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1 Evaluation of 3-hydroxy fatty acids as a pH and temperature proxy in soils from  
2 temperate and tropical altitudinal gradients  
3

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20  
21 ABSTRACT

22  
23 3-OH FAs containing 10 to 18 C and a hydroxyl group in third position are characteristic  
24 components of Gram-negative bacteria. These compounds were previously used to detect and  
25 quantify Gram-negative bacterial communities in various types of samples, from terrestrial,  
26 aquatic and atmospheric environments. The relative abundance of 3-OH FAs in soils was  
27 recently shown to vary with mean annual air temperature (MAAT) and pH in soils from Mt.  
28 Shennongjia (China). In the current study, the concentrations and abundances of 3-OH FAs  
29 were determined in soils from altitudinal transects under tropical and temperate climates – Mt.  
30 Rungwe (SW Tanzania) and Mt. Majella (Central Italy), respectively. The aim was to (i)  
31 examine and compare the distributions of 3-OH FAs in soils from different climatic zones and  
32 to (ii) investigate the potential of 3-OH FAs as temperature and pH proxies. When combined  
33 with previously published data, a moderate correlation ( $R^2$  0.62) between the summed *iso* and  
34 *anteiso* to the total amount of normal 3-OH FAs (RIAN index) and pH is obtained. We show

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35 that Gram-negative bacteria respond in the same way to temperature variations in soils from  
36 different latitudes, with a relative increase of the *anteiso* to normal 3-OH FA ratios of the C<sub>15</sub>  
37 and C<sub>17</sub> compounds (RAN<sub>15</sub> and RAN<sub>17</sub> indices, respectively) with decreasing temperature.  
38 Nevertheless, the intercepts of the relationships between RAN<sub>15</sub> and MAAT are mountain-  
39 dependent, suggesting that regional calibrations may be required to use RAN<sub>15</sub> as a  
40 temperature proxy. In contrast with RAN<sub>15</sub>, the statistical similarity of the local relationships  
41 between RAN<sub>17</sub> and MAAT leads to a combined calibration ( $R^2$  0.60, residual mean error 5.1  
42 °C) covering a wide range of temperature (ca. 0 – 25 °C). As 3-OH FAs seem well-preserved  
43 in sedimentary archives, this strengthens the potential of these compounds as a temperature  
44 and pH proxy for paleoreconstructions in terrestrial settings.

45

46 *Keywords:* 3-Hydroxy fatty acids; soils; altitudinal transects; environmental proxies

47

## 48 **1. Introduction**

49

50 Investigating past climatic variations is essential to understand and predict future  
51 environmental changes. Paleoclimate studies are chiefly carried out for marine environments  
52 because environmental proxies were mainly developed and used for oceanic settings. The  
53 composition and mechanism of formation of marine sediments are indeed less complex than  
54 the highly heterogeneous continental settings. Several environmental proxies based on  
55 organic (e.g. the alkenone unsaturation index – U<sup>k</sup><sub>37</sub>; Brassell et al., 1986; the tetraether index  
56 of 86 carbon atoms – TEX<sub>86</sub>; Schouten et al., 2002) and inorganic (e.g. Mg/Ca ratio and  
57 <sup>18</sup>O/<sup>16</sup>O ratio of foraminifera; Erez and Luz, 1983; Elderfield and Ganssen, 2000) fossil  
58 remains were notably developed for the reconstruction of sea surface temperatures. However,  
59 it is essential to develop new proxies also applicable in continental environments to assess  
60 climatic variability over the continents and improve the understanding of past global climate.

61 Membrane lipids produced by some microorganisms can be used to this aim.  
62 Environmental stresses can alter membrane fluidity and permeability, thus affecting the  
63 proper functioning of the cell. Microorganisms (bacteria, archaea and some eukaryotes) are  
64 able to adjust their membrane composition in response to the prevailing environmental  
65 conditions in order to maintain an appropriate fluidity and ensure the optimal state of the  
66 cellular membrane (e.g. Hazell and Williams, 1990; Denich et al., 2003), so-called  
67 “homeoviscous adaptation” (Sinensky, 1974). Bacteria can thus change the number of  
68 unsaturations, ramifications or chain length of their membrane lipids – mainly fatty acids – in  
69 response to varying environmental conditions (e.g. Prado et al., 1988; Suutari and Laakso,  
70 1992). In the same way, the structure of glycerol dialkyl glycerol tetraethers (GDGTs), which  
71 are membrane lipids biosynthesised by archaea and some bacteria, is known to be related to  
72 environmental parameters (Schouten et al., 2013 and references therein). Bacterially-derived  
73 branched glycerol dialkyl glycerol tetraethers (brGDGTs) have especially been the object of  
74 growing interest, as they are, to date, the only available organic proxies which can be used for  
75 terrestrial temperature and pH reconstructions (e.g. Peterse et al., 2014; Zheng et al., 2015).  
76 Nevertheless, paleoenvironmental data derived from brGDGTs have to be interpreted with  
77 care, as their source microorganisms remain unknown, although some might belong to the  
78 phylum *Acidobacteria* (Sinninghe Damsté et al., 2011, 2014, 2018). Each environmental  
79 proxy has its strengths and limitations, related to several factors (biological, environmental,  
80 physical etc.). The development of new environmental proxies, independent of and  
81 complementary to those derived from brGDGTs, is required to improve the reliability and  
82 accuracy of terrestrial environmental reconstructions and to provide constraints on brGDGT  
83 applications.

84 Recently, Wang et al. (2016) suggested that other bacterial lipids, so called 3-hydroxy fatty  
85 acids (3-OH FAs), could be used as such a proxy. These compounds, containing 10 to 18 C

86 and a hydroxyl group in third position, are characteristic components of the Gram-negative  
87 bacterial lipopolysaccharide (LPS). The LPS is a major constituent of the external surface  
88 layer of these microorganisms. It is composed of three parts: (i) a polysaccharide chain (O  
89 antigen); (ii) a core oligosaccharide and (iii) the lipid A, the most internal region of the LPS,  
90 mainly constituted of 3-OH FAs (Raetz et al., 2007). Gram-negative bacteria are ubiquitous  
91 in terrestrial and aquatic environments. 3-OH FAs were previously used to detect and quantify  
92 Gram-negative bacterial communities in various types of samples, such as marine dissolved  
93 organic matter (Wakeham et al., 2003), atmospheric aerosols (Cheng et al., 2012), fresh snow  
94 (Tyagi et al., 2015) or soils (Zelles et al., 1995). Nevertheless, only little information is  
95 available regarding the effect of environmental parameter variations on the 3-OH FA  
96 structures. Recently, 3-OH FAs were analysed in 26 soils located between 315 and 2840 m  
97 elevation along Mt. Shennongjia (China; Wang et al., 2016). Significant correlations were  
98 obtained between the relative abundance of these compounds and temperature or pH. The  
99 ratio of the summed *iso* and *anteiso* to the total amount of *normal* 3-OH FAs (defined as the  
100 RIAN index) correlated with soil pH, and the *anteiso* to *normal* 3-OH FA ratio for the C<sub>15</sub> and  
101 C<sub>17</sub> compounds (RAN<sub>15</sub> and RAN<sub>17</sub> indices, respectively) correlated with MAAT (Wang et  
102 al., 2016). The RAN<sub>15</sub> and RIAN were very recently used to reconstruct Holocene  
103 temperature and hydrological changes from a stalagmite collected in Central China (Wang et  
104 al., 2018). This suggests that 3-OH FA-based proxies are promising tools for  
105 paleoenvironmental reconstructions. Nevertheless, to date, the applicability of 3-OH FAs as  
106 temperature and pH proxies has only been investigated in Central China so should be tested in  
107 other regions of the world.

108 In the present study, concentrations and abundances of 3-OH FAs were determined in soils  
109 from two contrasting altitudinal transects: one under tropical climate – Mt. Rungwe, SW  
110 Tanzania, and the other one under temperate climate – Mt. Majella, Central Italy. The aims

111 were (i) to examine and compare the distributions of 3-OH FAs in soils from different  
112 climatic zones, including the Chinese samples previously analysed by Wang et al. (2016) and  
113 (ii) to investigate the potential of 3-OH FAs as temperature and pH proxies.

114

## 115 **2. Material and methods**

116

### 117 *2.1. Sites and sampling*

118

119 Soil samples were collected along two different altitudinal gradients. The first site is Mt.  
120 Rungwe (2960 m) located in the southwest of Tanzania, described in detail by *e.g.*  
121 Williamson et al. (2014) and Coffinet et al. (2017). Briefly, it experiences a tropical climate,  
122 with an alternating hot humid season (November to May) and a cold dry season (June to  
123 October), the precipitation reaching 2400 mm/yr on average with similar amounts from the  
124 base to the top of the mountain (Bergonzini, 1998). Natural vegetation along the altitudinal  
125 transect includes Zambezian Miombo-type woodland at low altitude and Afromontane  
126 vegetation at higher altitude. Various soil types derived from geological bedrock materials  
127 (volcanic ash and pumice) are encountered along Mt. Rungwe, eutric Leptosols, umbric  
128 Nitisols and haplic Lixisols being predominant (Tilumanywa, 2013). Soils in the high  
129 grasslands are thin and quite rocky, while those on the steep slopes of Mt. Rungwe are  
130 predominantly dark greyish and dark brown with sandy and clay loams, and those of the main  
131 arable lands are coarse or medium-textured, ranging from sandy loams to alluvial  
132 (Tilumanywa, 2013). MAAT measurements with temperature loggers during 1 yr (Davis  
133 Instruments, Hayward, CA, USA) at three altitudinal points showed a decrease from 25.6 °C  
134 at 540 m to 22.6 °C at 920 m and 16.9 °C at 1720 m. MAAT at other altitudes were linearly  
135 extrapolated from these recorded temperature data (Table 1). 3-OH FAs were analysed in 21  
136 surface soils (A horizon; 0-5 cm depth) collected between ca. 500 and 2800 m along Mt.  
137 Rungwe in April and December 2012 and November 2016. Samples were kept at room

138 temperature for a limited amount of time (ca. 2 weeks) before being sent to France by  
139 airplane.

140 The second site is Mt. Majella (2794 m elevation) located in Abruzzo, Central Italy and  
141 was described in detail by *e.g.* Bemigisha et al. (2009) and Bonanomi et al. (2016). Climate is  
142 Mediterranean at the lower part of the gradient and changes to subalpine-alpine humid type at  
143 the upper part. Along the southern side of the massif, where samples were collected, mean  
144 annual precipitation (MAP) changes (Table 1 and Supp. Table 1), ranging between ca. 800-  
145 900 mm/year at the lower part of the gradient (0-1000 m a.s.l.) and ca. 1200-1300 mm/year at  
146 the upper part (> 2000 m a.s.l.). A sharp vertical zonation of vegetation type is observed,  
147 including olive orchards at the bottom (ca. 300 m a.s.l.), forest and low elevation grasslands in  
148 between (800 – 1850 m a.s.l) and high altitudinal grasslands above (Bonanomi et al., 2016).  
149 MAAT decreases from 14.6 °C at 300 m to 3.1 °C at 2405 m and 0.03 °C at 2730 m (Stanisci  
150 et al., 2005). Soil is spatially variable from shallow rendzic Leptosol with xeromoder humus  
151 and basic pH to skeletal Leptosol, the latter covered with flat frost-shattered gravel stones on  
152 the plateau and bare solifluxed steep slopes (Bonanomi et al., 2016). 11 surficial soil samples  
153 (0-5 cm depth) were collected between 400 and 2800 m along Mt. Majella in December 2016  
154 and were immediately frozen at -20 °C after sampling. Samples from Mt. Rungwe and Mt.  
155 Majella were stored at -20 °C and then freeze-dried, ground and homogenized.

156

## 157 2.2. *pH and elemental analysis*

158

159 The pH of freeze-dried samples was measured in ultrapure water (Milli-Q, Millipore with a  
160 1:2.5 soil water ratio (Table 1). The decarbonation of soil samples from Mt. Rungwe collected  
161 in 2012 and the subsequent organic carbon content ( $C_{org}$ ) elemental analysis was performed at  
162 the Service Central d'Analyse du CNRS for soil samples, as reported in Coffinet et al. (2014).  
163 All the other samples (Mt. Rungwe and Mt. Majella soils collected in 2016) were

164 decarbonated with HCl and neutralized with deionized water (cf. Huguet et al., 2013a).  $C_{org}$   
165 content was then determined using an Elementar Vario Pyrocube.

166

### 167 2.3. *Sample preparation*

168

169 Preparation of samples for 3-OH FA analysis followed the procedure of Wang et al. (2016)  
170 with slight modifications. Briefly, ca. 10 g of freeze-dried and ground soil were subjected to  
171 acid hydrolysis. Whereas only free 3-OH FAs are obtained upon direct solvent extraction,  
172 additional compounds bound to LPS of Gram-negative bacteria can be released through the  
173 hydrolysis step (Wang et al., 2012, 2016). Acid hydrolysis was shown to be more efficient in  
174 extracting 3-OH FAs from soil samples than saponification, suggesting that most of these  
175 molecules are linked to macromolecules by amide bonds in soils, the latter being difficult to  
176 cleave off by saponification (Yang et al., 2016). Consequently, soil samples were subjected to  
177 acid digestion rather than saponification in this study. They were refluxed with 3M HCl aq.  
178 for 3 h using a silicon oil bath heated at 130 °C. After cooling, the suspension was centrifuged  
179 for 10 min at 15 °C and 3500 rpm and the supernatant transferred to a separation funnel. The  
180 residue was then sequentially extracted with a mixture of dichloromethane (DCM):MeOH  
181 (1:1, v/v; 2×) and DCM (2×). Each extraction was followed by centrifugation and  
182 combination of all extracts in the separation funnel. The DCM phase was separated from the  
183 MeOH/H<sub>2</sub>O layer, which was again extracted with DCM (3×). The DCM fractions were  
184 combined and rotary-evaporated. The lipid extract obtained after acid hydrolysis of the soil  
185 was methylated with HCl-MeOH 1 M at 80 °C for 1 h and then separated into 3 fractions over  
186 a silica column (activated at 150 °C overnight) using (i) 30 mL of heptane/EtOAc (98:2), (ii)  
187 30 mL of EtOAc and (iii) 30 mL of MeOH. OH-FA methyl esters (FAMES) eluted only in the  
188 second fraction. The OH-FAMES, diluted in DCM, were derivatised with a solution of *N,O*-  
189 bis(trimethylsilyl)trifluoroacetamide (BSTFA) – Trimethylchlorosilane (TMCS) 99:1 (Grace



190 Davison Discovery Science, USA) at 70 °C for 20 min before gas chromatography-mass  
191 spectrometry (GC-MS) analysis.

192

#### 193 2.4. 3-OH FA analysis

194

195 3-OH FAs were analysed using an Agilent Network 6890 GC System coupled with a 5973  
196 Mass Selective Detector, with electron impact at 70 eV. A Restek RXI-5 Sil MS silica column  
197 (30 m × 0.25 mm i.d., 0.50 µm film thickness) was used with He as the carrier gas at 1  
198 ml/min. The GC oven program was: 70 °C to 200 °C at 10 °C/min, then to 310 °C (held 45  
199 min) at 3°C/min. Samples were injected in splitless mode and the injector was at 280 °C.  
200 Quantification of 3-OH FAs was performed by comparing the integrated signal from the  
201 respective compound with that from an analogous deuterated internal standard (3-  
202 hydroxytetradecanoic acid, 2,2,3,4,4-d<sub>5</sub>; Sigma-Aldrich, France).

203 A 1:1 mixture of the internal standard and non-deuterated C<sub>12</sub>, C<sub>14</sub> and C<sub>18</sub> 3-OH FA  
204 standards (Larodan Chemicals, Sweden) was injected to determine the response factor of the  
205 internal standard relative to 3-OH FAs before soil sample analyses. The standard mixture was  
206 methylated and silylated as described above before injection. Non-deuterated TMSi methyl  
207 ester derivatives of 3-OH FAs showed an intense diagnostic fragment ion ( $m/z$  175) as  
208 observed by e.g. Wang et al. (2016), whereas that of the deuterated internal standard is  $m/z$   
209 178. GC-MS analysis of the mixture resulted in an equivalent response factor for the  
210 deuterated standard relative to the non-deuterated 3-OH FA compounds, whatever their chain  
211 lengths.

212 In soil samples from Mt. Rungwe and Mt. Majella, the TMSi methyl ester derivatives of 3-  
213 OH FAs were identified from their retention times and mass spectra. In addition to  $m/z$  175,  
214 other characteristic ions include  $M^+ - 15$  (base peak),  $M^+ - 31$ , and  $m/z$  89, 103, 133 and 159  
215 (e.g. Volkman et al., 1999; Wang et al., 2016). A solution of the deuterated 3-OH FA internal

216 standard, methylated as described above (cf. section 2.3.), was added to each sample after acid  
217 hydrolysis, solvent extraction and separation on silica column. Typically, 1.5  $\mu\text{g}$  of  
218 methylated internal standard was added to 5 mg of the EtOAc fraction from the extract. This  
219 mixture was then silylated with BSTFA-TMCS, as previously described, and then analysed by  
220 GC-MS. The coefficient of variation (CV) for triplicate injections of 5 samples was 0.7% for  
221 3-OH FAs.

222 The RIAN index was calculated as follows (Wang et al., 2016):

$$223 \text{RIAN} = -\log [(I+A)/N] \quad (1)$$

224 where  $I$ ,  $A$  and  $N$  represent the sum of all the *iso*, *anteiso* and *normal* 3-OH FAs respectively.

225 In the same way, the  $\text{RAN}_{15}$  and  $\text{RAN}_{17}$  indices were defined as follow (Wang et al., 2016)

$$226 \text{RAN}_{15} = [\text{anteiso-C}_{15}] / [\text{normal-C}_{15}] \quad (2)$$

$$227 \text{RAN}_{17} = [\text{anteiso-C}_{17}] / [\text{normal-C}_{17}] \quad (3)$$

228 and correspond to the ratios of *anteiso* to *normal*  $\text{C}_{15}$  and  $\text{C}_{17}$  3-OH FAs, respectively. The  
229 analytical error based on triplicate injections was 0.006 for RIAN, 0.18 for  $\text{RAN}_{15}$  and 0.05  
230 for  $\text{RAN}_{17}$ .

### 231 2.5. *BrGDGT analysis*

232  
233 BrGDGT data have been previously published for (i) 19 of the 26 soil samples from Mt.  
234 Shennongjia (Yang et al., 2015) and for (ii) 16 of the 21 soil samples from Mt. Rungwe  
235 collected in 2012 (Coffinet et al., 2014). BrGDGT analysis of the aforementioned samples  
236 was performed using the traditional method based on a normal phase separation with a cyano  
237 column, which does not allow the separation of 5- and 6-methyl isomers.

238 Regarding soils from Mt. Majella, sample preparation was similar to that detailed by  
239 Coffinet et al. (2014). In these samples, brGDGTs were analysed with a Shimadzu LCMS-

240 2020 in selected ion monitoring mode using a procedure modified from Yang et al. (2015)  
 241 and Hopmans et al. (2016) allowing the separation of 5- and 6-methyl isomers. Separation  
 242 was achieved with two silica columns in tandem (150 mm × 2.1 mm, 1.9 μm, Thermo  
 243 Finnigan; USA) thermostated at 40 °C. Injection volume was 10 μl. GDGTs were first eluted  
 244 isocratically with 82% A/18% B for 25 min (A = hexane, B = hexane/isopropanol 9/1, v/v).  
 245 The following linear gradient was subsequently used: 82% A/18% B to 65% A/35% B in 25  
 246 min, followed by 65% A/35 % B to 100% B, maintained for 10 min and then back to 82%  
 247 A/18% B in 10 min, maintained for 30 min. The flow rate was set at 0.2 ml/min. Semi-  
 248 quantification of brGDGTs was performed by comparing the integrated signal of the  
 249 respective compound with the signal of a C<sub>46</sub> synthesized internal standard (Huguet et al.,  
 250 2006) assuming their response factors to be identical.

251 The MBT and CBT indices were calculated as follows (Weijers et al., 2007):

$$252 \quad MBT = \frac{[Ia+Ib+Ic]}{[Ia+Ib+Ic]+[IIa+IIb+IIc]+[IIIa+IIIb+IIIc]+[II'a+II'b+II'c]+[III'a+III'b+III'c]} \quad (4)$$

253

$$254 \quad CBT = -\log\left(\frac{[Ib]+[IIb+II'b]}{[Ia]+[IIa+II'a]}\right) \quad (5)$$

255 The Roman numerals correspond to the structures presented in De Jonge et al. (2014). The  
 256 6-methyl brGDGTs are denoted by an accent after the roman numerals for their corresponding  
 257 5-methyl isomers.

258 MBT' was calculated according to Peterse et al. (2012):

$$259 \quad MBT = \frac{[Ia+Ib+Ic]}{[Ia+Ib+Ic]+[IIa+IIb+IIc]+[IIIa]+[II'a+II'b+II'c]+[III'a]} \quad (6)$$

260 MBT'<sub>5Me</sub> was calculated using the equation of De Jonge et al. (2014):

$$261 \quad MBT'_{5Me} = \frac{[Ia+Ib+Ic]}{[Ia+Ib+Ic]+[IIa+IIb+IIc]+[IIIa]} \quad (7)$$

262 For soils from Mt. Shennongjia, MAAT was previously estimated by Wang et al. (2016)  
263 using a local calibration (Yang et al., 2015):

$$264 \text{ MAAT} = 2.06 + 28.13 \times \text{MBT} - 9.06 \times \text{CBT} \quad (8)$$

265 Along Mt. Rungwe, MAAT was estimated using the East African regional calibration  
266 proposed by Coffinet et al. (2017):

$$267 \text{ MAAT} = -8.76 \times \text{CBT} + 24.24 \times \text{MBT}' + 9.60 \quad (8)$$

268 Regarding Mt. Majella, MAAT was reconstructed using the global soil calibration by De  
269 Jonge et al. (2014) as no local or regional calibration exists for this mountain:

$$270 \text{ MAAT} = -8.57 + 31.45 \times \text{MBT}'_{5\text{Me}} \quad (9)$$

271 The 6-methyl brGDGTs are excluded from the calibration by De Jonge et al. (2014),  
272 improving the accuracy of MAT reconstructions compared to previous global soil calibrations  
273 including both 5- and 6-methyl brGDGTs (Weijers et al., 2007; Peterse et al., 2012).

## 274 2.6. *Statistical analyses*

275

276 All statistical analyses were performed using the software Graph Pad Prism 7.04 (Graph Pad  
277 Software Inc.). Relationships of 3-OH FA distribution with MAAT and soil pH in specific  
278 altitudinal transects as well as in the complete dataset were evaluated based on the Pearson  
279 coefficient of determination  $r^2$  and were considered as significant when the  $p$ -value of the  
280 correlation coefficient was lower than 0.05. Whatever the correlation, samples with  
281 standardized residuals higher than 2 or lower than -2 were identified as outliers. Comparison  
282 of the regressions obtained from the different mountains was performed using homogeneity of  
283 slope test followed by an analysis of co-variance in order to compare the slopes and intercepts

284 of the correlations, respectively. Differences between soil datasets were considered significant  
285 when *p*-value was lower than 0.05.

286

### 287 **3. Results and discussion**

#### 288 *3.1. Distribution and concentration of 3-OH FAs*

289

290 3-OH FAs were detected in all soil samples from Mt. Majella and Mt. Rungwe (Supp.  
291 Table 1), as shown for example in the chromatogram of a sample collected at 520 m altitude  
292 along Mt. Rungwe (Fig. 1). The carbon number of the 3-OH FAs in soils from these two  
293 mountains varied between C<sub>10</sub> and C<sub>18</sub>, with even and *normal* chain compounds dominant  
294 over even, *iso* and *anteiso* homologs (Fig. 1; Supp. Table 2), as observed for soils from Mt.  
295 Shennongjia (Wang et al., 2016). It should be noted that 3-OH FAs with 19 to 26 carbon  
296 atoms were also detected in soils from Mt. Rungwe and Mt. Majella and represented < 10 %  
297 of total 3-OH FAs (i.e. C<sub>10</sub> to C<sub>26</sub> homologs; data not shown). These long chain 3-OH FAs  
298 were previously shown to be widely distributed in microorganisms, being produced by *e.g.*  
299 yeasts, fungi, and Gram-positive bacteria in addition to Gram-negative ones (Zelles, 1997;  
300 Kock et al., 1999). In this paper, only 3-OH FAs with 10 to 18 C, typical for Gram-negative  
301 bacteria (Wilkinson et al., 1988), will be considered.

302 About 60 to 90 % of total 3-OH FAs (with a chain length of 10 to 18 C) were of even  
303 carbon number (Supp. Table 2), as typically observed in the LPS of Gram-negative bacteria  
304 (Wilkinson, 1988). *n*-C<sub>14</sub>, C<sub>16</sub> and C<sub>18</sub> were the most abundant 3-OH FAs in soils from both  
305 Mt. Rungwe and Mt. Majella (*ca.* 50 to 60 % and 40 to 50 % of total 3-OH FAs for Mt.  
306 Rungwe and Mt. Majella, respectively; Supplementary Table 2). Odd 3-OH FAs were  
307 dominated by C<sub>15</sub> and C<sub>17</sub> homologs, with *iso* compounds being *ca.* 2 to 10 times more  
308 abundant than those with *anteiso* and normal chains (Supplementary Table 2). Odd short-  
309 chain homologs (C<sub>11</sub> and C<sub>13</sub>) were generally present at low concentration in soil samples  
310 (each representing less than 5% of total 3-OH FAs), and some of them were even not detected

311 in some soils (e.g. *anteiso* C<sub>11</sub> and *n*-C<sub>11</sub> along Mt. Majella; Supp. Table 2). The different 3-  
312 OH FAs reported in this study were similarly observed in a wide range of environmental  
313 samples, e.g. atmospheric dust (Saraf et al., 1997; Tyagi et al., 2015), marine dissolved  
314 organic matter (Wakeham et al., 2003), stalagmites (Wang et al., 2012, 2018), sediments  
315 (Mendoza et al., 1986; Wakeham, 1999) as well as in biomass from Gram-negative bacterial  
316 strains (e.g. Pinkart and White, 1998). The dominant homologs were shown to differ from  
317 sample to sample in all environmental settings, including soils from Mt. Majella and Mt.  
318 Rungwe (Supp. Table 2). This likely reflects variations in distribution of 3-OH FAs in the  
319 LPS of the Gram-negative bacterial species (e.g. *Pseudomonas* species, Pinkart and White,  
320 1998; *Geobacter* species, Hedrick et al., 2009). Thus, 3-OH FAs with either 10, 12 or 14 C  
321 were shown to be predominant among the different *Pseudomonas* species (Oyaizu and  
322 Komagata, 1982), while the most abundant homologs of the genera *Bacteroides* and  
323 *Fusobacterium* were either C<sub>14</sub> or C<sub>16</sub> 3-OH FAs (Miyagawa et al., 1979).

324 Concentrations of Gram-negative bacterial 3-OH FAs with respect to dry soil weight are  
325 similar for soils from Mt. Rungwe (1.1 to 24.2 µg/g soil; mean 7.8 µg/g soil) and Mt. Majella  
326 (0.8 to 21.3 µg/g soil, mean 10.2 µg/g soil; Supp. Table 1) and are in the same range as those  
327 previously observed in one surficial Finnish soil (16.8 µg/g soil; Keinänen et al., 2003) and  
328 soils from various locations under different cultivation types (0.4 to 6.3 µg/g ; Zelles, 1999  
329 and references therein). Nevertheless, when normalised to total organic carbon, 3-OH FA  
330 concentrations were significantly higher ( $p < 0.05$ ) in soils from Mt. Rungwe (17.9 to 1062.5  
331 µg/g C<sub>org</sub>; mean 197.41 µg/g C<sub>org</sub>) than in those from Mt. Majella (2.1 to 103.9 µg/g C<sub>org</sub>;  
332 mean 43.2 µg/g C<sub>org</sub>; Table 1), due the higher C<sub>org</sub> contents in Mt. Majella (29 ± 9.0 %) than in  
333 Mt. Rungwe samples (5.0 ± 3.0 %; Table 1). Several environmental parameters, such as  
334 vegetation cover or annual amount of precipitation, which differ significantly between Mt.  
335 Majella and Mt. Rungwe (cf. section 2.1.), might impact the growth of Gram-negative

336 bacteria and thus the production of 3-OH FAs in soils. The higher concentrations of Gram-  
337 negative bacterial 3-OH FA concentrations along Mt. Rungwe than along Mt. Majella might  
338 at least be partly due to both quantitative and qualitative differences in soil organic matter  
339 (SOM) composition between the two mountains. The abundance and composition of bacterial  
340 communities in soils were indeed reported to be directly influenced by the amount and quality  
341 of soil organic carbon, the proportions of Gram-negative bacteria generally increasing in soil  
342 in the presence of fresh and labile organic substrates (e.g. Nemergut et al., 2010; Fanin et al.,  
343 2014). In any case, the variability of 3-OH FA concentrations along each mountain is  
344 independent of elevation, as 3-OH FA concentrations do not correlate with elevation neither  
345 along Mt. Rungwe ( $R^2$  0.11) nor along Mt. Majella ( $R^2$  0.09).

346

### 347 3.2. 3-OH FA-derived proxies

348

349 The correlations between the relative abundance of the 3-OH FAs in soils from Mt.  
350 Rungwe and Majella and environmental parameters were investigated using the different  
351 indices recently defined by Wang et al. (2016) – RIAN,  $RAN_{15}$  and  $RAN_{17}$ .

352

#### 353 3.2.1. RIAN index

354 The ratio of the sum of *iso* and *anteiso* 3-OH FAs from Gram-negative bacteria to the total  
355 amount of *normal* 3-OH FAs in the  $C_{12}$ - $C_{18}$  range, defined as the RIAN index (Eq. 10) was  
356 previously shown to correlate negatively with soil pH along Mt. Shennongjia (Wang et al.,  
357 2016):

$$358 \text{RIAN} = 1.11 - 0.10 \times \text{pH} \quad (n = 26; R^2 0.70; p < 0.001) \quad (10)$$

359 This index was calculated for all soil samples from Mt. Rungwe and Mt. Majella. RIAN  
360 ranged between 0.31 and 0.80 along Mt. Rungwe and between 0.16 and 0.43 along Mt.  
361 Majella (Table 1). In contrast with Mt. Shennongjia, RIAN does not correlate with measured

362 pH neither along Mt. Rungwe ( $R^2$  0.10) nor along Mt. Majella ( $R^2$  0.16; Fig. 2a). The absence  
363 of local correlation between RIAN and pH along Mt. Majella and Mt. Rungwe may be due to  
364 the fact that pH varies in a much smaller range (ca. 1.5 units; Table 1) in soils from these two  
365 mountains than in those from Mt. Shennongjia (ca 3.5 units; Wang et al., 2016). A similar  
366 hypothesis may be put forward to explain (i) the moderate / very poor correlations between  
367 brGDGT-based CBT and pH along Mts. Rungwe ( $R^2$  0.31,  $p = 0.025$ ; data not shown) and  
368 Majella ( $R^2$  0.03,  $p = 0.70$ ; cf. Supp. Table 3 for CBT values), respectively and (ii) the  
369 contrast with Mt. Shennongjia, where CBT was significantly correlated with pH ( $R^2$  0.74,  
370  $p < 0.001$ ; Yang et al., 2015). As CBT and RIAN are poorly correlated with soil pH along Mts.  
371 Rungwe and Majella, no significant correlation between RIAN and CBT can be observed  
372 along these two mountains, in contrast with Mt. Shennongjia (Fig. 2b).

373 So as to overcome the limitation associated with the restricted pH range in a given  
374 mountain, RIAN data from the three mountains were combined. In the resulting extended  
375 dataset, with 65 samples covering a large range of pH (4.5 to 8.7), each soil was considered as  
376 an independent and individual sample. This combination of data resulted in a significant  
377 negative correlation between RIAN and pH (Fig. 2a):

$$378 \quad \text{RIAN} = 1.16 - 0.12 \times \text{pH} \quad (11)$$

379 Eq. 11 presents similar slope and intercept as the initial relationship between RIAN and pH  
380 observed along Mt. Shennongjia (Eq. 10; Wang et al., 2016).

381 Reconstruction of pH using RIAN can be performed using Eq. 12:

$$382 \quad \text{pH} = 8.63 - 5.36 \times \text{RIAN} \quad (n = 63; R^2 0.62; \text{RMSE } 0.56; n_{\text{outliers}} = 2; p < 0.001) \quad (12)$$

383 Two outliers (one from Mt. Rungwe and one from Mt. Majella; Fig. 2a), with standardized  
384 residuals higher than 2, are excluded from the relationship.

385 Bacteria have developed various adaptation mechanisms to cope with pH variations in the  
386 environment, the challenge being to maintain near neutral intracellular pH (Siliakus et al.,



387 2017 and references therein). Thus, brGDGT-producing bacteria remodel their membrane in  
388 response to changing pH by modifying brGDGT cyclisation degree (reflected in the CBT  
389 index; Schouten et al., 2013). Similarly, the correlation between RIAN and pH (Eq. 12)  
390 supports (i) the effect of pH on the relative distribution of Gram-negative bacterial 3-OH FAs  
391 in soils previously observed along Mt. Shennongjia by Wang et al. (2016) and (ii) the fact that  
392 the relative abundance of branched homologues vs. straight ones tends to decrease with pH,  
393 allowing Gram-negative bacteria to reduce the permeability and fluidity of their membrane. A  
394 moderate but significant correlation between RIAN and CBT was also obtained when  
395 combining the data from the three mountains (Fig. 2b), consistent with the pH dependence of  
396 these two indices.

397 Despite the potential of the RIAN as a pH proxy, the relatively large amount of scatter of  
398 the RIAN-pH relationship has to be taken into account. The RMSE associated with the  
399 relationship between RIAN and pH is 0.56, similar to the uncertainty associated with the  
400 latest CBT'-pH calibration (RMSE 0.52) developed by De Jonge et al. (2014), even though  
401 the latter is based on a larger number of soils ( $n = 222$ ). Reconstruction of pH using the  
402 RIAN-pH calibration (Eq. 12) should therefore be undertaken with special caution (i) in soils  
403 with  $\text{pH} < 5$ , the pH in these soils being significantly overestimated and (ii) in soils with  $\text{pH}$   
404  $> 8$ , where pH is significantly underestimated (Fig. 2c).

405

### 406 3.2.2. RAN<sub>15</sub> index

407 Wang et al. (2016) showed the influence of MAAT on the relative abundance of bacterial  
408 3-OH FAs via the calculation of the RAN<sub>15</sub> index based on *anteiso* and *normal* C<sub>15</sub> 3-OH  
409 FAs. RAN<sub>15</sub> was negatively correlated with MAAT along Mt. Shennongjia (Wang et al.,  
410 2016), leading to the following equations:

$$411 \text{RAN}_{15} = 6.84 - 0.26 \times \text{MAAT} \quad (13)$$

412  $MAAT = 17.95 - 1.99 \times RAN_{15}$  ( $n = 25$ ;  $R^2 0.52$ ;  $RMSE 2.5 \text{ }^\circ\text{C}$ ;  $n_{outliers} = 2$ ;  $p < 0.001$ ) (14)

413 In the present study, one outlier with standardized residuals higher than 3 was excluded  
414 from the relationships between MAAT and  $RAN_{15}$  observed along Mt. Shennongjia, slightly  
415 modifying the correlations initially published by Wang et al. (2016).

416  $RAN_{15}$  varies in the same range along Mt. Rungwe (1.25 – 5.73), Mt. Majella (0.68 – 6.43)  
417 and Mt. Shennongjia (0.68 – 8.09, excluding the outlier; Wang et al., 2016). As previously  
418 observed along Mt. Shennongjia (Eqs. 13 and 14; Wang et al., 2016), the  $RAN_{15}$  index shows  
419 a moderate and negative correlation with MAAT along both Mt. Majella ( $R^2 0.54$ ) and Mt.  
420 Rungwe ( $R^2 0.70$ ). This is reflected in Eqs. 15 and 16 for Mt. Majella, and Eqs. 17 and 18 for  
421 Mt. Rungwe, respectively (Fig. 3a):

422  $RAN_{15} = 5.34 - 0.28 \times MAAT$  (15)

423  $MAAT = 13.54 - 1.94 \times RAN_{15}$  ( $n = 11$ ;  $R^2 0.52$ ;  $RMSE 2.9 \text{ }^\circ\text{C}$ ;  $p = 0.01$ ) (16)

424  $RAN_{15} = 8.16 - 0.25 \times MAAT$  (17)

425  $MAAT = 30.50 - 3.19 \times RAN_{15}$  ( $n = 28$ ;  $R^2 0.52$ ;  $RMSE 1.6 \text{ }^\circ\text{C}$ ;  $p < 0.001$ ) (18)

426 This shows that MAAT has a similar effect on the distribution of  $C_{15}$  3-OH FAs in soils  
427 from different locations,  $RAN_{15}$  increasing with decreasing temperature, likely as a result of  
428 homeoviscous adaptation. Such a temperature effect was previously reported on the relative  
429 distribution of (i) archaeal-derived isoprenoid GDGTs, the number of cyclopentane moieties  
430 increasing with temperature (Schouten et al., 2013 and references therein) and of (ii)  
431 branched-GDGT, the amount of methyl groups on the carbon chains increasing at low  
432 temperatures, enabling the membrane to remain in a liquid crystalline state (Weijers et al.,  
433 2007; De Jonge et al., 2014). Similarly, increases in the amount of branched chain fatty acids,  
434 saturated fatty acids, long-chain fatty acids and polar carotenoid content were observed in  
435 mesophilic and thermophilic bacteria (Siliakus et al., 2017 and references therein). Regarding  
436 Gram-negative bacteria, the present results combined with those from Wang et al. (2016)

437 suggest that the relative abundance of *anteiso* vs. *normal* C<sub>15</sub> 3-OH FA increases with  
438 decreasing temperature to maintain membrane fluidity, consistent with the fact that *anteiso*  
439 fatty acids have a lower melting point than *normal* ones (Suutari and Lakso, 1994).

440 The correlations between RAN<sub>15</sub> and MAAT along Mts. Shennongjia, Rungwe and  
441 Majella (Fig. 3) do not significantly differ in slope (homogeneity of slope test: df = 2, F =  
442 0.072, *p* = 0.931), but display significantly different intercepts (df = 2, F = 22.25, *p* < 0.0001).  
443 The present results suggest that regional calibrations with MAAT might be necessary to use  
444 RAN<sub>15</sub> as a temperature proxy. Nevertheless, further application of RAN<sub>15</sub> to other altitudinal  
445 transects distributed worldwide is now needed to confirm this statement.

446 The hydroxy fatty acid profile of Gram-negative bacteria is dependent on environmental  
447 conditions but can also greatly vary between genus and even species of bacteria (e.g.  
448 *Pseudomonas sp*; Pinkart et al., 1998). Therefore, at a given temperature and independently of  
449 other environmental conditions, the ratio of *anteiso* vs. *normal* C<sub>15</sub> 3-OH FA may strongly  
450 vary from one site to another depending on the Gram-negative bacterial communities present  
451 in soil. This may notably explain why a given RAN<sub>15</sub> value corresponds to different MAAT  
452 depending on the sampling site. Despite these potential differences in absolute RAN<sub>15</sub> values  
453 between microbial species, the present results suggest that a given range of variation in  
454 temperature has the same effect on Gram-negative bacteria whatever the sampling site. In  
455 addition, RAN<sub>15</sub> was shown to be significantly correlated with the MAAT estimates derived  
456 from the established GDGT-based proxies (MBT/CBT) along the three investigated  
457 mountains (Fig. 3b). This confirms that temperature is the dominant control on RAN<sub>15</sub> for the  
458 three sites.

459

460

461

462 3.2.3. RAN<sub>17</sub> index

463 Similarly to RAN<sub>15</sub>, RAN<sub>17</sub>, based on the ratio of *anteiso* and *normal* C<sub>17</sub> 3-OH FAs, was  
464 previously shown to be negatively correlated with MAAT (R<sup>2</sup> 0.50) along Mt. Shennongjia  
465 (Fig. 4a; Wang et al., 2016). RAN<sub>17</sub> values are generally lower along Mt. Rungwe (0.33 –  
466 1.62; Table 1) than along Mt. Shennongjia (1.11 – 3.56; Wang et al., 2016) and show high  
467 variability along Mt. Majella (0.65 – 4.75; Fig. 4a and Table 1). A significantly negative  
468 correlation between RAN<sub>15</sub> and MAAT is observed along Mt. Rungwe (Fig. 4a), in line with  
469 results obtained from Mt. Shennongjia (Wang et al., 2016), leading to the following  
470 equations:

471 
$$\text{RAN}_{17} = 2.39 - 0.065 \times \text{MAAT} \quad (19)$$

472 
$$\text{MAAT} = 30.12 - 8.24 \times \text{RAN}_{17} \quad (n = 28; R^2 0.54; \text{RMSE } 2.5 \text{ } ^\circ\text{C}; p < 0.001) \quad (20)$$

473 The relationship between RAN<sub>17</sub> and MAAT along Mt. Majella, expressed in Eqs. 21 and 22,  
474 is weaker than the one observed along Mts. Rungwe and Shennongjia and cannot be  
475 considered as significant, even though the *p*-value, in the range 0.05-0.10, indicates a trend  
476 towards significance:

477 
$$\text{RAN}_{17} = 3.16 - 0.15 \times \text{MAAT} \quad (21)$$

478 
$$\text{MAAT} = 10.80 - 1.84 \times \text{RAN}_{17} \quad (n = 11; R^2 0.28; \text{RMSE } 3.8 \text{ } ^\circ\text{C}; p = 0.092) \quad (22)$$

479 The absence of statistical significance in the relationship between RAN<sub>17</sub> and MAAT is  
480 likely due to the high variability of RAN<sub>17</sub> values along Mt. Majella, leading to a weak  
481 correlation. The latter might have several explanations. First, RAN<sub>17</sub> is weakly (but not  
482 significantly) correlated with soil pH (R<sup>2</sup> 0.2, *p* = 0.16), in contrast with Mts. Rungwe and  
483 Shennongjia, where no correlation between RAN<sub>17</sub> and pH is observed (R<sup>2</sup> < 0.020; data not  
484 shown). The alkalinity of Mt. Majella soils (7.8 ± 0.1; Table 1) may lead to a higher influence  
485 of pH on RAN<sub>17</sub>, and in turn, to a higher scattering in the RAN<sub>17</sub>-MAAT relationship than in

486 acidic and neutral soils, such as those of Mt. Rungwe ( $6.1 \pm 0.5$ ; Table 1) and Mt.  
487 Shennongjia ( $6.2 \pm 1.0$ ; Wang et al., 2016).

488 Second, it might also not be excluded that precipitation/soil moisture affects 3-OH FA  
489 distribution, including RAN<sub>17</sub> and RAN<sub>15</sub> indices. Soil moisture is indeed a major factor  
490 influencing microbial community structure and characteristics (Brockett et al., 2012). It may  
491 therefore affect the diversity of Gram-negative bacteria and thus their membrane lipids, i.e. 3-  
492 OH FAs. In the present study RAN<sub>17</sub> as well as RAN<sub>15</sub> hint at a linear relationship with MAP  
493 along Mt. Majella ( $R^2$  0.28 and  $R^2$  0.54, respectively; data not shown), as also observed along  
494 Mt. Shennongjia ( $R^2$  0.48 and  $R^2$  0.50, respectively; Wang et al., 2016). Correlations between  
495 RAN<sub>17</sub>/RAN<sub>15</sub> and MAP are similar to those observed between these indices and MAAT, as  
496 MAAT and MAP strongly co-vary with elevation along both Mt. Majella ( $R^2$  0.93, Supp.  
497 Table 2) and Mt. Shennongjia (Wang et al., 2016). Nevertheless, Wang et al. (2016)  
498 suggested that MAAT was the dominant parameter influencing RAN<sub>15</sub> and RAN<sub>17</sub> along Mt.  
499 Shennongjia, as (i) the climate in this region is moist to humid ( $> 1000$  mm/year) making  
500 MAP an unlikely limiting ecological factor for the growth of Gram-negative bacteria and (ii)  
501 only weak correlations between RAN<sub>17</sub>/RAN<sub>15</sub> and punctual soil humidity measurements  
502 were observed ( $R^2 < 0.20$ ; Wang et al., 2016). A similar assumption regarding the  
503 predominant influence of MAAT over precipitation/soil humidity on RAN<sub>15</sub> and RAN<sub>17</sub> can  
504 be made for Mt. Majella, subjected to a moderately humid climate (ca. 800 – 1300 mm  
505 precipitation/year; Table 1). Unfortunately, direct soil humidity measurements are not  
506 available for Mt. Majella samples and cannot be used to strengthen this hypothesis.

507 In line with the observations made from the relationships between RAN<sub>17</sub> and recorded  
508 MAAT (Fig. 4a), RAN<sub>17</sub> is moderately and significantly correlated with brGDGT-derived  
509 MAAT estimates along Mts. Rungwe and Shennongjia, whereas no significant correlation is  
510 observed along Mt. Majella (Fig. 4b). This may reflect the fact that 3-OH FAs and brGDGTs

511 are produced by different microorganisms, and that the ecological and physiological  
512 responses of Gram-negative bacteria and brGDGT-producing bacteria to other environmental  
513 factors are not necessarily the same and are site-dependent.

514 In any case, statistical tests revealed no significant differences in either slopes ( $df = 2$ ,  $F =$   
515  $1.801$ ,  $p = 0.174$ ) or in the intercepts of the relationships between  $RAN_{17}$  and recorded MAAT  
516 along Mts. Rungwe, Majella and Shennongjia ( $df = 2$ ,  $F = 0.678$ ,  $p = 0.512$ ; Fig. 4a). In  
517 addition, most data from Mt. Majella fall within the 95 % prediction interval of the  $RAN_{17}$ -  
518 MAAT correlations along Mts. Rungwe and Shennongjia. This shows that the distribution of  
519 Gram-negative bacterial  $C_{17}$  3-OH FAs is similarly affected by temperature variations in soils  
520 from different latitudes, with a relative decrease of *normal* vs. *anteiso* homologs with  
521 decreasing temperature, as previously observed for  $C_{15}$  3-OH FAs. This suggests that different  
522 Gram-negative bacteria species might present the same range of  $RAN_{17}$  values at one given  
523 temperature, even though microbial diversity can strongly vary from one soil to another,  
524 leading to a natural variability of the  $RAN_{17}$ .

525 The statistical similarity of the local relationships between  $RAN_{17}$  and recorded MAAT  
526 along Mts. Rungwe, Majella, and Shennongjia allows the combination of the three datasets,  
527 resulting in a significant and moderate negative correlation (Eqs. 23 and 24):

$$528 \quad RAN_{17} = 2.70 - 0.081 \times MAAT \quad (23)$$

$$529 \quad MAAT = 25.74 - 7.38 \times RAN_{17} \quad (n = 65; R^2 0.60; RMSE 5.1 \text{ } ^\circ\text{C}; p = < 0.001) \quad (24)$$

530 This calibration covers a wide range of temperature (ca. 0 – 25 °C; Fig. 4a) and confirms  
531 the potential of  $RAN_{17}$  as a temperature proxy in terrestrial settings. When combining the data  
532 from the three mountains,  $RAN_{17}$  also appear to be significantly correlated with brGDGT-  
533 derived MAAT (Fig. 4b), consistent with the observations made from recorded MAAT.

534 Even though almost all the data points from Mts. Rungwe, Shennongjia and Majella are  
535 within the 95 % prediction interval of the combined calibration (Eq. 24; Fig. 4a), the

536 substantial uncertainty in temperature reconstruction based on  $RAN_{17}$  (RMSE 5.1 °C; Fig. 4c)  
537 has to be taken into account, with the largest residuals observed for soils from Mt. Majella, as  
538 expected from the high variability of the  $RAN_{17}$  values (Fig. 4a). The RMSE of the  $RAN_{17}$ -  
539 MAAT calibration is similar to that reported for the latest correlation between brGDGT  
540 distribution and MAAT (4.6 °C; De Jonge et al., 2014), even though the latter is based on a  
541 much larger number of soils ( $n = 222$ ).

542

### 543 *3.3. Implications for the use of 3-OH FAs as environmental proxies*

544

545 This study espouses the potential of 3-OH FAs as new temperature and pH proxies in soils,  
546 in line with the first and recent application of these compounds as paleoproxies in a stalagmite  
547 from central China (Wang et al., 2018). Both  $RAN_{15}$  and  $RAN_{17}$  can be used independently to  
548 reconstruct temperature from soils. However, the presently considered data of samples from  
549 three different regions suggest that  $RAN_{15}$  might only be relevant for temperature  
550 reconstructions using independent local calibrations. Analysis of 3-OH FAs in a larger  
551 number of soils from over the world is now required to determine to which extent the RIAN,  
552  $RAN_{15}$  and  $RAN_{17}$  indices can be used as temperature and pH proxies at a global scale.

553 The remaining scatter in relationships between RIAN and pH (Fig. 2) as well as  
554  $RAN_{15}/RAN_{17}$  and MAAT (Figs. 3 and 4) is likely partly due to the intrinsic heterogeneity of  
555 soils, and also to variations in the diversity of Gram-negative bacterial communities between  
556 soils. Indeed, bacterial community composition, including Gram-negative bacteria, was  
557 observed to vary with elevation (Margesin et al., 2008) and 3-OH FA lipid profile is strongly  
558 dependent on Gram-negative bacterial species, as previously discussed (e.g. Parker et al.,  
559 1982; Bhat and Carlson, 1992).

560 Additionally, several other factors, such as amount of precipitation/soil moisture, type of  
561 vegetation or seasonality, may also have an effect on the relative distribution of 3-OH FAs

562 and thus on RIAN, RAN<sub>15</sub> and RAN<sub>17</sub> values in soils. Similar hypotheses are put forward to  
563 explain the large uncertainty remaining in global correlations between brGDGT distributions  
564 and MAAT (e.g. Peterse et al., 2012) despite recent analytical improvement (De Jonge et al.,  
565 2014). Soil humidity (e.g. Dirghangi et al., 2013; Menges et al., 2014) as well as soil type  
566 (Davtian et al., 2016) were shown to have an influence on the distribution of brGDGTs in  
567 soils. A bias in brGDGT distribution towards the warmest months of the year, when bacterial  
568 growth is higher, has also been reported in some soils (e.g. Pautler et al., 2014) and peat  
569 (Huguet al., 2013b). More generally, distinct seasonal changes in soil microbial community  
570 composition and biomass were previously observed in different settings (e.g. Smit et al.,  
571 2001; Habekots et al., 2008; Buckeridge et al., 2013). The specific activity and diversity of  
572 Gram-negative bacteria may vary seasonally, leading to preferential production of 3-OH FAs  
573 during certain periods of the year.

574 The use of air temperature instead of *in situ* soil temperature could also account for part of  
575 the scatter in the RAN<sub>15</sub> and RAN<sub>17</sub> correlations, as previously discussed for brGDGT-  
576 derived proxies (Weijers et al., 2007; Peterse et al., 2012). As soil temperatures are largely  
577 driven by air temperatures, MAAT is assumed to be a good approximation of soil  
578 temperature. Nevertheless, an offset may exist between MAAT and soil temperature, as the  
579 latter is dependent on numerous factors, such as soil properties (e.g. water content, chemical  
580 composition or granulometry), land relief and soil cover (Lehnert, 2014). Soil temperature  
581 data may help in partly reducing the scatter in the RAN<sub>15</sub> and RAN<sub>17</sub> calibrations. This is  
582 hampered by the fact that soil temperatures are much less documented than air temperatures  
583 and most of the time not available in the long-term, with only punctual soil temperature  
584 measurements when sampling.

585 Additional geochemical and microbiological studies are now necessary to better  
586 understand and constrain the influence of environmental parameters on Gram-negative



587 bacterial diversity and 3-OH FA distribution in soils. 3-OH FAs are present in a wide range of  
588 terrestrial and aquatic settings, and are likely well preserved in sedimentary archives, as they  
589 have been detected in several recent lacustrine sediments (Mendoza et al., 1987; Goosens et  
590 al., 1988; Wang et al., 2016), in a Chinese stalagmite spanning the last 9 kyrs (Wang et al.,  
591 2018) as well as in a last glacial marine sediment sample from the Baltic Sea and several Late  
592 Quaternary sapropels (deposited between 125,000 and 200,000 yrs B.P.; Ten Haven et al.,  
593 1987) from the eastern Mediterranean. Therefore, they could be used as paleoenvironmental  
594 proxies in various archives, such as paleosols, marine and lacustrine sediments, providing (i) a  
595 similar preservation of the C<sub>10</sub> to C<sub>18</sub> suite of 3-OH FA homologues and (ii) the development  
596 of calibrations specific to each type of environment.

597 Different organic proxies may provide conflicting information, as observed for example in  
598 marine environments for alkenone-based U<sup>k</sup><sub>37</sub> and isoprenoid GDGT-based TEX<sub>86</sub> (e.g.  
599 Lopes dos Santos et al., 2010; McClymont et al., 2012). Independent proxies are therefore  
600 required to ensure the reliability of paleoenvironmental reconstructions. Nevertheless, to  
601 date, the only organic proxies available for pH and temperature reconstruction in terrestrial  
602 environments are those based on brGDGTs. As such, RIAN, RAN<sub>15</sub> and RAN<sub>17</sub> may  
603 represent independent and complementary temperature and pH proxies to the well-established  
604 MBT and CBT indices. In the present study, the pH (Fig. 2b) and temperature (Figs. 3b and  
605 4b) proxies derived from 3-OH FAs on the one hand and brGDGTs on the other hand were  
606 poorly to moderately correlated and the corresponding relationships were observed to be site-  
607 dependent. This may reflect the differences in physiological responses of 3-OH FA- and  
608 brGDGT-source microorganisms to environmental changes and/or the variations in microbial  
609 community composition along the altitudinal gradients investigated. Such hypotheses remain  
610 speculative, as, in contrast with 3-OH FAs, a precise microbial source for brGDGTs has not  
611 been identified yet, even though some members of the Gram-negative phylum Acidobacteria

612 may produce these lipids (e.g. Sinninghe Damsté et al., 2018). In any case, the environmental  
613 factors impacting 3-OH-FA and brGDGT distribution in soils and the relationships between  
614 the temperature and pH proxies derived from these two families of lipids require investigation  
615 in a larger set of samples.

616

#### 617 **4. Conclusions**

618

619 The abundance and distribution of 3-OH FAs were determined in surficial soils collected  
620 along altitudinal gradients from two different regions – Mt. Rungwe, Tanzania and Mt.  
621 Majella, Italy – and were compared with data previously published from Mt. Shennongjia  
622 (China; Wang et al., 2016). Gram-negative bacterial 3-OH FAs, with 10 to 18 C, are detected  
623 in all soils with even and *normal* chain compounds dominant over even, *iso* and *anteiso*  
624 homologs. 3-OH FA distribution is shown to vary with elevation, as reflected in the general  
625 decrease in the RIAN, RAN<sub>15</sub> and RAN<sub>17</sub> when elevation increases.

626 The combination of data from Mts. Rungwe, Majella and Shennongjia resulted in a  
627 significant negative correlation between RIAN and pH, further highlighting the potential of  
628 this index as a pH proxy. The correlations between RAN<sub>15</sub> and MAAT along Mts.  
629 Shennongjia, Rungwe and Majella do not significantly differ in slope, suggesting that Gram-  
630 negative bacteria respond in the same way to temperature variations in soils from different  
631 latitudes, with a relative decrease in *normal* vs. *anteiso* homologs with decreasing  
632 temperature. Nevertheless, the relationships between RAN<sub>15</sub> and MAAT along the three  
633 mountains display significantly different intercepts, suggesting that regional calibrations may  
634 be required to use RAN<sub>15</sub> as a temperature proxy. In contrast with RAN<sub>15</sub>, the statistical  
635 similarity of the local relationships between RAN<sub>17</sub> and MAAT along Mts. Rungwe, Majella,  
636 and Shennongjia led to the establishment of a combined calibration covering a wide range of  
637 temperature (ca. 0 – 25 °C). Analysis of 3-OH FAs in a large number of soils distributed

638 worldwide is now required to investigate the applicability of the proxies derived from these  
639 lipids at the global scale. As 3-OH FAs are anticipated to be well preserved in sedimentary  
640 archives, these compounds should be promising pH and temperature proxies, that would be  
641 independent of and complementary to those based on well-established brGDGTs  
642 (MBT/CBT). More studies based on natural samples and cultures of Gram-negative bacteria  
643 are now needed to better constrain the environmental factors influencing the relative  
644 abundance of 3-OH FAs in soils, to investigate the limits and applicability of the RIAN,  
645 RAN<sub>15</sub> and RAN<sub>17</sub> as paleoproxies and to improve and/or develop the calibrations between  
646 these indices and temperature/pH at regional and global scales.

647

648

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656

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## Figure and table captions

**Figure 1.** ~~Extracted-Ion mass ( $m/z$  175)~~ chromatogram showing distribution of 3-OH FAs in sample 1 (520 m altitude) from Mt. Rungwe. *Normal*, *iso* and *anteiso* 3-OH FAs are represented by red circles, green triangles and blue squares, respectively.

**Figure 2. (a)** Relationship between RIAN and soil pH along Mt. Rungwe and Mt. Majella (this study) and Mt. Shennongjia (Wang et al., 2016). Data for Mt. Rungwe samples are in blue, those for Mt. Majella in green and those from Mt. Shennongjia in orange. Black dashed line represents correlation based on combined data from the three mountains. Dotted lines represent 95% prediction interval for the combined correlation. The empty symbols are outliers as defined in the text. ~~(b) Offset between the measured and calculated pH values based on Eq. 6 plotted versus measured pH values. Symbols are the same as those used in panel (a).~~ **(b)** Relationship between RIAN and CBT along Mt. Rungwe and Mt. Majella (this study) and Mt. Shennongjia (Wang et al., 2016). Black dashed line represents correlation based on combined data from the three mountains. **(c)** Offset between the measured and calculated pH values based on Eq. 12 plotted versus measured pH values. Symbols in ~~panels (b) and (c)~~ are the same as those used in ~~panel (a)~~.

**Figure 3. (a)** Relationship between  $RAN_{15}$  and mean annual air temperature (MAAT) along Mt. Rungwe and Mt. Majella (this study) and Mt. Shennongjia (Wang et al., 2016). Data and correlations for Mt. Rungwe samples are in blue, those for Mt. Majella in green and those from Mt. Shennongjia in orange. Dotted lines represent 95% prediction interval for each local correlation. The empty symbols are outliers as defined in the text. **(b)** Relationship between  $RAN_{15}$  and brGDGT-derived MAAT estimates along Mt. Rungwe and Mt. Majella (this study) and Mt. Shennongjia (Wang et al., 2016). BrGDGT-derived MAAT estimates for soils from Mts. Rungwe and Shennongjia were obtained using local soil calibrations developed by Coffinet et al. (2017) and Yang et al. (2015), respectively, whereas the global soil calibration by De Jonge et al. (2014) was applied to soils from Mt. Majella. Symbols are the same as those used in panel (a).

**Figure 4. (a)** Relationship between  $RAN_{17}$  and MAAT along Mt. Rungwe and Mt. Majella (this study) and Mt. Shennongjia (Wang et al., 2016). Data and correlations for Mt. Rungwe samples are in blue, those for Mt. Majella in green and those for Mt. Shennongjia in orange. Black dashed line represents correlation based on combined data from the three mountains. Dotted lines represent 95% prediction interval for local and global correlations. ~~(b) Offset between the measured and calculated MAAT values based on Eq. 17 plotted versus measured MAAT values.~~ **(b)** Relationship between  $RAN_{17}$  and brGDGT-derived MAAT estimates along Mt. Rungwe and Mt. Majella (this study) and Mt. Shennongjia (Wang et al., 2016). BrGDGT-derived MAAT estimates for soils from Mts. Rungwe and Shennongjia were obtained using local soil calibrations developed by Coffinet et al. (2017) and Yang et al. (2015), respectively, whereas the global soil calibration by De Jonge et al. (2014) was applied to soils from Mt. Majella. Black dashed line represents correlation based on combined data from the three mountains. **(c)** Offset between the measured and calculated MAAT values based on Eq. 24 plotted versus measured MAAT values. Symbols in panels (b) and (c) are the same as those used in panel (a). Symbols are the same as those used in panel (a).

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~~Figure 5. Relationships between (a) RAN<sub>15</sub> and brGDGT derived MAAT estimates, (b) RAN<sub>17</sub> and brGDGT derived MAAT estimates and (c) RIAN and CBT along Mt. Rungwe and Mt. Majella (this study) and Mt. Shennongjia (Wang et al., 2016). Data and correlations for Mt. Rungwe samples are in blue, those for Mt. Majella in green and those for Mt. Shennongjia in orange. BrGDGT derived MAAT estimates for soils from Mts. Rungwe and Shennongjia were obtained using local soil calibrations developed by Coffinet et al. (2017) and Yang et al. (2015), respectively, whereas the global soil calibration by De Jonge et al. (2014) was applied to soils from Mt. Majella.~~

**Table 1.** Location and bulk features of soil samples from Mt. Rungwe and Mt. Majella collected in 2012 and 2016, abundance and distribution of 3-OH FAs plus indices derived from them as well. n.a., not available.

**Supplementary Table 1.** Mean annual precipitation (MAP) recorded along Mt. Majella.

**Supplementary Table 2.** Relative abundances (%) and total concentration ( $\mu\text{g/g}$  dry soil) of the *normal*, *iso* and *anteiso* 3-OH FAs in soils from Mt. Rungwe and Mt. Majella (n.d., not detected).

~~**Supplementary Table 3.** Indices derived from brGDGTs for soils from Mt. Majella and MAAT estimates calculated using the global soil calibration by De Jonge et al. (2014).~~

~~**Supplementary Table 3.** Indices derived from brGDGTs for soils from Mt. Majella and MAAT estimates calculated using the global soil calibration by De Jonge et al. (2014).~~

Mis en forme : Justifié

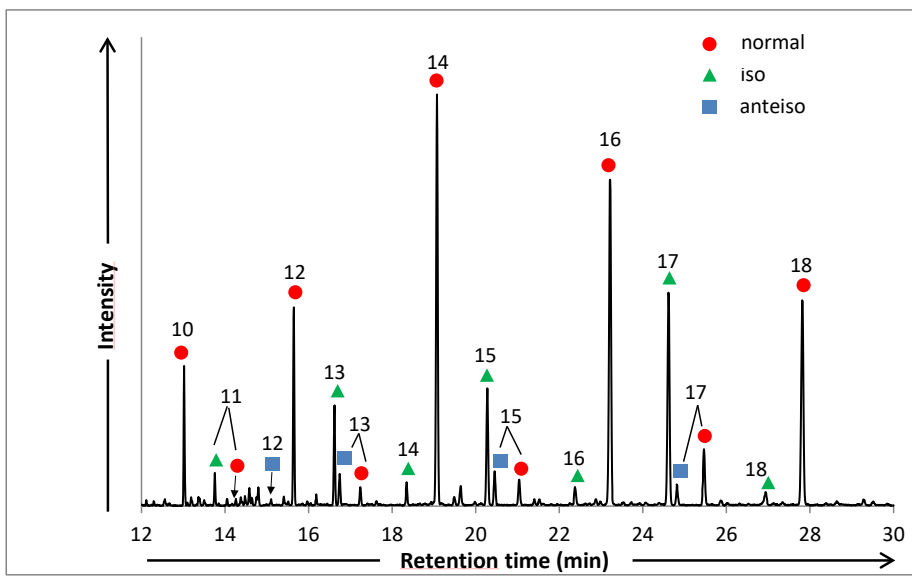


Fig. 1

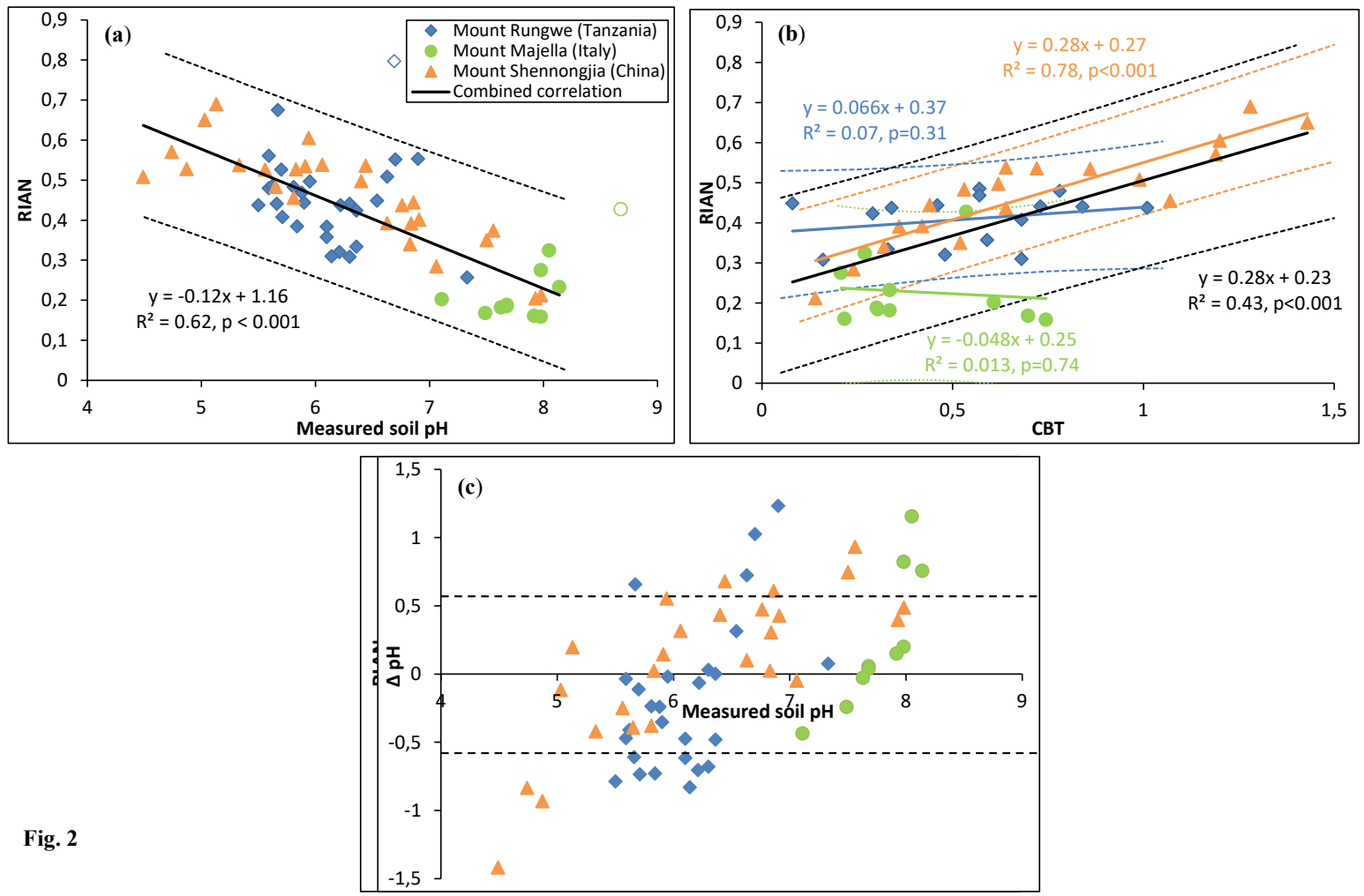
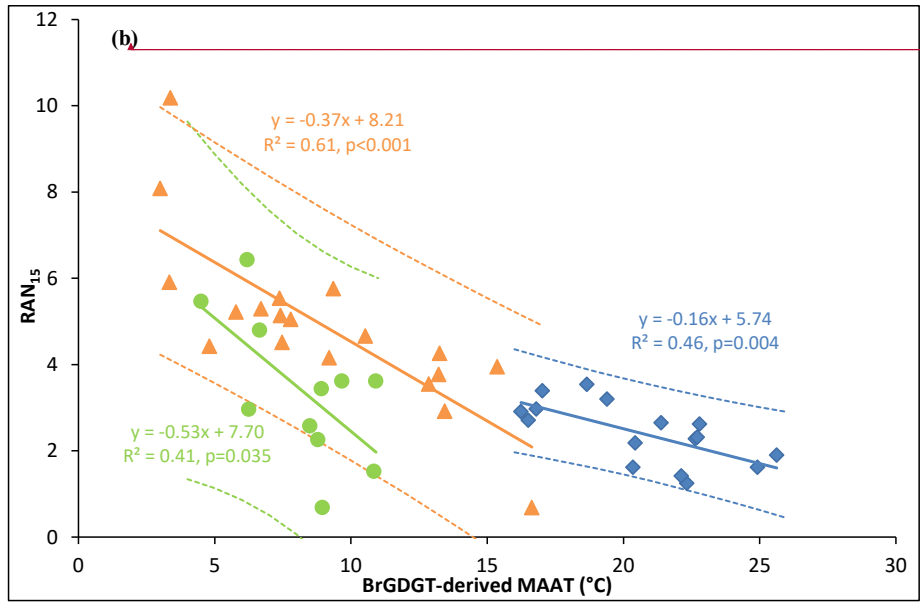
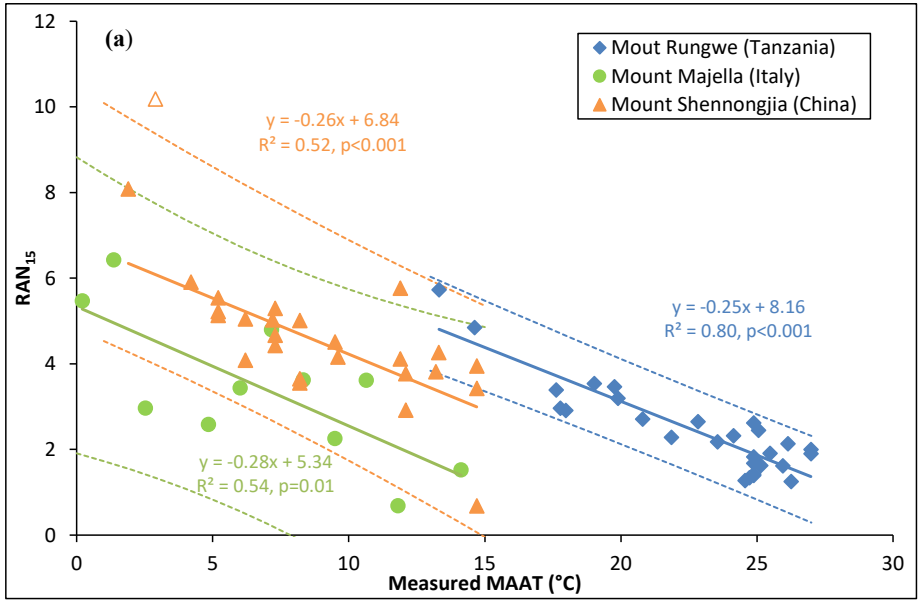


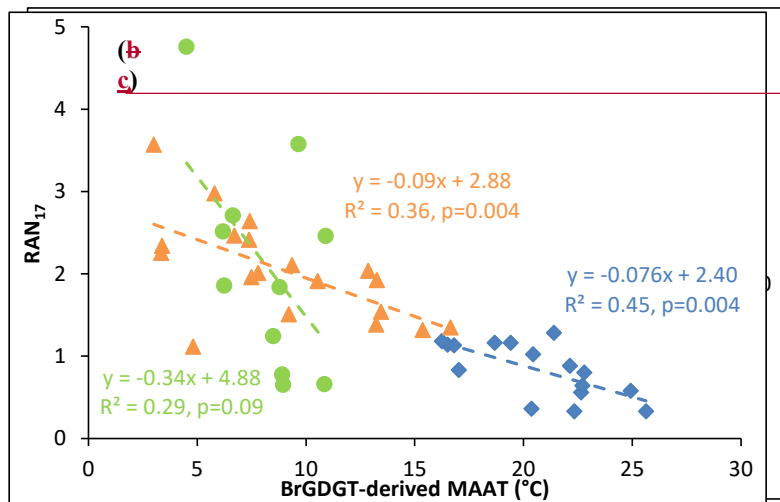
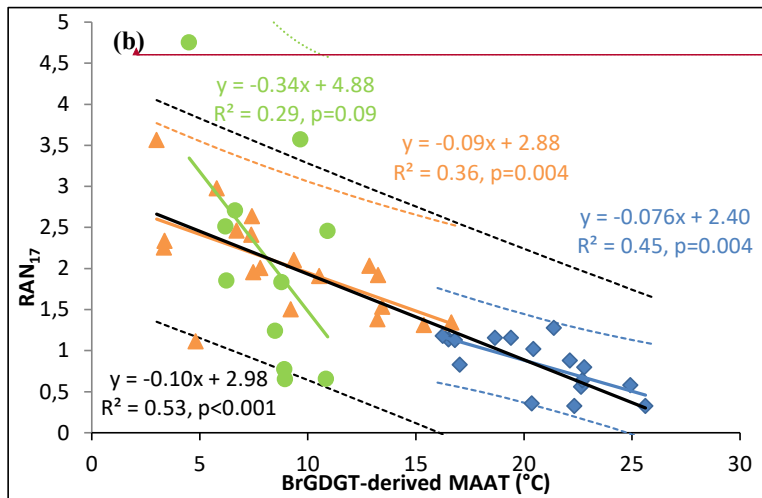
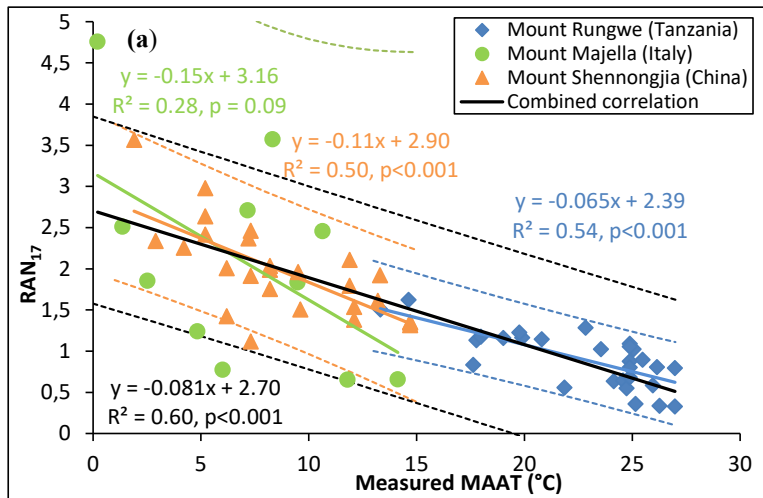
Fig. 2





Mis en forme : Police :Gras

Fig. 3



Mis en forme : Police :Gras

Mis en forme : Police :Gras

Fig. 4

**Table 1**

Campaign	Sample	Altitude (m)	Coordinates	MAAT <sup>a</sup> (°C)	MAP <sup>b</sup> (mm/year)	Soil pH	Corg (%)	Total 3-OH FAs (µg/g C <sub>org</sub> )	RIAN	RAN <sub>15</sub>	RAN <sub>17</sub>
Mt. Rungwe (2012)	1	520	S 09.40492° E 33.90782°	25.7	n.a.	6.5	4.9	314.9	0.45	1.90	0.33
	2	520	S 09.40450° E 33.90870°	25.7	n.a.	5.6	1.5	77.3	0.56	2.00	0.79
	3	640	S 09.37025° E 33.79949°	24.8	n.a.	6.4	6.2	344.2	0.42	1.25	0.33
	4	660	S 09.22000° E 33.47000°	24.7	n.a.	6.1	5.2	79.8	0.38	2.14	0.81
	5	692	S 09.35699° E 33.79721°	24.4	n.a.	6.3	2.6	105.8	0.31	1.62	0.58
	6	826	S 09.35270° E 33.82296°	23.4	n.a.	5.9	5.8	205.7	0.44	1.62	0.36
	7	840	S 09.35150° E 33.82280°	23.3	n.a.	5.8	5.6	56.4	0.38	2.45	1.02
	8	869	S 09.33512° E 33.76300°	23.1	n.a.	5.9	5.2	28.7	0.50	1.83	1.05
	9	869	S 09.33008° E 33.75884°	23.1	n.a.	7.3	4.8	64.2	0.26	1.42	0.88
	10	869	S 09.33640° E 33.75590°	23.1	n.a.	6.2	7.1	17.9	0.32	1.68	1.09
	11	869	S 09.33008° E 33.75884°	23.1	n.a.	6.1	4.4	25.5	0.31	2.62	0.80
	12	994	S 09.33470° E 33.81031°	22.2	n.a.	6.4	3.6	191.4	0.33	2.32	0.64
	13	1092	S 09.32068° E 33.80958°	21.5	n.a.	6.1	2.3	141.3	0.36	2.18	1.02
	14	1211	S 09.30013° E 33.80783°	20.6	n.a.	5.7	8	96.8	0.44	2.65	1.28
	15	1374	S 09.28262° E 33.81349°	19.4	n.a.	5.6	6.3	116.0	0.48	2.28	0.56
	16	1550	S 09.26021° E 33.82095°	18.2	n.a.	5.5	4.9	260.0	0.44	2.71	1.14
	17	1702	S 09.35993° E 33.81637°	17.1	n.a.	5.7	2.9	226.9	0.41	3.20	1.16
	18	1846	S 09.22716° E 33.81249°	16.0	n.a.	5.6	3.2	111.3	0.49	3.54	1.16
	19	2020	S 09.02202° E 33.56576°	14.7	n.a.	5.9	2.5	580.4	0.47	2.91	1.18
	20	2055	S 09.02124° E 33.56478°	14.5	n.a.	6.2	5.3	458.1	0.44	2.97	1.13
	21	2080	S 09.07322° E 33.40782°	14.3	n.a.	6.3	0.8	1062.5	0.44	3.39	0.83
Mt. Rungwe (2016)	22	770	S 09.28966° E 33.90248°	23.9	n.a.	7.3	5.2	121.3	0.33	1.89	0.88
	23	870	S 09.33512° E 33.76305°	23.1	n.a.	6.7	2.7	270.7	0.49	1.39	0.45
	24	895	S 09.33646° E 33.75608°	22.9	n.a.	6.6	7.1	123.7	0.51	1.34	0.55
	25	923	S 09.33295° E 33.75195°	22.7	n.a.	6.7	3.6	193.1	0.80	1.27	0.64
	26	1723	S 09.12449° E 33.83101°	16.9	n.a.	5.8	3.3	79.7	0.48	3.46	1.23
	27	2580	S 09.03545° E 33.77150°	10.6	n.a.	6.9	16.7	52.4	0.55	4.84	1.62
	28	2797	S 09.02571° E 33.78635°	9.1	n.a.	5.7	9.3	121.6	0.53	5.73	1.50
Mt. Majella (2016)	29	400	N 42.08696° E 14.20067°	14.1	789	8.1	18.5	14.5	0.32	1.52	0.65
	30	800	N 42.08391° E 14.17626°	11.8	882	8.7	30.2	6.9	0.43	0.68	0.65
	31	1000	N 42.07850° E 14.16762°	10.6	929	7.7	18.3	103.9	0.19	3.61	2.46
	32	1200	N 42.07572° E 14.1628°	9.5	976	7.7	28.0	62.9	0.18	2.25	1.83
	33	1400	N 42.07427° E 14.15488°	8.3	1022	7.6	28.6	49.8	0.18	3.62	3.57
	34	1600	N 42.07617° E 14.14216°	7.2	1069	7.1	38.6	22.4	0.20	4.79	2.71
	35	1800	N 42.07965° E 14.13433°	6.0	1116	8.0	42.3	2.3	0.16	3.43	0.77
	36	2000	N 42.08598° E 14.12597°	4.8	1162	8.1	37.3	2.1	0.23	2.58	1.24
	37	2400	N 42.09066° E 14.09672°	2.5	1256	7.5	36.7	32.8	0.17	2.96	1.85
	38	2600	N 42.08882° E 14.08881°	1.4	1303	8.0	16.2	92.0	0.27	6.43	2.51
	39	2800	N 42.08780° E 14.0867°	0.2	1350	7.9	24.8	85.8	0.16	5.46	4.75

<sup>a</sup>Calculated based from climatic data available from 3 meteorological stations along Mt. Rungwe and Mt. Majella (cf. Section 2).

<sup>b</sup>Extrapolated from precipitation data available along Mt. Majella (cf. Supp. Table 1).

**Supplementary Table 1. Mean annual precipitation (MAP) recorded along Mt. Majella.**

Altitude (m)	MAP (mm/year)
325	813
650	779
767	973
1000	898
1251	928
1500	1035
2000	1163
2500	1291
2800	1368

Supplementary Table 2. Relative abundances (%) and total concentration ( $\mu\text{g/g}$  dry soil) of the normal, iso and anteiso 3-OH FAs in soils from Mt. Rungwe and Mt. Majella (n.d., not detected).

Campaign	Sampling month	Sample	Altitude	n-C10	iso-C11	anteiso-C11	n-C11	anteiso-C12	n-C12	iso-C13	anteiso-C13	n-C13	iso-C14	n-C14	iso-C15	anteiso-C15	n-C15	iso-C16	n-C16	iso-C17	anteiso-C17	n-C17	iso-C18	n-C18	Concentration ( $\mu\text{g/g}$ soil)	
Mt. Rungwe (2012)	April	1	520	2.73	0.48	0.08	0.19	0.16	6.44	3.97	0.58	0.57	1.12	19.79	5.20	2.11	1.11	1.08	21.31	9.48	1.26	3.85	0.75	17.76	15.43	
		2	520	0.44	n.d.	n.d.	n.d.	n.d.	4.68	2.31	0.29	0.35	0.86	27.68	5.71	2.03	1.01	1.77	19.38	10.59	1.34	1.69	1.05	18.81	1.16	
		3	640	3.97	0.95	0.04	0.23	0.22	7.06	3.64	1.33	0.77	1.02	19.64	5.84	1.62	1.30	0.99	19.74	12.65	1.15	3.44	0.99	13.43	21.34	
	December	4	660	2.09	0.52	0.02	0.19	0.16	5.50	3.35	0.53	0.64	1.14	22.19	6.51	2.17	1.02	1.32	22.55	11.34	1.30	1.61	0.89	14.98	4.15	
		5	692	2.28	0.92	0.05	0.22	0.15	5.74	3.18	0.97	0.63	1.11	21.71	8.14	1.99	1.23	1.62	20.10	12.12	1.55	2.66	1.17	12.46	2.75	
	December	6	826	3.31	0.64	0.09	0.26	0.18	7.10	4.14	0.66	0.71	1.12	19.92	5.23	2.07	1.27	1.07	20.53	9.34	1.32	3.69	0.62	16.75	11.93	
		7	840	5.96	1.33	0.07	0.40	0.30	10.14	4.80	1.39	0.82	1.49	31.41	7.79	2.98	1.22	1.28	22.34	12.90	1.63	1.60	1.37	16.61	3.16	
	April	8	869	2.02	0.93	0.05	0.34	n.d.	6.66	2.88	0.87	0.90	1.02	29.16	5.89	1.83	1.00	0.91	18.39	8.62	1.40	1.32	n.d.	15.80	1.49	
		9	869	1.80	0.71	0.08	0.20	0.37	4.74	2.46	2.16	0.67	1.25	21.17	8.56	1.92	1.35	1.86	20.03	13.25	1.83	2.09	1.18	12.32	3.08	
	April	10	869	1.95	0.55	0.09	0.20	0.23	5.97	3.65	0.63	1.21	1.42	22.91	7.42	2.27	1.35	1.63	19.18	12.07	1.62	1.48	0.76	13.40	1.12	
		11	869	0.84	0.50	0.03	0.19	0.34	5.27	3.55	1.03	0.72	1.77	29.00	9.30	3.27	1.25	1.35	19.22	8.70	1.05	1.31	1.99	9.33	6.89	
	April	12	994	2.00	0.52	0.04	0.21	0.26	6.61	4.02	0.93	0.64	1.47	20.98	6.67	2.58	1.11	1.31	20.69	11.92	1.24	1.95	0.70	14.13	6.89	
		13	1092	6.05	2.06	0.17	0.59	0.43	10.51	5.22	0.87	0.72	1.20	23.67	6.18	2.07	0.95	1.17	17.06	9.56	1.32	1.29	0.25	8.64	3.25	
	April	14	1211	8.91	0.80	0.04	0.36	0.23	8.88	2.89	1.59	0.74	1.20	24.46	5.51	2.32	0.87	1.10	15.78	7.56	1.68	1.31	1.27	12.09	7.74	
		15	1374	6.74	0.50	n.d.	0.25	0.59	8.22	5.07	1.71	0.79	1.75	21.28	4.07	2.20	0.96	1.02	18.88	6.24	1.04	1.86	0.71	16.13	7.31	
	April	16	1550	3.69	0.57	0.04	0.24	0.18	9.05	3.03	1.04	0.65	1.17	22.90	5.05	2.26	0.83	1.24	19.14	9.62	1.35	1.18	1.21	15.55	12.74	
		17	1702	4.53	0.70	0.07	0.26	0.21	8.70	3.33	0.69	0.55	1.31	20.85	6.05	2.83	0.89	1.06	17.64	9.89	1.50	1.29	0.48	17.18	6.58	
	April	18	1846	3.78	0.49	0.06	0.20	0.19	10.58	3.34	0.73	0.67	1.41	23.86	4.92	2.93	0.83	0.95	18.70	7.80	1.40	1.20	0.42	15.53	3.56	
		19	2020	3.59	0.39	0.05	0.22	0.12	9.51	3.81	0.65	0.63	1.31	23.52	5.67	2.70	0.93	0.93	19.33	7.82	1.40	1.19	0.53	15.70	14.51	
	April	20	2055	3.24	0.41	0.07	0.19	0.14	9.02	3.72	0.71	0.61	1.46	23.41	5.25	2.75	0.93	0.96	19.07	9.20	1.40	1.23	0.68	15.56	24.28	
		21	2080	2.01	0.22	0.03	0.16	0.15	6.41	2.59	1.46	0.68	1.43	19.76	4.75	3.41	1.01	1.06	20.19	9.20	1.46	1.76	0.83	21.41	8.50	
Mt. Rungwe (2016)	November	22	770	2.86	0.49	0.06	0.11	0.10	5.15	2.71	0.44	0.54	0.99	18.48	5.89	1.78	0.94	1.07	20.71	15.60	1.67	1.91	1.11	17.39	6.31	
		23	870	5.76	0.69	0.08	0.35	0.19	6.43	2.46	0.37	0.79	0.69	19.74	4.48	1.50	1.44	1.26	16.39	9.35	1.20	1.72	0.97	24.12	7.31	
		24	895	7.11	0.78	n.d.	n.d.	0.08	5.67	3.03	0.29	0.74	0.77	20.78	4.65	1.72	1.24	1.35	17.76	9.60	1.01	2.25	1.16	20.02	8.78	
		25	923	4.06	0.45	0.04	0.31	n.d.	5.90	1.78	0.03	0.84	0.70	18.29	3.11	1.11	0.87	0.13	13.74	5.80	0.51	0.80	0.12	41.43	6.95	
		26	1723	8.25	0.49	0.04	0.48	0.09	9.77	2.97	0.42	0.64	1.13	22.55	5.10	2.66	0.77	1.12	16.20	8.55	1.28	1.04	1.01	30.87	2.63	
		27	2580	15.63	0.42	n.d.	n.d.	0.17	8.04	2.36	0.40	0.93	1.58	24.00	3.84	2.67	0.55	1.19	14.93	6.70	1.30	0.80	1.24	13.25	8.75	
		28	2797	12.10	0.68	n.d.	n.d.	0.24	0.28	9.65	2.71	1.16	0.47	1.24	19.99	4.32	2.74	0.48	0.45	15.15	8.45	1.59	1.05	1.04	16.48	11.31
		December	29	400	0.95	0.14	n.d.	n.d.	0.29	3.55	2.79	0.78	2.21	1.11	20.21	6.54	2.63	1.26	4.24	20.32	8.97	2.23	2.81	5.56	13.40	2.69
	30		800	6.30	0.56	n.d.	n.d.	0.22	4.99	1.88	0.39	1.71	1.45	18.14	3.19	1.81	2.54	2.91	18.95	7.83	1.89	2.58	3.42	19.23	2.09	
	Mt. Majella (2016)	December	31	1000	4.03	0.81	n.d.	n.d.	0.36	8.83	4.30	0.90	0.59	1.83	19.08	6.71	3.21	0.92	2.35	16.07	14.52	3.17	1.29	1.12	9.84	19.01
32			1200	4.48	0.77	n.d.	n.d.	0.32	6.91	4.18	0.67	0.69	1.67	19.25	8.01	3.26	1.44	2.59	16.40	13.74	3.14	1.71	1.19	9.57	17.60	
33			1400	2.23	0.39	n.d.	n.d.	0.17	9.81	2.96	0.72	0.40	1.96	20.86	5.75	3.02	0.84	2.79	16.34	16.77	4.40	1.23	0.79	8.58	14.25	
34			1600	4.94	0.85	n.d.	n.d.	0.39	10.83	4.23	1.41	0.64	1.43	21.11	6.70	2.74	0.57	1.68	14.93	15.44	2.71	1.00	1.00	7.41	8.65	
35			1800	2.99	n.d.	n.d.	n.d.	n.d.	5.70	4.00	1.75	n.d.	1.62	24.03	9.71	3.03	0.88	2.79	16.74	9.56	2.40	3.10	6.12	5.57	0.98	
36			2000	n.d.	n.d.	n.d.	n.d.	n.d.	4.95	3.51	0.63	1.32	1.62	25.01	8.25	3.04	1.18	1.99	18.92	9.96	2.81	2.27	5.12	9.41	0.78	
37			2400	2.53	0.68	n.d.	n.d.	0.29	6.10	4.19	0.89	0.84	1.63	20.85	8.95	3.33	1.12	2.75	17.07	13.33	3.06	1.65	1.37	9.37	12.05	
38			2600	2.47	0.55	n.d.	n.d.	0.18	8.75	3.55	0.80	0.68	1.69	19.38	4.33	4.11	0.64	3.05	18.92	12.16	3.18	1.27	1.09	13.20	14.91	
39			2800	3.62	0.59	n.d.	n.d.	0.14	10.80	3.42	0.87	1.05	1.82	19.14	4.38	3.38	0.62	3.35	14.86	16.04	3.77	0.79	3.12	8.23	21.28	

**Supplementary Table 3. Indices derived from brGDGTs for soils from Mt. Majella and MAAT estima**

Altitude	MBT	MBT'5Me	CBT	MAAT estimate (°C)
400	0,21	0,62	0,27	10,9
800	0,15	0,56	0,54	9,0
1000	0,18	0,62	0,30	10,9
1200	0,16	0,55	0,30	8,8
1400	0,17	0,58	0,34	9,7
1600	0,20	0,48	0,61	6,6
1800	0,16	0,56	0,74	8,9
2000	0,17	0,54	0,34	8,5
2400	0,15	0,47	0,70	6,2
2600	0,20	0,47	0,21	6,2
2800	0,14	0,42	0,22	4,5

tes calculated using the global soil calibration by De Jonge et al. (2014).