *Lipids.* 1988;23(12):1139–
619 45.
- 620 42. Nechev J, Christie WW, Robaina R, De Diego F, Popov S, Stefanov K. Lipid
621 composition of the sponge *Verongia aerophoba* from the Canary islands. *Eur J Lipid*
622 *Sci Technol.* 2002;104(12):800–7.
- 623 43. Hochmuth T, Niederkrüger H, Gernert C, Siegl A, Taudien S, Platzer M, et al. Linking
624 chemical and microbial diversity in marine sponges: Possible role for poribacteria as
625 producers of methyl-branched fatty acids. *ChemBioChem.* 2010;11(18):2572–8.
- 626 44. Schöttner S, Hoffmann F, Cárdenas P, Rapp HT, Boetius A, Ramette A. Relationships
627 between host phylogeny, host type and bacterial community diversity in cold-water
628 coral reef sponges. *PLoS One.* 2013;8(2):1–11.
- 629 45. Fieseler L, Horn M, Wagner M, Hentschel U. Discovery of the novel candidate

- 630 phylum “Poribacteria” in marine sponges. *Appl Environ Microbiol.* 2004;70(6):3724–
631 32.
- 632 46. Radax R, Rattei T, Lanzen A, Bayer C, Rapp HT, Urich T, et al. Metatranscriptomics
633 of the marine sponge *Geodia barretti* : tackling phylogeny and function of its
634 microbial community. 2012;14:1308–24.
- 635 47. Luter HM, Bannister RJ, Whalan S, Kutti T, Pineda MC, Webster NS. Microbiome
636 analysis of a disease affecting the deep-sea sponge *Geodia barretti*. *FEMS Microbiol*
637 *Ecol.* 2017;93(6):1–6.
- 638 48. Busch K, Hanz U, Mienis F, Mueller B, Franke A, Martyn Roberts E, et al. On giant
639 shoulders: How a seamount affects the microbial community composition of seawater
640 and sponges. *Biogeosciences.* 2020;17(13):3471–86.
- 641 49. Kool DM, Zhu B, Rijpstra WIC, Jetten MSM, Ettwig KF, Sinninghe Damsté JS. Rare
642 branched fatty acids characterize the lipid composition of the intra-aerobic methane
643 oxidizer “*Candidatus Methylomirabilis oxyfera*.” *Appl Environ Microbiol.*
644 2012;78(24):8650–6.
- 645 50. Kerger B, Nichols PD, Antworth CP, Sand W, Bock E, Cox JC, et al. Signature fatty
646 acids in the polar lipids of acid-producing *Thiobacillus* spp .: methoxy , cyclopropyl ,
647 alpha-hydroxy-cyclopropyl and branched and normal monoenoic fatty acids. *FEMS*
648 *Microbiol Ecol.* 1986;2(2):67–77.
- 649 51. Schwab VF, Herrmann M, Roth VN, Gleixner G, Lehmann R, Pohnert G, et al.
650 Functional diversity of microbial communities in pristine aquifers inferred by PLFA-
651 and sequencing-based approaches. *Biogeosciences.* 2017;14(10):2697–714.
- 652 52. Lipski A, Spieck E, Makolla A, Altendorf K. Fatty acid profiles of nitrite-oxidizing
653 bacteria reflect their phylogenetic heterogeneity. *Syst Appl Microbiol.*
654 2001;24(3):377–84.

- 655 53. Hoffmann F, Radax R, Woebken D, Holtappels M, Lavik G, Rapp HT, et al. Complex
656 nitrogen cycling in the sponge *Geodia barretti*. *Environ Microbiol.* 2009;11(9):2228–
657 43.
- 658 54. Radax R, Hoffmann F, Rapp HT, Leininger S, Schleper C. Ammonia-oxidizing
659 archaea as main drivers of nitrification in cold-water sponges. *Environ Microbiol.*
660 2012;14(4):909–23.
- 661 55. van Duyl FC, Lengger SK, Schouten S, Lundälv T, van Oevelen D, Müller CE. Dark
662 CO₂ fixation into phospholipid-derived fatty acids by the cold-water coral associated
663 sponge *Hymedesmia (Stylopus) coriacea* (Tisler Reef, NE Skagerrak). *Mar Biol Res.*
664 2020;16(1):1–17.
- 665 56. Siegl A, Kamke J, Hochmuth T, Piel J, Richter M, Liang C, et al. Single-cell genomics
666 reveals the lifestyle of Poribacteria, a candidate phylum symbiotically associated with
667 marine sponges. *ISME J.* 2011;5(1):61–70.
- 668 57. Pape T. Lipidbiomarker Schwammassoziierter Bakterien Und Archaeen. Doctoral
669 dissertation. University of Hamburg. 2004 Available from: [https://ediss.sub.uni-](https://ediss.sub.uni-hamburg.de/volltexte/2004/2112/)
670 [hamburg.de/volltexte/2004/2112/](https://ediss.sub.uni-hamburg.de/volltexte/2004/2112/)
- 671 58. Veuger B, Pitcher A, Schouten S, Sinninghe Damsté JS, Middelburg JJ. Nitrification
672 and growth of autotrophic nitrifying bacteria and Thaumarchaeota in the coastal North
673 Sea. *Biogeosciences.* 2013;10(3):1775–85.
- 674 59. Boon JJ, De Leeuw JW, Van De Hoek GJ, Vosjan JH. Significance and taxonomic
675 value of iso and anteiso monoenoic fatty acids and branched β hydroxy acids in
676 *Desulfovibrio desulfuricans*. *J Bacteriol.* 1977;129(3):1183–91.
- 677 60. Griffith DR, McNichol AP, Xu L, McLaughlin FA, MacDonald RW, Brown KA, et al.
678 Carbon dynamics in the western Arctic Ocean: Insights from full-depth carbon isotope
679 profiles of DIC, DOC, and POC. *Biogeosciences.* 2012;9(3):1217–24.

- 680 61. Leys SP, Kahn AS, Fang JKH, Kutti T, Bannister RJ. Phagocytosis of microbial
681 symbionts balances the carbon and nitrogen budget for the deep-water boreal sponge
682 *Geodia barretti*. *Limnol Oceanogr.* 2018;1–16.
- 683 62. Bart M, Mueller B, Rombouts T, van de Ven C, Tompkins G, Osinga R, et al.
684 Dissolved organic carbon (DOC) is essential to balance the metabolic demands of
685 North-Atlantic deep-sea sponges. *BioRxiv [Preprint].*2020 bioRxiv 305086 [posted
686 2020 Sep 21]. Available from:
687 <https://www.biorxiv.org/content/10.1101/2020.09.21.305086v1>
- 688 63. Wassmann P, Slagstad D, Ellingsen I. Primary production and climatic variability in
689 the European sector of the Arctic Ocean prior to 2007: Preliminary results. *Polar Biol.*
690 2010;33(12):1641–50.
- 691 64. Koga Y, Morii H. Biosynthesis of ether-type polar lipids in archaea and evolutionary
692 considerations. *Microbiol Mol Biol Rev.* 2007;71(1):97–120.
- 693 65. Hahn S, Stoilov IL, Tam Ha TB, Raederstorff D, Doss GA, Li HT, et al. Biosynthetic
694 studies of marine lipids. 17. The course of chain elongation and desaturation in long-
695 chain fatty acids of marine sponges. *J Am Chem Soc.* 1988;110(24):8117–24.
- 696 66. Barnathan G, Kornprobst JM, Doumenq P, Miralles J. New unsaturated long-chain
697 fatty acids in the phospholipids from the Axinellida sponges *Trikenrion loeve* and
698 *Pseudaxinella cf. lunaecharta*. *Lipids.* 1996;31(2):193–200.
- 699 67. Hougaard L, Christophersen C, Nielsen PH, Klitgaard A, Tendal O. The chemical
700 composition of species of *Geodia*, *Isops* and *Stryphnus* (Choristida: Demospongia:
701 Porifera)-A comparative study with some taxonomical implications. *Biochem Syst*
702 *Ecol.* 1991;19(3):223–35.
- 703 68. Makarieva TN, Santalova Ie, Gorshkova IA, Dmitrenok AS, Guzii AG, Gorbach VI, et
704 al. A new cytotoxic fatty acid (5Z,9Z)-22-methyl-5,9-tetracosadienoic acid and the

- 705 sterols from the far Eastern sponge *Geodinella robusta*. *Lipids*. 2006;37(1):75–80.
- 706 69. Morales RW, Litchfield C. Incorporation of ^{14}C -Acetate into C26 Fatty Acids of the
707 Marine Sponge *Microciona prolifera*. *Lipids*. 1977;12(7):570-76.
- 708 70. Walkup RD, Jamieson GC, Ratcliff MR, Djerassi C. Phospholipid studies of marine
709 organisms: 2.¹ Phospholipids, phospholipid-bound fatty acids and free sterols of the
710 sponge *Aplysina fistularis* (Pallas) forma *fulva* (Pallas) (= *Verongia thiona*)². Isolation
711 and structure elucidation of unprecedented branched. *Lipids*. 1981;16(9):631–46.

712

713 **Supplementary information**

714 **S1 Fig. Mass spectra of DMDS conducts of C₂₆ (a,b) and C₂₈ (c,d) LCFA with $\Delta^{9,19}$ (a,c)
715 and $\Delta^{11,21}$ (b,d) unsaturation**

716 **S1 Table. All fatty acid compositional data.** This excel file contains fatty acid data ($\mu\text{g g}$
717 DW^{-1} and relative abundance (%), in PL and TL) of individual specimens. The excel file also
718 contains the fragments of Me-branched C₁₆ and C₁₈, the relative positions of saturated
719 (branched and linear) FAMES in hydrogenated samples and the isotope data.

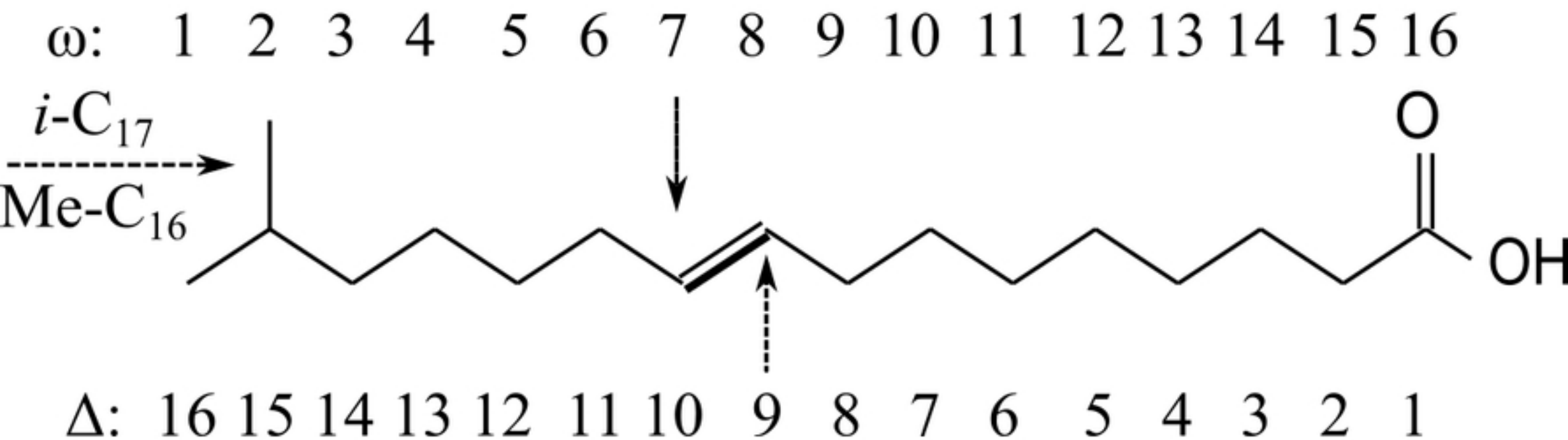
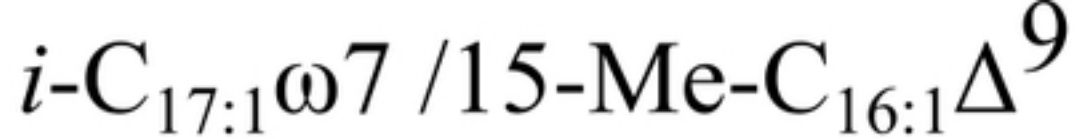


Figure 1

Geodia hentscheli

Langseth Ridge

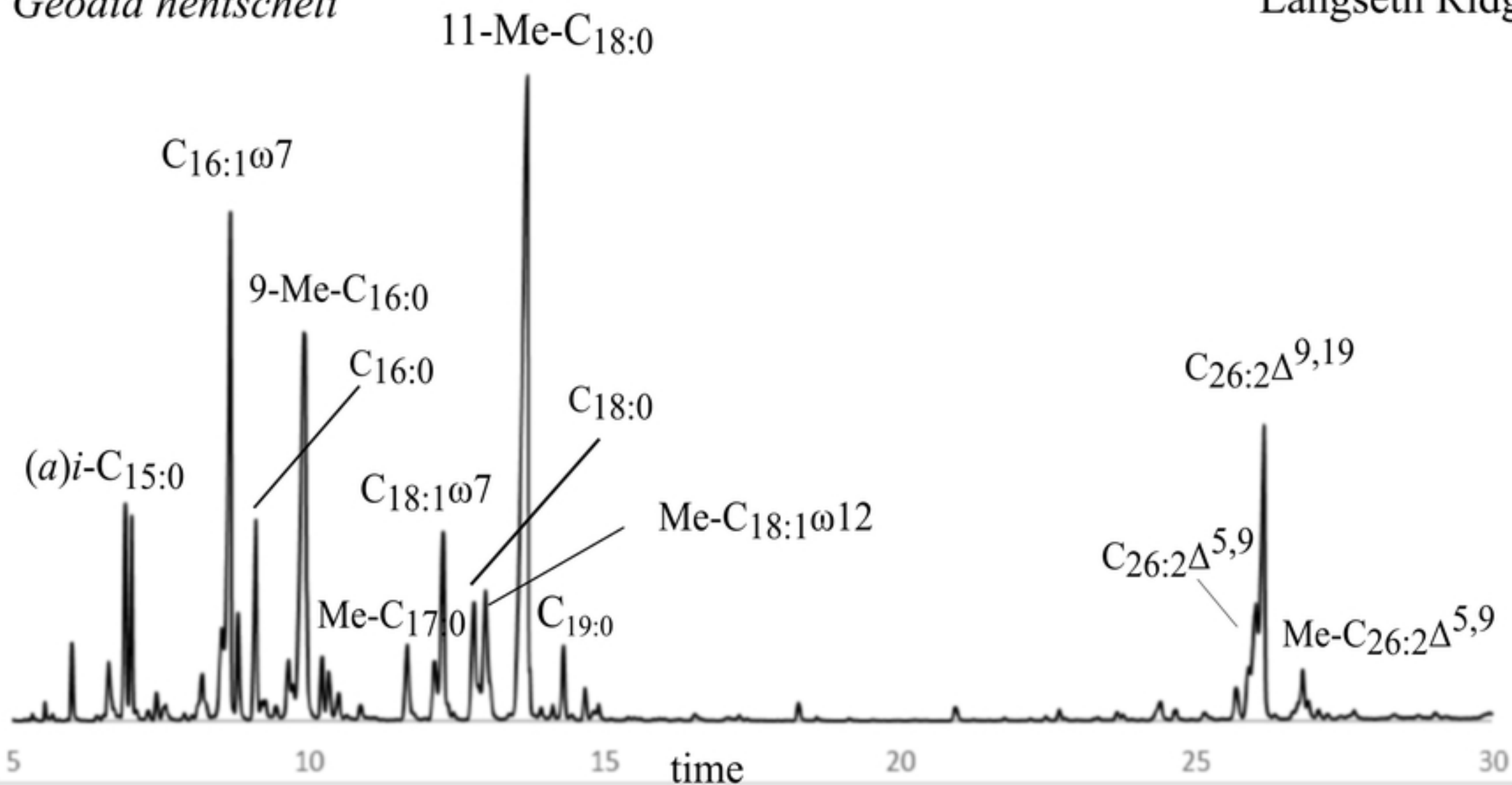


Figure 2

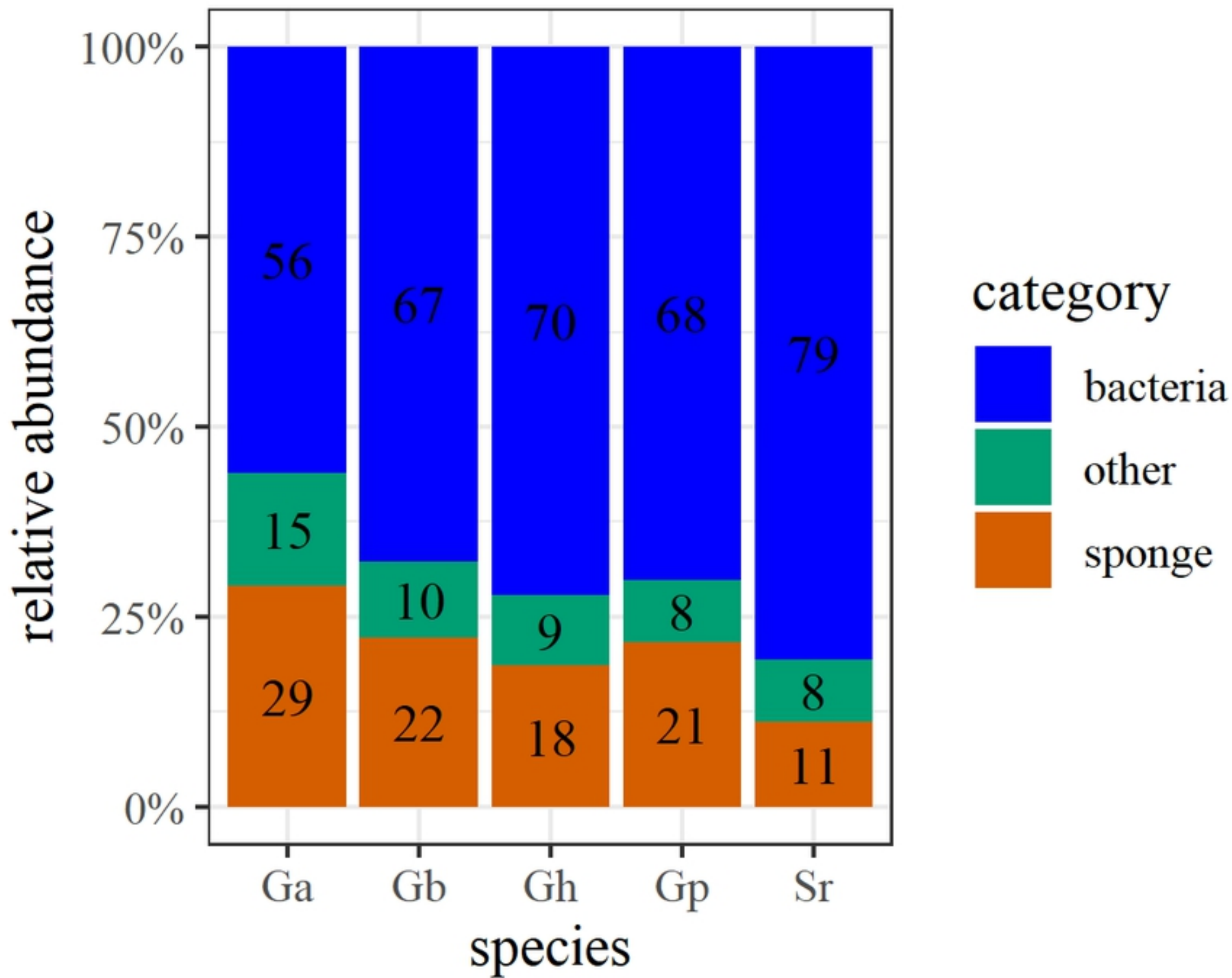


Figure 3

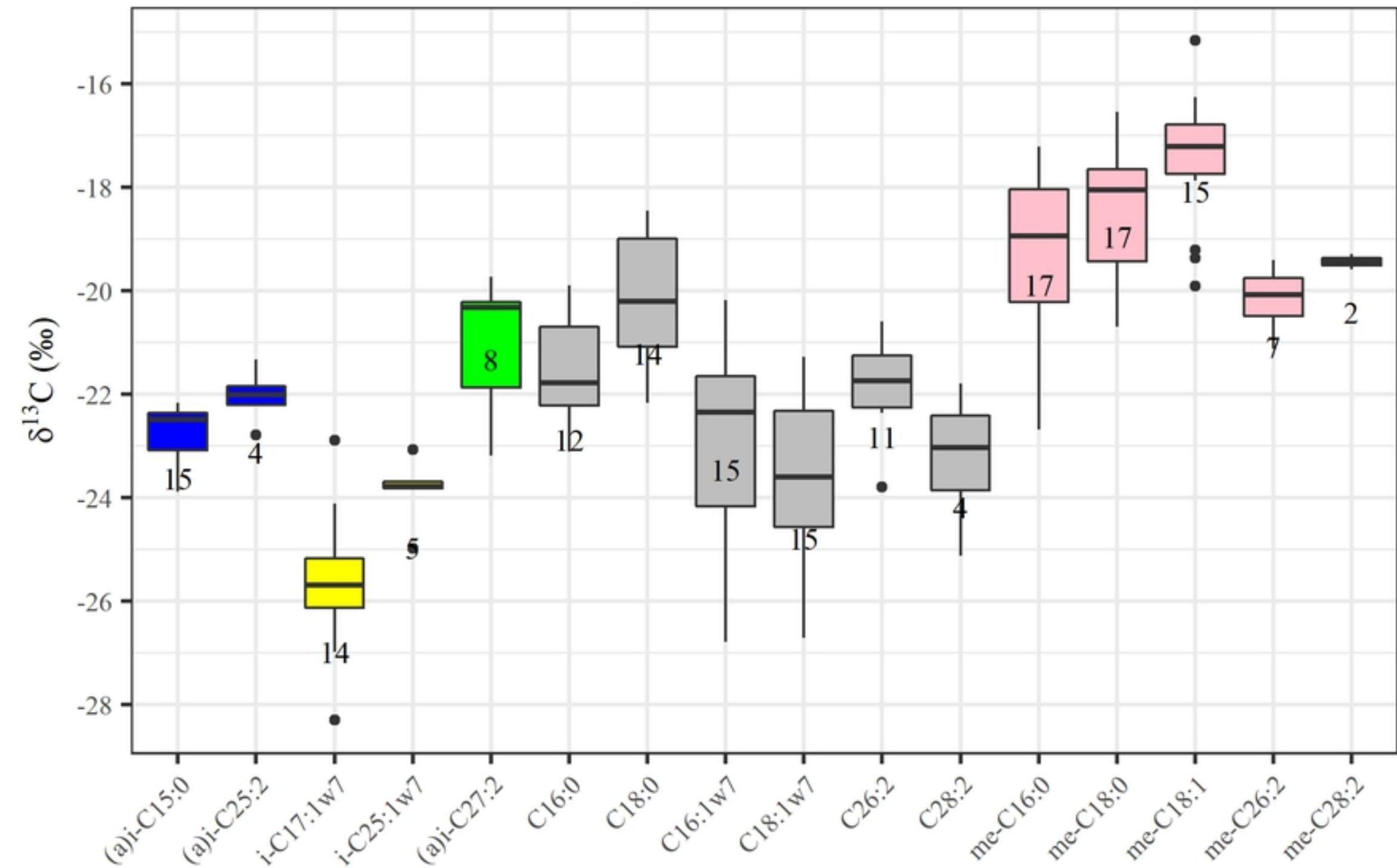


Figure 4

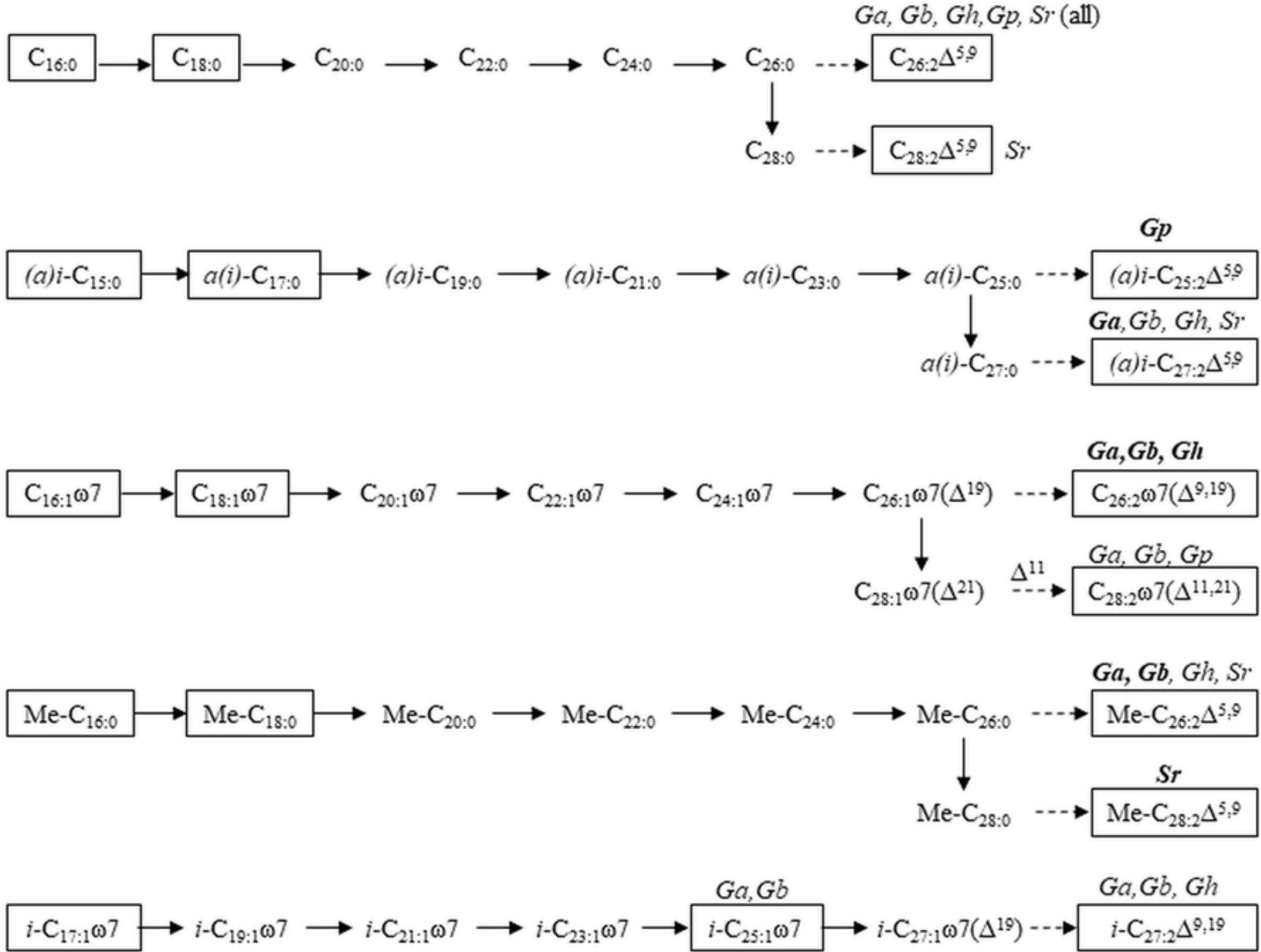


Figure 5