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DOI:
10.1016/j.orggeochem.2016.01.010

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

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Abstract: Gram-negative bacterial 3-hydroxy fatty acids (3-OH-FAs) biomarkers are widespread in a variety of environments including both marine and terrestrial sediments (including speleothems). In this study we analysed the hydroxylated membrane lipids of 26 soil samples from an altitudinal transect of Shennongjia Mountain (Mt.) in central China to study the environmental factors controlling the relative distribution of 3-OH-FAs. Our results show that both the ratio of the summed *iso* and *anteiso* to the total amount of *normal* 3-OH-FAs (RIAN), and the ratio of summed *iso* and *anteiso* to the total amount of all 3-OH-FAs (Branched Index) were primarily related to the pH of soil (R^2 = 0.70 and 0.70, respectively). Additionally, the *anteiso* to *normal* 3-hydroxy fatty acids ratio of the C_{15} and C_{17} homologues (RAN_{15} and RAN_{17}) shows a significant negative correlation with mean annual air temperature (MAAT) (R^2=0.51 and 0.48, respectively). When comparing the 3-OH-FA based indices with established glycerol dialkyl glycerol tetraether (GDGT) based indices from the same soil samples, the RIAN and Branched Index show strong linear correlations with the cyclisation ratio of branched tetraethers (CBT) (R^2 = 0.77 and 0.74, respectively), and the RAN_{15} and RAN_{17} show negative correlations with the MBT/CBT-MAAT (MBT, methylation index of branched tetraethers) (R^2 = 0.61 and 0.36, respectively). Our new field-based correlations demonstrate the physiological response of Gram-negative bacterial 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, $U^{K}_{37'}$ (Brassell et al., 1986; Prahl and Wakeham, 1987; Sachs et al.,

2001; Haug et al., 2005) and TEX_{86} (Schouten et al., 2002; Kim et al., 2008), based on C_{37} alkenones and GDGTs, respectively, have been widely employed to calculate sea surface temperatures (SST) as far back as the Jurassic (Jenkyns et al., 2012).

Numerous lipid biomarkers derived from terrestrial organic matter are preserved in lacustrine (e.g. Castañeda and Schouten, 2011) and marine (Pancost and Boot, 2004) archives. Commonly utilised biomarker groups include higher plant derived *n*-alkyl compounds, terpenoids and lignins (Pancost and Boot, 2004) and soil bacterial branched-GDGTs (Weijers et al., 2007a). Such compounds can be used to reconstruct general changes in inputs and provenance of terrestrial material (Pancost and Boot, 2004, Seki et al., 2014). Compound specific isotopic analyses, particularly on higher plant waxes have expanded the range of palaeoclimatic applications, for example, D/H analysis is used to infer changes in past hydrological regimes (Sachse et al., 2012 and reference therein) and the $\delta^{13}C$ analysis of higher plant biomarkers is a powerful tool to constrain changes in C_3 vs C_4 vegetation (e.g. Hughen et al., 2004). More recently, the bacterial GDGT based cyclization of branched tetraether (CBT) proxy has been developed and applied for the reconstruction of soil pH in terrestrial settings (Weijers et al., 2007b). In parallel, the combination of CBT with the methylation of branched tetraethers (MBT) index may be deployed to estimate past variations in mean annual air temperature (MAAT) (Weijers et al., 2007b). However, overall, relatively less attention has

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

Lipopolysaccharide (LPS) is the main component of the outer membrane of Gram-negative bacteria. Lipid A, a constituent part of LPS, consists of glucosamine units and fatty acids, many of the latter are 3-hydroxy fatty acids (3-OH-FAs), also known as □-hydroxy fatty acids, with carbon numbers from C_{10} to C_{18} (Fig. 1) (Wollenweber and Rietschel, 1990; Szponar et al., 2002; Szponar et al., 2003). These are bound to the glucosamine unit either by ester bonds or amide bonds (Wollenweber et al., 1982; Kumar et al., 2002). A significant body of literature demonstrates that the dominant precursors for C_{10}-C_{18} 3-OH-FAs compounds in the environment are Gram-negative bacteria (Wollenweber and Rietschel, 1990; Saraf et al., 1997; Szponar et al., 2002; Keinänen et al., 2003; Szponar et al., 2003). Such that 3-OH-FAs in the C_{10}-C_{18} range are accepted as diagnostic markers for the characterisation and quantification of Gram-negative bacterial LPS (i.e. endotoxins) in clinical and 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; Kra□nik et al., 2006; Lee et al., 2007).
108 However, one study suggests C_{10}-C_{18} 3-OH-FAs are also produced by Gram-
109 positive *Lactobacillus plantarum* (Sjogren et al., 2003). Additionally, long chain
110 3-OH-FAs (C_{26}-C_{30}) are reportedly derived from microalgae of the class
111 Eustigmatophyceae (Volkman et al., 1998).

112 3-OH-FAs with carbon chain lengths from C_{10} to C_{18} 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

2. Methods

2.1 Sampling site

Mt. Shennongjia, with an altitude of 3105 m above sea level (m.a.s.l.), is located in a climatic region dominated by the Asian monsoon. Five meteorological stations established at different altitudes in this region provide a precise altitudinal record of meteorological conditions. Moreover, a large gradient of soil pH, MAAT and mean annual precipitation (MAP) prevails on Mt. Shennongjia, making it a natural laboratory to test the relationship between 3-OH-FAs and environmental parameters. Average climatic conditions trend from warm and dry conditions at the base (315 m.a.s.l.) to cool and wet conditions at the highest sampling site (2840 m.a.s.l.), with MAAT varying from 1.9 °C to 14.7 °C; MAP from 1226mm to 3313mm and soil humidity from 11.6% to 55.6% (Supplementary data Table 1). Soil pH varies from 4.49 to 7.98, however it has no causal relationship with altitude, MAAT, MAP or soil humidity (Fig. 3), indicating the pH is an independent environmental factor, likely controlled by changes in bedrock geology. Both MAAT (R^2=0.995) and MAP (R^2= 0.951) are highly correlated to altitude (and thus co-vary), according to the linear regressions between altitude and climatic factors reported by Li and Manfred (2002) based on the climatic data from the local meteorological station (Songpei, 930 m.a.s.l.) and the four subsidiary stations in the Mt. Shennongjia area (Yangriwan, 460 m.a.s.l.; Dajiuhu, 1700 m.a.s.l.; 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 ±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 ℃ 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 ℃ 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 BF_3-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 × 0.25 mm × 0.25
204 □m) at the China University of Geosciences (Wuhan). The GC oven
205 temperature was ramped from 70 ℃ to 200 ℃ at 10 ℃/min, then to 310 ℃ at
206 3 ℃/min, held at 310 ℃ 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 × 0.32 mm × 0.25 □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

fragment ions, m/z 175 ($[CH_3]_3SiO = CHCH_2CO_2CH_3$), due to the cleavage between C_3 and C_4, and M-15 (base peak) results from a loss of a CH_3 group. Other characteristic ions include m/z 103, 89, 133, 159, and M^+-31 (Eglinton et al., 1968; Mielniczuk et al., 1993; Volkman et al., 1999). Samples were analysed in duplicate or triplicate to obtain the analytical errors of the proxies. The analytical errors are graphically illustrated in the relevant figures with error bars.

3. Results and discussion:

3.1 Distribution of 3-OH-FAs

A total of 26 soil samples from Mt. Shennongjia were analysed. The carbon number of the 3-OH-FAs ranges from C_{10} to C_{18}, including *iso*- C_{11}, C_{13}, C_{15}, C_{16}, C_{17} and *anteiso*- C_{13}, C_{15}, C_{17} 3-OH-FAs. *n*-C_{14} is the dominant homologue (Fig. 5). The distribution of the Mt. Shennongjia 3-OH-FAs is akin to that derived from the LPS component of the outer bacterial membrane of Gram-negative bacteria (Klok et al., 1988). Thus we assume that the 3-OH-FAs measured in the Mt. Shennongjia soils originate from the soil dwelling consortia of Gram-negative bacteria. Furthermore, the suite of 3-OH-FAs compounds detected is similar to that reported from stalagmites (Blyth et al., 2006; Huang et al., 2008; Wang et al., 2012), marine DOM (Wakeham et al., 2003) and lake sediments (Matsuda and Koyama, 1977; Zhang et al., 2014), although the dominant homologue varies between C_{12}, C_{14} to 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 Branching Ratio = (I + A)/N (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 RIAN = -log (Branching Ratio) (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):

RIAN= 1.11-0.10 × pH (R^2= 0.70, p<0.001) (3)

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

pH= 11.10-10.00 × RIAN (R^2= 0.70, p<0.001, RMSE= 0.54) (4)

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

Branched Index= (I+A)/(I+A+N) (5)

RIN= I/N (6)

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

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

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

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

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

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

343 pH = 0.60 + 20.00 × Branched Index (R^2= 0.70, p<0.001, RMSE= 0.54) (9)

344 pH = 2.63 + 12.50 × RIN (R^2= 0.67, p<0.001, RMSE= 0.56) (10)

345 At this early stage of development of 3-OH-FA based proxies for
346 palaeoenvironmental applications, we recommend that the RIAN, 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 (RAN_{15}) and the ratio of *anteiso* to *normal* C_{17} 3-OH-FA (RAN_{17}).
355 RAN_{15} and RAN_{17} are defined as follows:

356 RAN_{15}= *a*-C_{15} /*n*-C_{15} 3-OH-FA (11)

357 RAN_{17}= *a*-C_{17} /*n*-C_{17} 3-OH-FA (12)

358 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 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 RAN_{15} and 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

being a function of MAAT and pH, recent work has highlighted that the GDGT based MBT/CBT-MAAT index is significantly influenced by precipitation/ soil moisture in the semi-arid western USA, where MAP is below 700-800 mm yr^{-1} (Dirghangi et al., 2013), in the semi-arid Iberian Peninsula (Menges et al., 2014) and in China (Yang et al., 2014). Yang et al. (2014) found complexities in the relationship of the MBT and CBT indices to MAAT in alkaline and arid soils in China, in contrast to their positive correlation in more acidic soils in the complete Chinese, or global, datasets. Our research area is characterised by relatively acidic to neutral soils (pH 4.5 - 8.0), and a moist-humid climate, where MAP is above 1000 mm yr^{-1}, even on the drier, lower slopes of the mountain. Therefore, we suggest precipitation/soil moisture is unlikely to be an ecologically limiting factor that significantly affects the distribution of the membrane lipids. In support of this assumption we found that both RAN_{15} and RAN_{17} showed very weak correlations with soil humidity measurements (R^2= 0.19 and 0.16, respectively, see Supplementary data Table 3), although we note that such measurements only represent the conditions at the time of sampling and not necessarily the average, mean annual conditions. Furthermore, RAN_{15} and RAN_{17} show significant correlations with the GDGT-based MBT/CBT-MAAT proxy published by Yang et al. (2015) on the same soil samples (R^2= 0.61 and 0.36, respectively) (Fig. 9a, b). Thus we assume that MAAT is the dominant parameter that affects these ratios even though the impact of MAP could not be entirely excluded. The ratios of both RAN_{15} and RAN_{17} 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_{15} 3-OH-FA and temperature, but a much less significant
394 relationship between *iso* to *normal* C_{15} 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 $RAN_{15} = 7.60\text{-}0.33 \times MAAT$ (13)

403 $MAAT = 23.03 - 3.03 \times RAN_{15}$ (R^2= 0.51, p<0.001, RMSE= 2.6 °C) (14)

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

405 $MAAT = 26.36 - 9.09 \times RAN_{17}$ (R^2= 0.48, p<0.001, RMSE= 2.7 °C) (16)

406 The relationships of both RAN_{15} and RAN_{17} (equations 13 and equation 15)
407 to MAAT are similar (see Fig. 8), although RAN_{17} has somewhat more scatter.

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

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

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

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

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

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

5. Conclusion

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

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

Acknowledgements

This work was supported by the Natural Science Foundation of China (grant No. 41330103, 41130207, 41201203), and the 111 project (grant No. B08030). We thank the China Scholarship Council (CSC) (File NO. 201306410031) for supporting Canfa Wang's study at the University of Birmingham. We thank the University of Birmingham Dynamic Investment Fund (DIF) for supporting the establishment of the new Birmingham Molecular Climatology laboratory. Two anonymous reviewers and the associate editor Dr. Klaas G.J. Nierop are thanked for their constructive comment which helped to improve the manuscript.

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729

Figure captions

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

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

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

751

752 **Fig. 4** Mass spectrum of the C_{16} 3-OH-FA TMSi ester. The m/z 175 fragment is
753 due to the cleavage between C_3 and C_4, and the [M-15] base peak results from a
754 loss of a CH_3 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 date 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_{10} to C_{18}, including *iso* C_{11}, C_{13}, C_{14}, C_{15},
762 C_{16} and *anteiso* C_{11}, C_{13} C_{15} C_{17}.

763

764 **Fig. 6** The relationship between 3-OH-FAs indices and pH. (a) Exponential
765 correlation between the Branching Ratio and pH (R^2= 0.76, $p<0.001$). (b) Linear
766 correlation between RIAN and soil pH (R^2=0.70, $p<0.001$). (c) Linear correlation
767 between Branched Index and pH (R^2= 0.70, $p<0.001$). (d) Linear correlation
768 between RIN and pH (R^2= 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_{15} 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_{17} 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