

# Impacts of pH and temperature on soil bacterial 3-hydroxy fatty acids: development of novel terrestrial proxies

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1 **Impacts of pH and temperature on soil bacterial 3-hydroxy fatty acids:**  
2 **development of novel terrestrial proxies**

3

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19

20 **Abstract:** Gram-negative bacterial 3-hydroxy fatty acids (3-OH-FAs)  
21 biomarkers are widespread in a variety of environments including both marine  
22 and terrestrial sediments (including speleothems). In this study we analysed  
23 the hydroxylated membrane lipids of 26 soil samples from an altitudinal  
24 transect of Shennongjia Mountain (Mt.) in central China to study the  
25 environmental factors controlling the relative distribution of 3-OH-FAs. Our  
26 results show that both the ratio of the summed *iso* and *anteiso* to the total  
27 amount of *normal* 3-OH-FAs (RIAN), and the ratio of summed *iso* and *anteiso*  
28 to the total amount of all 3-OH-FAs (Branched Index) were primarily related to  
29 the pH of soil ( $R^2 = 0.70$  and  $0.70$ , respectively). Additionally, the *anteiso* to  
30 *normal* 3-hydroxy fatty acids ratio of the  $C_{15}$  and  $C_{17}$  homologues (RAN<sub>15</sub> and  
31 RAN<sub>17</sub>) shows a significant negative correlation with mean annual air  
32 temperature (MAAT) ( $R^2=0.51$  and  $0.48$ , respectively). When comparing the 3-  
33 OH-FA based indices with established glycerol dialkyl glycerol tetraether  
34 (GDGT) based indices from the same soil samples, the RIAN and Branched  
35 Index show strong linear correlations with the cyclisation ratio of branched  
36 tetraethers (CBT) ( $R^2 = 0.77$  and  $0.74$ , respectively), and the RAN<sub>15</sub> and RAN<sub>17</sub>  
37 show negative correlations with the MBT/CBT-MAAT (MBT, methylation index  
38 of branched tetraethers) ( $R^2 = 0.61$  and  $0.36$ , respectively). Our new field-based  
39 correlations demonstrate the physiological response of Gram-negative bacterial  
40 cell membranes to the external environment and suggest that 3-hydroxy fatty

41 acids can be applied in palaeoenvironmental studies to estimate past MAAT  
42 and soil pH.

43

44 **Keywords:** proxy, 3-hydroxy fatty acid, soil, temperature, soil pH,  
45 palaeoclimate

46

## 47 **1. Introduction**

48 A wide range of environmental information from both terrestrial and  
49 marine realms is required from palaeoclimate archives to better understand  
50 the climate system and to provide a palaeoclimatic context for predictions of  
51 future rates of climate change, impact and Earth System sensitivity. To date,  
52 various geochemical proxies based on inorganic and organic fossil remains have  
53 been applied in order to reconstruct past environmental parameters. Organic  
54 biomarkers have become widely deployed tools in the reconstruction of past  
55 environmental conditions, due in part to: a) the sensitive physiological  
56 responses of cell membranes and structural lipids to the external environment  
57 and b) their relatively high preservation potential (Summons, 1993; Eglinton  
58 and Eglinton, 2008). Since the 1960's a large array of lipid biomarkers with  
59 applications in palaeoclimatology have been identified, including plant waxes,  
60 hopanes, alkanes and glycerol dialkyl glycerol tetraethers (GDGTs). Two  
61 proxies,  $U_{37}^K$ , (Brassell et al., 1986; Prahl and Wakeham, 1987; Sachs et al.,

62 2001; Haug et al., 2005) and TEX<sub>86</sub> (Schouten et al., 2002; Kim et al., 2008),  
63 based on C<sub>37</sub> alkenones and GDGTs, respectively, have been widely employed  
64 to calculate sea surface temperatures (SST) as far back as the Jurassic  
65 (Jenkyns et al., 2012).

66 Numerous lipid biomarkers derived from terrestrial organic matter are  
67 preserved in lacustrine (e.g. Castañeda and Schouten, 2011) and marine  
68 (Pancost and Boot, 2004) archives. Commonly utilised biomarker groups  
69 include higher plant derived *n*-alkyl compounds, terpenoids and lignins  
70 (Pancost and Boot, 2004) and soil bacterial branched-GDGTs (Weijers et al.,  
71 2007a). Such compounds can be used to reconstruct general changes in inputs  
72 and provenance of terrestrial material (Pancost and Boot, 2004, Seki et al.,  
73 2014). Compound specific isotopic analyses, particularly on higher plant waxes  
74 have expanded the range of palaeoclimatic applications, for example, D/H  
75 analysis is used to infer changes in past hydrological regimes (Sachse et al.,  
76 2012 and reference therein) and the  $\delta^{13}\text{C}$  analysis of higher plant biomarkers is  
77 a powerful tool to constrain changes in C<sub>3</sub> vs C<sub>4</sub> vegetation (e.g. Hughen et al.,  
78 2004). More recently, the bacterial GDGT based cyclization of branched  
79 tetraether (CBT) proxy has been developed and applied for the reconstruction  
80 of soil pH in terrestrial settings (Weijers et al., 2007b). In parallel, the  
81 combination of CBT with the methylation of branched tetraethers (MBT) index  
82 may be deployed to estimate past variations in mean annual air temperature  
83 (MAAT) (Weijers et al., 2007b). However, overall, relatively less attention has

84 been paid to terrestrial environments, compared to the marine realm, due to  
85 the historical paucity of ubiquitous biomarkers with quantitative  
86 palaeoclimatic utility. Thus the discovery and development of new quantitative  
87 terrestrial proxies is of major significance. Targets of particular value are  
88 compounds preserved in both aquatic and terrestrial sediments, as this  
89 facilitates the correlation and comparison of palaeoclimatic records between  
90 marine and terrestrial environments (Pancost and Boot, 2004; Castañeda and  
91 Schouten, 2011).

92 Lipopolysaccharide (LPS) is the main component of the outer membrane of  
93 Gram-negative bacteria. Lipid A, a constituent part of LPS, consists of  
94 glucosamine units and fatty acids, many of the latter are 3-hydroxy fatty acids  
95 (3-OH-FAs), also known as  $\omega$ -hydroxy fatty acids, with carbon numbers from  
96 C<sub>10</sub> to C<sub>18</sub> (Fig. 1) (Wollenweber and Rietschel, 1990; Szponar et al., 2002;  
97 Szponar et al., 2003). These are bound to the glucosamine unit either by ester  
98 bonds or amide bonds (Wollenweber et al., 1982; Kumar et al., 2002). A  
99 significant body of literature demonstrates that the dominant precursors for  
100 C<sub>10</sub>-C<sub>18</sub> 3-OH-FAs compounds in the environment are Gram-negative bacteria  
101 (Wollenweber and Rietschel, 1990; Saraf et al., 1997; Szponar et al., 2002;  
102 Keinänen et al., 2003; Szponar et al., 2003). Such that 3-OH-FAs in the C<sub>10</sub>-C<sub>18</sub>  
103 range are accepted as diagnostic markers for the characterisation and  
104 quantification of Gram-negative bacterial LPS (i.e. endotoxins) in clinical and  
105 environmental studies (Sonesson et al., 1990; Mielniczuk et al., 1993; Saraf et

106 al., 1997; Szponar et al., 2002; Keinänen et al., 2003; Wakeham et al., 2003;  
107 Lee et al., 2004; Ferrando et al., 2005; Kračnik et al., 2006; Lee et al., 2007).  
108 However, one study suggests C<sub>10</sub>-C<sub>18</sub> 3-OH-FAs are also produced by Gram-  
109 positive *Lactobacillus plantarum* (Sjogren et al., 2003). Additionally, long chain  
110 3-OH-FAs (C<sub>26</sub>-C<sub>30</sub>) are reportedly derived from microalgae of the class  
111 Eustigmatophyceae (Volkman et al., 1998).

112 3-OH-FAs with carbon chain lengths from C<sub>10</sub> to C<sub>18</sub> have been used to  
113 quantify and characterize the Gram-negative bacterial community in samples  
114 from a diverse array of environments, including atmospheric aerosols (Lee et  
115 al., 2004) and marine dissolved organic matter (DOM) (Wakeham et al., 2003).  
116 However, thus far, the relationship between 3-OH-FAs and environmental  
117 parameters has not been systematically investigated in soils or sediments with  
118 the aim of exploring the possible utility of these ubiquitous fatty acids as  
119 quantitative environmental proxies.

120 We explore the distribution of these microbial biomarkers on Mt.  
121 Shennongjia, a national reserve located at the northwest of Hubei province,  
122 central China (31°15'-31°57'N, 109°59'-110°58'E) (Fig. 2), to test whether 3-OH-  
123 FAs record a signal of sensitive and differential physiological responses, by  
124 Gram-negative bacteria, to ambient environmental conditions, and if novel  
125 quantitative proxies could be independently established for  
126 palaeoenvironmental reconstruction.

127

## 128 2. Methods

### 129 2.1 Sampling site

130 Mt. Shennongjia, with an altitude of 3105 m above sea level (m.a.s.l.), is  
131 located in a climatic region dominated by the Asian monsoon. Five  
132 meteorological stations established at different altitudes in this region provide  
133 a precise altitudinal record of meteorological conditions. Moreover, a large  
134 gradient of soil pH, MAAT and mean annual precipitation (MAP) prevails on  
135 Mt. Shennongjia, making it a natural laboratory to test the relationship  
136 between 3-OH-FAs and environmental parameters. Average climatic conditions  
137 trend from warm and dry conditions at the base (315 m.a.s.l.) to cool and wet  
138 conditions at the highest sampling site (2840 m.a.s.l.), with MAAT varying  
139 from 1.9 °C to 14.7 °C; MAP from 1226mm to 3313mm and soil humidity from  
140 11.6% to 55.6% (Supplementary data Table 1). Soil pH varies from 4.49 to 7.98,  
141 however it has no causal relationship with altitude, MAAT, MAP or soil  
142 humidity (Fig. 3), indicating the pH is an independent environmental factor,  
143 likely controlled by changes in bedrock geology. Both MAAT ( $R^2=0.995$ ) and  
144 MAP ( $R^2= 0.951$ ) are highly correlated to altitude (and thus co-vary), according  
145 to the linear regressions between altitude and climatic factors reported by Li  
146 and Manfred (2002) based on the climatic data from the local meteorological  
147 station (Songpei, 930 m.a.s.l.) and the four subsidiary stations in the Mt.  
148 Shennongjia area (Yangriwan, 460 m.a.s.l.; Dajiuhu, 1700 m.a.s.l.;  
149 Changyanwu, 2300 m.a.s.l.; the mountain observation tower, 2930 m.a.s.l.).

150 The vertical vegetation distribution on Shennongjia Mountain is very distinct.  
151 Based on the latest investigation by Zhao et al., (2005), the vegetation zones  
152 along the elevation gradient were described as follows: evergreen broadleaved  
153 forest zone at altitudes below 900 m.a.s.l.; mixed evergreen and deciduous  
154 broadleaved forest between 900 and 1500 m.a.s.l.; deciduous broadleaved forest  
155 zone between 1500 and 2000 m.a.s.l.; mixed conifer and deciduous broadleaved  
156 forest between 2000 and 2400 m.a.s.l.; and sub-alpine conifer forest zone  
157 (including sub-alpine shrubs and meadows) above altitudes of 2400 m.a.s.l.  
158 (Zhao et al., 2005).

## 159 *2.2 Sample collection*

160 Twenty-six soil samples were collected along an altitude transect of Mt.  
161 Shennongjia between 315 and 2840 m.a.s.l. at altitudinal intervals of ca. 200 m.  
162 The topmost leaf-litter layer was removed before sampling. Samples from each  
163 soil are derived from the depth intervals between 0 to 10 cm. The samples were  
164 wrapped in pre-combusted aluminium foil and then stored with ice bags. Upon  
165 arrival at the laboratory, the soils were stored at -20°C in a freezer before  
166 freeze drying. The location of sampling sites was measured by a portable GPS  
167 instrument (Supplementary data Table 1). Soil moisture was determined by  
168 measuring the weight difference before and after freeze drying. Then the dry  
169 samples were ground into powder with a pestle and mortar. A late Holocene  
170 lake sediment sample was taken from a core collected from Tianchi Lake in

171 Gansu Province, China (Zhou et al., 2010) (Fig. 2). A stalagmite sub-sample  
172 was obtained from the HS4 stalagmite which was collected from Heshang Cave,  
173 Hubei province, China (Hu et al., 2008) (Fig. 2). A marine sediment sample was  
174 collected from IODP Site M0060, in the Baltic Sea.

### 175 *2.3 Soil pH measurement*

176 Soil pH data either comes from or was measured following the method of  
177 Yang et al. (2015). Soil samples were mixed with ultrapure water in a ratio of  
178 1:2.5 (g/mL). After standing for 30 min, the supernatant pH was measured,  
179 using a meter with a precision of  $\pm 0.01$ . The pH was measured three times and  
180 the mean value was taken as the final pH.

### 181 *2.4 Extraction and clean-up methods*

182 The soil, stalagmite and marine sediment samples were subjected to acid  
183 hydrolysis following an optimized acid digestion method (Wang et al., 2012).  
184 10g of homogenized sample was mixed with 30 mL pre-cleaned HCl (3M), and  
185 then refluxed under 130 °C for 3h. After cooling, the solution was extracted x3  
186 with DCM, to yield the Total Lipid Extract (TLE). The lake sediment was  
187 hydrolysed by 0.3M KOH methanolic solution containing 5% water, heating  
188 under 70 °C for 2h in a closed test tube. The neutral fraction was extracted  
189 with *n*-hexane:DCM (9:1, v/v) and then the acid fraction was extracted with  
190 DCM after adjusting the pH of the residues below 2 with pre-cleaned HCl. The  
191 TLE (soils, stalagmite and marine sediment) and acid fraction (lake sediment)

192 was methylated by  $\text{BF}_3\text{-MeOH}$  solution at 70 °C for 1.5h. The resulting fatty  
193 acid methyl esters (FAMEs) were separated into non-OH-FAMEs and OH-  
194 FAMEs following the method described by Jenske and Vetter (2008). Non-OH-  
195 FAMEs were eluted in the first fraction with a solvent mixture of n-hexane and  
196 ethyl acetate (v/v =98:2), whereas OH-FAMEs were obtained by elution with  
197 100% ethyl acetate. The OH-FAME fraction was further derivatised by BSTFA  
198 (N, O-bis (trimethylsilyl) trifluoroacetamide) at 70 °C for 1.5h before further  
199 analysis by gas chromatogram-mass spectrometer (GC-MS).

## 200 *2.5 Instrumentation*

201 The 3-OH-FAs from soils, stalagmite and marine sediment were analysed  
202 by an Agilent 7890A gas chromatogram and 5975C mass spectrometer (GC-MS)  
203 equipped with a ZB-5MS fused silica capillary column (60 m  $\times$  0.25 mm  $\times$  0.25  
204  $\mu\text{m}$ ) at the China University of Geosciences (Wuhan). The GC oven  
205 temperature was ramped from 70 °C to 200 °C at 10 °C/min, then to 310 °C at  
206 3 °C/min, held at 310 °C for 47 min. The carrier gas was Helium (99.999%) and  
207 the gas flow was 1.0 mL/min. The 3-OH-FAs from Tianchi Lake were analysed  
208 by a 7890B gas chromatogram and 5977A mass spectrometer equipped with a  
209 BP5MS fused silica capillary column (60 m  $\times$  0.32 mm  $\times$  0.25  $\mu\text{m}$ ) at the  
210 University of Birmingham. The ionization energy of the mass spectrometer was  
211 set at 70 eV. The 3-OH-FAs were identified based on their mass spectra and  
212 relative retention times (Fig. 4). All the 3-OH-FAs TMSi esters show diagnostic

213 fragment ions,  $m/z$  175 ( $[\text{CH}_3]_3\text{SiO} = \text{CHCH}_2\text{CO}_2\text{CH}_3$ ), due to the cleavage  
214 between  $\text{C}_3$  and  $\text{C}_4$ , and M-15 (base peak) results from a loss of a  $\text{CH}_3$  group.  
215 Other characteristic ions include  $m/z$  103, 89, 133, 159, and  $\text{M}^+ - 31$  (Eglinton et  
216 al., 1968; Mielniczuk et al., 1993; Volkman et al., 1999). Samples were analysed  
217 in duplicate or triplicate to obtain the analytical errors of the proxies. The  
218 analytical errors are graphically illustrated in the relevant figures with error  
219 bars.

## 220 **3. Results and discussion:**

### 221 *3.1 Distribution of 3-OH-FAs*

222 A total of 26 soil samples from Mt. Shennongjia were analysed. The carbon  
223 number of the 3-OH-FAs ranges from  $\text{C}_{10}$  to  $\text{C}_{18}$ , including *iso*-  $\text{C}_{11}$ ,  $\text{C}_{13}$ ,  $\text{C}_{15}$ ,  $\text{C}_{16}$ ,  
224  $\text{C}_{17}$  and *anteiso*-  $\text{C}_{13}$ ,  $\text{C}_{15}$ ,  $\text{C}_{17}$  3-OH-FAs. *n*- $\text{C}_{14}$  is the dominant homologue (Fig.  
225 5). The distribution of the Mt. Shennongjia 3-OH-FAs is akin to that derived  
226 from the LPS component of the outer bacterial membrane of Gram-negative  
227 bacteria (Klok et al., 1988). Thus we assume that the 3-OH-FAs measured in  
228 the Mt. Shennongjia soils originate from the soil dwelling consortia of Gram-  
229 negative bacteria. Furthermore, the suite of 3-OH-FAs compounds detected is  
230 similar to that reported from stalagmites (Blyth et al., 2006; Huang et al., 2008;  
231 Wang et al., 2012), marine DOM (Wakeham et al., 2003) and lake sediments  
232 (Matsuda and Koyama, 1977; Zhang et al., 2014), although the dominant  
233 homologue varies between  $\text{C}_{12}$ ,  $\text{C}_{14}$  to  $\text{C}_{16}$  in these different sample types, and

234 the relative abundance of each individual compound fluctuates from sample to  
235 sample.

### 236 *3.2 pH impact on 3-OH-FAs and potential proxies*

237 Organic geochemical method development work on acid digestion of  
238 speleothem and cave samples from Heshang cave, located ca. 120 km from Mt.  
239 Shennongjia in central China (Wang et al., 2012; Huang et al., 2008), revealed  
240 that a suite of 3-OH-FAs were readily extractable and relatively abundant  
241 compared to established palaeoclimate biomarkers (e.g. plant waxes). This  
242 prompted an investigation of the distributions of these compounds along the Mt.  
243 Shennongjia altitudinal gradient and the current study of their empirical  
244 relationship to environmental parameters. Below we discuss in more detail the  
245 most promising 3-OH-FA indices we have identified. In Table 3 in the  
246 Supplementary data we include a list of all the 3-OH-FA based indices we  
247 tested, including those which showed low or insignificant correlations with  
248 environmental parameters (MAAT, soil pH, MAP, soil moisture and altitude).

249 The first group of indices we discuss are those which show relatively high  
250 correlations with soil pH. Recent work has demonstrated that pH is a key  
251 environmental parameter in controlling soil bacterial community structure and  
252 diversity (Bååth and Anderson, 2003; Lauber et al., 2009; Griffiths et al., 2011;  
253 Shen et al., 2013; Zhang et al., 2015). In particular, Giotis et al. (2007) found  
254 that a strain of Gram-negative bacterium increased/decreased the proportion of  
255 branched-chain fatty acids in higher pH/lower pH conditions. Our results from

256 the Mt. Shennongjia transect show that the ratio of the total sum of *iso* and  
257 *anteiso* 3-OH-FAs to the total amount of *normal* 3-OH-FAs i.e., the Branching  
258 Ratio (equation 1), has a positive correlation with the pH value of soils (Fig. 6a).  
259 The Branching Ratio is defined as follows:

$$260 \text{ Branching Ratio} = (I + A)/N \quad (1)$$

261 Where I represents the sum of all the *iso* 3-OH-FAs, A represents the sum  
262 of all the *anteiso* 3-OH-FAs, and N represents the sum of all the *normal* 3-OH-  
263 FAs.

264 When plotting the Branching Ratio against the pH value of the soils, there  
265 is an exponential relationship between the two ( $R^2= 0.76$ ), with the Branching  
266 Ratio increasing significantly from 0.31 at pH 4.49 to 0.61 at pH 7.98 (Fig. 6a).  
267 Notably, the Branching Ratio shows no obvious correlation with MAAT, MAP  
268 or soil humidity (Fig. 7a-c, Supplementary data Table 3).

269 The fact that pH on Mt. Shennongjia does not correlate with other  
270 measured parameters (MAAT, MAP, soil humidity) precludes problems of co-  
271 variance and gives us confidence that the Branching Ratio does primarily  
272 record a signal of environmental pH.

273 Equation (1) and Figure 6a clearly indicate proportionally less branched 3-  
274 OH-FAs, including *iso* and *anteiso* isomers, when pH decreases, and thus a  
275 lower pH yields a lower Branching Ratio value. This is consistent with the  
276 general observation that bacteria can alter the branching and cyclicity of their  
277 fatty acid membrane lipids in response to ambient environmental factors

278 (Denich et al., 2003). Branching in fatty acids increases the fluidity (Russell  
279 and Fukunaga, 1990) and permeability (McElhaney et al., 1973) of the  
280 cytoplasmic membrane.

281 We suggest that the observation of a decreasing Branching Ratio at lower  
282 pH reflects chemiosmotic coupling, i.e. the production of fewer branched  
283 homologues, producing a less fluid / more impermeable membrane to  
284 counteract steeper proton gradients. The existence and maintenance of a  
285 proton gradient over bacterial cell membranes is vital for the energy supply of  
286 a cell (Mitchell, 1966) and involves the trapping of proton conducting water  
287 molecules in the lipid core of the membranes (Nagle and Morowitz, 1978;  
288 Wikström et al., 2015). The high significance of the exponential regression  
289 supports this hypothesis. The proton gradient over the bacterial cell  
290 membranes will be largely determined by ambient proton concentrations and  
291 pH is a nonlinear function, being the negative logarithm of ambient proton  
292 concentrations. Given the exponential relationship between pH and the  
293 Branching Ratio (Fig. 6a) and the definition of pH as the negative logarithm of  
294 the proton concentration, it is possible to obtain a linear relationship between  
295 the two by defining an alternative index:

$$296 \text{RIAN} = -\log(\text{Branching Ratio}) \quad (2)$$

297 When plotting the ratio of the total sum of *iso* and *anteiso* 3-OH-FAs to the  
298 total amount of *normal* 3-OH-FAs (RIAN) against the pH of the soils resulted  
299 in the following linear correlation (Fig. 6b):

300  $RIAN = 1.11 - 0.10 \times pH$  ( $R^2 = 0.70$ ,  $p < 0.001$ ) (3)

301 Thus we propose the following novel pH proxy for application to terrestrial  
302 palaeoclimatic archives:

303  $pH = 11.10 - 10.00 \times RIAN$  ( $R^2 = 0.70$ ,  $p < 0.001$ ,  $RMSE = 0.54$ ) (4)

304 In addition to Branching Ratio and RIAN, we find that the ratio of  
305 summed branched homologues to the sum of all 3-OH-FA homologues  
306 (Branched Index) and the ratio of summed *iso* to summed *normal* 3-OH-FA  
307 homologues (RIN) also show strong correlations with soil pH ( $R^2 = 0.70$  and  
308  $R^2 = 0.67$ , respectively) (Fig. 6c, d, Supplementary data Table 3). The equations  
309 for the Branched Index and RIN are:

310  $Branched\ Index = (I + A) / (I + A + N)$  (5)

311  $RIN = I / N$  (6)

312 Where I represents the sum of all the *iso* 3-OH-FAs, A represents the sum  
313 of all the *anteiso* 3-OH-FAs, and N represents the sum of all the *normal* 3-OH-  
314 FAs. The possible advantages of these alternative indices are that the  
315 Branched Index is bounded at values between 0 and 1 (the Branching ratio and  
316 the RIAN are unbounded), whereas RIN only utilises the *normal* and *iso*  
317 homologues and does not require measurement of the *anteiso* homologues. RIN  
318 may prove to have a practical advantage as the *anteiso* homologues occur in the  
319 lowest abundance in our samples (see Figure 5) and may be hard to accurately  
320 integrate in some environmental samples where the overall abundance or  
321 preservation of 3-OH-FAs is lower.

322 All the ratios and indices presented show positive or negative correlations  
323 ( $R^2= 0.67$  to  $0.76$ ,  $p<0.001$ ) with pH (Fig. 6) but show no obvious correlation  
324 with MAAT, MAP or soil humidity (Fig. 7 and Supplementary data Table 3).  
325 All the ratios and indices appear to be independent measures of the  
326 decreased/increased degree of branching of 3-OH-FAs with lower/higher pH.  
327 As discussed above, for the Branching Ratio, this suggests a causal relationship  
328 with soil pH which we argue reflects chemiosmotic coupling, i.e. the production  
329 of fewer or more branched homologues to control membrane  
330 fluidity/permeability in response to proton gradients across bacterial cell  
331 membranes. This is comparable with the suggestion of Weijers et al. (2007b)  
332 that a lower/higher degree of methylation of branched GDGTs in lower/higher  
333 pH conditions reflects chemiosmotic coupling and is consistent with the finding  
334 of Bardy et al. (2009) that the contribution of branched  $C_{15}$  and  $C_{17}$  alkanolic  
335 acids relative to their linear homologues decreased with pH in a podzolic  
336 sequence in the Amazon basin.

337 Based on the linear correlations showed in Fig. 6c, d, we obtain the  
338 following equations with pH for the Branched Index and RIN:

339 Branched Index =  $-0.03 + 0.05 \times \text{pH}$  (7)

340 RIN =  $-0.21 + 0.08 \times \text{pH}$  (8)

341 Thus we propose the additional novel pH proxies for application to  
342 terrestrial palaeoclimatic archives:

343  $\text{pH} = 0.60 + 20.00 \times \text{Branched Index} (R^2= 0.70, p<0.001, \text{RMSE}= 0.54) \quad (9)$

344  $\text{pH} = 2.63 + 12.50 \times \text{RIN} (R^2= 0.67, p<0.001, \text{RMSE}= 0.56) \quad (10)$

345 At this early stage of development of 3-OH-FA based proxies for  
346 palaeoenvironmental applications, we recommend that the RAN, Branched  
347 Index and RIN should all be measured in samples, as all of them clearly have  
348 potential as pH proxies and only further work can constrain which may be  
349 most reliable or practicable.

350

### 351 *3.3 Temperature impact on 3-OH-FAs and potential proxies*

352 In addition to the novel pH proxies described above, we found two indices  
353 that have potential as novel temperature proxies, the ratio of *anteiso* to *normal*  
354  $C_{15}$  3-OH-FA ( $\text{RAN}_{15}$ ) and the ratio of *anteiso* to *normal*  $C_{17}$  3-OH-FA ( $\text{RAN}_{17}$ ).  
355  $\text{RAN}_{15}$  and  $\text{RAN}_{17}$  are defined as follows:

356  $\text{RAN}_{15} = \alpha\text{-}C_{15} / n\text{-}C_{15} \text{ 3-OH-FA} \quad (11)$

357  $\text{RAN}_{17} = \alpha\text{-}C_{17} / n\text{-}C_{17} \text{ 3-OH-FA} \quad (12)$

358  $\text{RAN}_{15}$  shows a linear relationship with MAAT and MAP ( $R^2 = 0.51$  and  
359  $0.50$ , respectively) (Fig. 8a, b). A similar result was also found in  $\text{RAN}_{17}$  ( $R^2 =$   
360  $0.48$  and  $0.48$ , respectively) (Fig. 8c, d). It is not surprising that both MAAT  
361 and MAP show a linear relationship with  $\text{RAN}_{15}$  and  $\text{RAN}_{17}$ , because both  
362 parameters strongly co-vary with elevation on Mt. Shennongjia. It has been  
363 suggested that precipitation could be an important environmental control on  
364 soil bacterial lipids in semi-arid to arid regions. Although initially proposed as

365 being a function of MAAT and pH, recent work has highlighted that the GDGT  
366 based MBT/CBT-MAAT index is significantly influenced by precipitation/ soil  
367 moisture in the semi-arid western USA, where MAP is below 700-800 mm yr<sup>-1</sup>  
368 (Dirghangi et al., 2013), in the semi-arid Iberian Peninsula (Menges et al., 2014)  
369 and in China (Yang et al., 2014). Yang et al. (2014) found complexities in the  
370 relationship of the MBT and CBT indices to MAAT in alkaline and arid soils in  
371 China, in contrast to their positive correlation in more acidic soils in the  
372 complete Chinese, or global, datasets. Our research area is characterised by  
373 relatively acidic to neutral soils (pH 4.5 - 8.0), and a moist-humid climate,  
374 where MAP is above 1000 mm yr<sup>-1</sup>, even on the drier, lower slopes of the  
375 mountain. Therefore, we suggest precipitation/soil moisture is unlikely to be an  
376 ecologically limiting factor that significantly affects the distribution of the  
377 membrane lipids. In support of this assumption we found that both RAN<sub>15</sub> and  
378 RAN<sub>17</sub> showed very weak correlations with soil humidity measurements ( $R^2$ =  
379 0.19 and 0.16, respectively, see Supplementary data Table 3), although we note  
380 that such measurements only represent the conditions at the time of sampling  
381 and not necessarily the average, mean annual conditions. Furthermore, RAN<sub>15</sub>  
382 and RAN<sub>17</sub> show significant correlations with the GDGT-based MBT/CBT-  
383 MAAT proxy published by Yang et al. (2015) on the same soil samples ( $R^2$ =  
384 0.61 and 0.36, respectively) (Fig. 9a, b). Thus we assume that MAAT is the  
385 dominant parameter that affects these ratios even though the impact of MAP  
386 could not be entirely excluded. The ratios of both RAN<sub>15</sub> and RAN<sub>17</sub> increase

387 with decreasing environmental temperature (Fig. 8a, c). It has been observed  
388 that *anteiso* fatty acids have a lower melting point than *normal* fatty acids  
389 (Kaneda, 1991; Suutari and Laakso, 1994). Thus in order to maintain  
390 membrane fluidity, bacteria may increase the proportion of *anteiso* 3-OH-FAs  
391 (increasing the RAN indices) with decreasing temperature. This hypothesis is  
392 supported by the fact that we found a significant relationship between ratio of  
393 *anteiso* to *normal* C<sub>15</sub> 3-OH-FA and temperature, but a much less significant  
394 relationship between *iso* to *normal* C<sub>15</sub> 3-OH-FA (see Supplementary data  
395 Table 3). *Anteiso*-branched fatty acids have greater fluidizing properties and  
396 disturb packing order to a greater extent than *iso*-branched fatty acids (Russell,  
397 1995). This is conferred by the *anteiso*-methyl branch being located on the third  
398 carbon from the methyl terminus while the *iso*-methyl branch is positioned on  
399 the second carbon from the end of the chain (Russell, 1984).

400 Based on the linear correlation showed in Fig. 8, we obtain the following  
401 equations:

$$402 \text{ RAN}_{15} = 7.60 - 0.33 \times \text{MAAT} \quad (13)$$

$$403 \text{ MAAT} = 23.03 - 3.03 \times \text{RAN}_{15} \text{ (R}^2 = 0.51, \text{ p} < 0.001, \text{ RMSE} = 2.6 \text{ }^\circ\text{C)} \quad (14)$$

$$404 \text{ RAN}_{17} = 2.90 - 0.11 \times \text{MAAT} \quad (15)$$

$$405 \text{ MAAT} = 26.36 - 9.09 \times \text{RAN}_{17} \text{ (R}^2 = 0.48, \text{ p} < 0.001, \text{ RMSE} = 2.7 \text{ }^\circ\text{C)} \quad (16)$$

406 The relationships of both RAN<sub>15</sub> and RAN<sub>17</sub> (equations 13 and equation 15)  
407 to MAAT are similar (see Fig. 8), although RAN<sub>17</sub> has somewhat more scatter.

408 GDGT data have been previously published from 19 of our 26 soil samples  
409 (Yang et al., 2015). Thus, we can directly compare our 3-OH-FA based proxies  
410 with established GDGT based proxies (CBT and MBT/CBT). Our RIAN and  
411 Branched Index proxies for pH show high linear correlation with the GDGT-  
412 based CBT (Fig. 9c, d) suggesting all three proxies have the same dominant  
413 control, namely pH. Furthermore RAN<sub>15</sub> and RAN<sub>17</sub> based on 3-OH-FA show a  
414 linear correlation with the GDGT-based MBT/CBT-MAAT proxy (Fig. 9a, b)  
415 although this is significantly higher for RAN<sub>15</sub>. It is important to note that,  
416 unlike the current MBT/CBT-MAAT proxy, our proposed 3-OH-FA derived  
417 temperature proxies are independent from pH.

418 In addition to the ratios, indices and proposed novel proxies presented  
419 above we explored a full range of 3-OH-FA distributions (e.g. Average Chain  
420 Length of 3-OH-FAs) versus environmental parameters in the samples  
421 obtained from Mt. Shennongjia. Above we present only the most significant  
422 correlations and findings, but include all results in the Supplementary Data,  
423 Table 3.

#### 424 **4. Wide occurrence of 3-OH-FAs in other settings**

425 We undertook an initial investigation to confirm the preservation of 3-OH-  
426 FAs on Quaternary time scales in several palaeoclimatic archives: a lake  
427 sediment sample dated to 1984±30 yr B.P. from Tianchi Lake, Gansu province,  
428 China, a speleothem sample dated to 8645±78 yr B.P. from Heshang Cave,  
429 China and a last glacial marine sediment sample from the 81 mbsf from IODP

430 Site M0060, Baltic Sea. The distribution of 3-OH-FAs varied between samples,  
431 but the suite of C<sub>10</sub> to C<sub>18</sub> *normal*, plus certain *iso*- and *anteiso*- 3-OH-FAs  
432 homologues, were all present in measurable concentrations (Fig. 10). Notably,  
433 monounsaturated 3-OH-FAs with even carbon numbers (C<sub>12</sub>, C<sub>14</sub>, C<sub>16</sub>, C<sub>18</sub>) were  
434 uniquely found in the Tianchi Lake sediment, suggesting either: a) a unique  
435 source of 3-OH-FAs in that lake environment or; b) greater preservation of the  
436 more labile unsaturated homologues (Fig. 10, Supplementary data Table 4).

437 The variations in the 3-OH-FA signatures between the different settings  
438 are likely due to controls by environmental and climatic parameters on  
439 membrane lipid production by bacteria (as suggested for the altitudinal  
440 transect of modern soils in this paper). Moreover, the origin and preservational  
441 pathways of 3-OH-FAs in some settings could be complex. For example, 3-OH-  
442 FAs in lake sediments may be produced *in situ* and/or may be derived from the  
443 surrounding soils, this may complicate the application of 3-OH-FAs as  
444 temperature/pH proxies in lakes. In general, we can not discount the influence  
445 on the 3-OH-FA signatures of unknown, site-specific, factors related to the  
446 differences in depositional setting or variations in populations of the Gram-  
447 negative bacterial producer. Thus specific calibrations are likely required for  
448 applications to a diverse range of palaeoclimatic archives. However, the  
449 preservation of the same suite of 3-OH-FAs in such different depositional  
450 environments, hints at a potentially wide applicability of these microbial  
451 proxies in a variety of environmental settings.

## 452 **5. Conclusion**

453 In summary, 3-OH-FAs in surface soils collected from an altitudinal  
454 transect on Mt. Shennongjia were examined to explore their relationships with  
455 environmental parameters. The RIAN, Branched index and RIN indices are  
456 highly correlated with soil pH. Furthermore, the RAN<sub>15</sub> and RAN<sub>17</sub> ratios  
457 exhibit significant correlations with MAAT and MAP. As precipitation is not  
458 likely to be an ecologically limiting factor in the moist-humid environment of  
459 Mt. Shennongjia we assume that MAAT is the dominant control. Notably, the  
460 3-OH-FA based temperature proxies RAN<sub>15</sub> and RAN<sub>17</sub>, are not pH dependent,  
461 which should be an advantage in environments where pH is highly variable  
462 and could be a confounding variable. Our discovery of new independent proxies  
463 for pH and MAAT from an altitudinal transect of surface soils from Mt.  
464 Shennongjia has potentially wide implications for palaeoclimatic and  
465 environmental studies. 3-OH-FA proxies could be used in a variety of  
466 environmental settings (See Fig. 10). Multi-proxy terrestrial reconstructions of  
467 pH and temperature could be established by comparing 3-OH-FAs with GDGT  
468 based proxies. Gram-negative bacteria have a wide distribution in natural  
469 environment (Gupta, 1998), and 3-OH-FAs have been identified in diverse  
470 environments, including marine and terrestrial settings and even in  
471 atmospheric aerosols (Wakeham et al., 2003; Lee et al., 2004; Huang et al.,  
472 2008). In particular, these compounds are easy to identify and precisely  
473 quantify using GC-MS and GC-FID systems. This makes it possible to utilize a

474 small amount of sample weight and to gain high-resolution palaeo-records, for  
475 example even from stalagmite archives (Blyth et al., 2006; Huang et al., 2008;  
476 Wang et al., 2012). Additionally, measurement of 3-OH-FAs requires only  
477 standard GC-MS and GC-FID systems and can be readily adopted by most  
478 organic geochemistry laboratories (without the need for investment in  
479 additional, expensive equipment). It is clear that 3-OH-FAs have hitherto  
480 unrealized potential as palaeoclimate proxies. We hope this paper opens up  
481 new avenues of research on 3-OH-FAs, including culture studies, empirical  
482 calibrations (both global and regional) and application to an array of  
483 palaeoclimatic archives (e.g. lakes, speleothems, marine records).

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729

730 **Figure captions**

731

732 **Fig. 1** General structure of lipopolysaccharide (LPS) from Gram-negative  
733 bacteria (Alexander and Rietschel, 2001). LPS is characterized by three main  
734 units: the O-polysaccharides chains, the core oligosaccharide and lipid A. The  
735 repeating subunits of the O-polysaccharides are composed of between one and  
736 eight glycosyl residues and differ between strains by virtue of differing sugars,  
737 sequence, chemical linkage, substitution and the ring forms utilised. The outer  
738 core is inclined to contain common sugars such as hexoses or hexosamines etc.  
739 The inner core contains the unusual sugars 3-deoxy-D-manno-octulosonic acid  
740 (Kdo) and D-glycero-D-manno-heptose (Hep) (Erridge et al., 2002). Lipid A, the  
741 innermost part of LPS, consists of two glucosamine (GlcN) moieties, with  
742 attached acyl chains ("fatty acids") by either amide bonds or ester bonds, and  
743 normally contains one phosphate group on each GlcN (Raetz et al., 2009).

744

745 **Fig. 2** Regional map, illustrating the location of Shennongjia Mountain,  
746 Heshang Cave and Tianchi Lake.

747

748 **Fig. 3** Cross plots showing the relationship of soil pH in samples from Mt.  
749 Shennongjia with soil humidity, Mean Annual Air Temperature (MAAT), Mean  
750 Annual Precipitation (MAP) and altitude.

751

752 **Fig. 4** Mass spectrum of the C<sub>16</sub> 3-OH-FA TMSi ester. The m/z 175 fragment is  
753 due to the cleavage between C<sub>3</sub> and C<sub>4</sub>, and the [M-15] base peak results from a  
754 loss of a CH<sub>3</sub> group.

755

756 **Fig. 5** Extracted ion chromatograph (m/z 175) showing the composition and  
757 distribution of 3-OH-FAs in the Mt. Shenongjia soil sample collected at 832  
758 m.a.s.l. (see sample SNJ 11-4 in the Supplementary data Table 1 for more  
759 detailed information). Red circles represent the *normal* 3-OH-FAs, yellow  
760 squares represent the *iso* 3-OH-FAs, grey triangles represent the *anteiso* 3-OH-  
761 FAs. The carbon numbers range from C<sub>10</sub> to C<sub>18</sub>, including *iso* C<sub>11</sub>, C<sub>13</sub>, C<sub>14</sub>, C<sub>15</sub>,  
762 C<sub>16</sub> and *anteiso* C<sub>11</sub>, C<sub>13</sub> C<sub>15</sub> C<sub>17</sub>.

763

764 **Fig. 6** The relationship between 3-OH-FAs indices and pH. (a) Exponential  
765 correlation between the Branching Ratio and pH (R<sup>2</sup>= 0.76, p<0.001). (b) Linear  
766 correlation between RIAN and soil pH (R<sup>2</sup>=0.70, p<0.001). (c) Linear correlation  
767 between Branched Index and pH (R<sup>2</sup>= 0.70, p<0.001). (d) Linear correlation  
768 between RIN and pH (R<sup>2</sup>= 0.67, p<0.001).

769

770 **Fig. 7** Cross plots showing the relationship between Branching Ratio and  
771 Branched Index to environmental parameters (MAT, MAP, and soil humidity).

772

773 **Fig. 8** The relationship between 3-OH-FA ratios and environmental factors. (a)  
774 The RAN<sub>15</sub> shows negative linear relationship with MAAT ( $R^2= 0.51$ ,  $p<0.001$ )  
775 and (b) positive linear relationship with MAP ( $R^2= 0.50$ ,  $p<0.001$ ). (c) The  
776 RAN<sub>17</sub> shows negative linear relationship with MAAT ( $R^2= 0.48$ ,  $p<0.001$ ) and  
777 (d) positive linear relationship with MAP ( $R^2= 0.48$ ,  $p<0.001$ ).

778

779 **Fig. 9** Cross plots showing the correlation between certain 3-OH-FA based and  
780 GDGT based proxies.

781

782 **Fig. 10** Extracted ion chromatogram (m/z 175) showing the distribution of 3-  
783 OH-FAs in contrasting geological samples. Red circles represent the *normal* 3-  
784 OH-FAs, yellow squares represent the *iso* 3-OH-FAs, grey triangles represent  
785 the *anteiso* 3-OH-FAs and white circles represent the monounsaturated 3-OH-  
786 FAs. (a) The composition and distribution of 3-OH-FAs in a sediment sample  
787 from Tianchi Lake. (b) The distribution of 3-OH-FAs in a Heshang Cave  
788 stalagmite sample. (c) The distribution of 3-OH-FAs in Baltic Sea sediment  
789 sample.

790