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Thanh Thuy Nguyen Tu, Alix Vidal, Katell Quenea, Mercedes Mendez-Millan, Sylvie Derenne. Influence of earthworms on apolar lipidfeatures in soils after 1 year of incubation. *Biogeochemistry*, 2020, 147 (3), pp.243-258. 10.1007/s10533-020-00639-w . hal-02478790

HAL Id: hal-02478790

<https://hal.science/hal-02478790>

Submitted on 18 Feb 2020

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1 **Influence of earthworms on apolar lipid features in soils after one year of incubation**

2

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9

10 **Abstract**

11 Molecular and compound specific isotope compositions of apolar lipids were characterized in soil
12 mesocosms incubated for one year with or without ¹³C-labelled plant residues and earthworms, in
13 order to investigate, at the molecular scale, the effect of earthworms on the fate of organic matter
14 (OM) in soils. Molecular and isotope composition of long chain alkanes in casts confirmed that
15 earthworms preferentially ingest soil fractions rich in plant debris. Apolar lipid specific isotope
16 composition allowed calculation of the proportion of carbon derived from the labelled residues (C_{lab}).
17 Casts displayed higher C_{lab} values than surrounding soil while soil without earthworm exhibited
18 intermediate C_{lab} . The odd-over-even predominance (OEP) of alkanes suggested they are probably
19 less degraded in casts than in the surrounding soil. Taken together, OEP and C_{lab} values suggested
20 that besides high incorporation of plant residues, earthworms may also favor the preservation of
21 plant apolar lipids in their casts. Additionally, chain length and isotope pattern of alkanes further
22 suggested root lipids were probably less degraded than shoot lipids. High ¹³C-incorporation level for
23 the bacterial biomarker hopene provided evidence for intense recycling of plant OM and suggested
24 further contribution of bacterial necromass to soil OM.

25

26 **Keywords**

27 Earthworms, Organic matter, Lipids, ¹³C-labelling, Soil, Cast

28

29 **Introduction**

30 Soil organic matter (OM) is a key pool in the biogeochemical cycle of carbon. In the current
31 global change context, precisely documenting the processes leading to OM decomposition and
32 sequestration in soils is crucial as it may help in mitigating atmospheric CO₂ levels (Jobbágy and
33 Jackson 2000; Walthall et al. 2012). Soil ecosystem engineers, including earthworms, not only drive
34 soil fertility and structure, but also OM decomposition (Bossuyt et al. 2005). They can enhance OM
35 decomposition in soil but may also incorporate OM into their dejections (i.e. casts) that leads to its
36 stabilization (Lavelle & Martin 1992; Zangerlé et al. 2011; Vidal et al. 2017). Casts correspond to a
37 mixture of minerals and OM at different degradation stages, which are fragmented and complexed
38 with intestinal mucus thanks to transit in the earthworm gut (Lee 1985; Six et al. 2004). Bacterial
39 activity promoted by earthworms in casts favors OM decomposition at the short-time scale (Brown
40 et al. 2000; Vidal et al. 2016a). Conversely, at the month or year scale OM may be stabilized in drying
41 casts (Brown et al. 2000; Martin 1991). Mechanisms behind OM decomposition in the presence of
42 earthworms have often been often explored at the short-term, while factors controlling OM
43 decomposition and stabilization at the larger time scale remain unclear. Indeed, recent studies have
44 highlighted the lack of experiments at larger time scales (> 200 days) to depict the impact of
45 earthworm of OM decomposition and carbon cycling (Lubbers et al. 2013; Angst et al. 2017).

46 So far, the influence of earthworms on OM decomposition in soils was mainly studied at the
47 bulk OM scale (Lavelle et al. 1998; Jégou et al. 2000; Six et al. 2004; Fonte et al. 2012; Stromberger et
48 al. 2012; Fahey et al. 2013). However, soil OM corresponds to a complex mixture of molecules with
49 diverse chemical structures and contrasted decomposition rate (Alexander 1981; Kelleher & Simpson
50 2006). Few authors have investigated the chemical modifications induced by earthworms on soil and
51 cast OM (Guggenberger et al. 1996; Filley et al. 2008; Crow et al. 2009; Hong et al. 2011; Frouz et al.
52 2015; Vidal et al. 2016a; Angst et al. 2017). These studies have notably pointed out: (i) the

53 predominance of microbial- and lignin-derived compounds in casts when compared to soil
54 (Guggenberger et al. 1996; Frouz et al. 2015;) and (ii) contrasted molecular evolution of shoot and
55 root residues in casts (Vidal et al. 2016a). Enhanced OM physical protection was also identified as a
56 major driving parameter for OM stabilization in casts (Angst et al. 2017). Incorporation and
57 decomposition of OM is a dynamic process that involves both newly incorporated OM and
58 indigenous OM. Differentiating these two pools of OM is also a main challenge in the study of carbon
59 cycling in soils and casts. For this purpose, natural ^{13}C -labelling is a powerful tool to monitor the fate
60 and dynamics of OM in soils (Mariotti & Balesdent 1990; Boutton 1996). Applied to the compound
61 specific level, ^{13}C -signatures in soils undergoing C_3/C_4 vegetation successions have shown that fatty
62 lipids, sugars, as well as lignin, cutin and suberin monomers have contrasting turnover times
63 (Wiesenberg et al. 2004; Bahri et al. 2006; Derrien et al. 2006; Mendez-Millan et al. 2010).

64 The present work aims at studying the influence of earthworms on the dynamics of
65 molecular constituents in soils. Lipids being of prime importance for soil fertility, water retention and
66 aggregate stability (Jambu et al. 1978; Dinel & Schnitzer 1990), the present study is focused on the
67 fate of these hydrophobic compounds. Lipids further include biomarkers typical of the different
68 sources of soil OM. This is notably the case of apolar lipids that generally contain long chain odd
69 alkanes that are characteristics of plant-derived OM and hopanoids indicative of bacterial OM
70 (Eglinton & Hamilton, 1967; Peters et al. 2005). Apolar lipids may also exhibit a higher preservation
71 potential than other lipidic molecules, especially compared with those comprising hydroxylated
72 chemical functions such as fatty acids or cutins and suberins (Gonzalez-Vila, 1995; Schmidt et al.
73 2011). Besides, specific lipid ^{13}C -measurements from natural or artificial labelling field experiments
74 were proven valuable for evidencing complex decomposition dynamics in soil OM or revealing fungal
75 contribution to its apolar lipid pool (Quénéa et al. 2006; Nguyen Tu et al. 2011; Mendez-Millan et al.
76 2014). The present study is based on a soil mesocosm experiment in which ^{13}C -labelled plant residues
77 were incubated in the presence or absence of earthworms. Shoot and root residues are the two key
78 sources of plant OM in soils. They differ in decomposition rate and palatability, probably due to

79 several factors including differences in chemical composition (Rasse et al. 2005). To selectively
80 address this effect, root and shoot residues were added onto distinct mesocosms. The anecic
81 earthworm *Lumbricus terrestris* was chosen for its ecological characteristics: it feeds on surface litter
82 and transfers it along vertical burrows eventually depositing its organo-mineral casts on burrow walls
83 and soil surface (Lee 1985; Jégou et al. 2001).

84 The experimental design allowed a first approach in investigating the influence of
85 earthworms on (apolar) lipids after one year of incubation. The aims of the study were notably to (1)
86 characterize apolar lipids in soil vs. casts and to (2) depict the contribution of shoot- and root-
87 residues to soil vs. casts apolar lipids, after one year of experiment. Distinguishing plant biomarkers
88 from microbial biomarkers, this work further aims at characterizing the transfer of plant-derived
89 carbon into microbial biomass in the presence or absence of earthworms.

90

91 **Material and methods**

92 *Experimental setup*

93 The samples used in the present study were collected from the mesocosm experiment
94 described in Vidal et al. (2017). Briefly, six containers were filled with approximately 75 L of a 4
95 mm-sieved loamy-sand soil, collected from permanent grassland in North of France (Oise, France).
96 Six *L. terrestris* earthworms per container were deposited onto the surface of three of the containers.
97 The amount of earthworms and plant residues deposited on the soil, as well as mesocosm size were
98 designed so as to optimize earthworm survival in condition closest to reality (Vidal et al. 2017). Plants
99 of Italian Ryegrass (*Lolium multiflorum*) were artificially labelled with ^{13}C at the *Commissariat à*
100 *l'Energie Atomique et aux Energies Alternatives* (CEA) in Cadarache (France). After three months of
101 growth under $^{13}\text{CO}_2$ enriched atmosphere, the mean $\delta^{13}\text{C}$ values were 1324‰ (± 43) and 1632‰
102 (± 16) for the roots and shoots, respectively (Vidal et al. 2017). Shoots and roots were separated,
103 dried and subsequently mixed and homogenized during 40 s. with a laboratory blender (Waring
104 Commercial) to obtain few millimeter sticks. 250 g of plant residue per container were deposited on

105 the soil surface of four containers: two with root residues and two with shoot residues. The last two
106 containers represent the mesocosm controls without plant residue, including one with earthworms.
107 The containers were then covered with coarse synthetic canvas to prevent earthworm escape and
108 cross contamination by labelled residues. The mesocosms were placed in a greenhouse where soil
109 humidity and temperature were maintained at 23% and 13°C, respectively.

110 Weekly throughout the incubation, each container was vaporized with water (1 L/week) and
111 seedlings or mosses developing in the mesocosms were manually eliminated. At the end of the
112 experiment, no more residues were observed on the soil surface in the mesocosms containing
113 earthworms, contrary to those without earthworm which exhibited remnant residues (ca. 30% of
114 initial weight; Vidal et al. 2017). After one year, several samples representative of each mesocosm
115 were collected, and pooled together to obtain a composite sample: (a) three soil cores (2.5 cm
116 diameter) were collected to a depth of 20 cm with an aluminum auger and (b) few grams of 3–4
117 earthworm cast fragments were randomly collected on the soil surface of earthworm containing
118 mesocosms. Although mesocosm size did not allow replication, these composite samples provide an
119 averaged estimation of the processes affecting plant lipids in the presence of earthworms. Samples
120 were freeze-dried and ground prior to analyses. All in all, two undegraded plant residues, six soil and
121 three cast samples were prepared for total lipid and lipid specific isotope analyses (Table 1). Bulk
122 isotope compositions of the samples were reported previously (Vidal et al. 2017).

123

124 *Analyses*

125 Depending on the amount of organic carbon in the samples, between 2 g for plant samples
126 and 20 g for soil and cast samples were used for lipid extraction with a solvent mixture of
127 dichloromethane (CH₂Cl₂):methanol (CH₃OH) (2:1, v:v) in an accelerated solvent extractor (ASE 100
128 Dionex) at 60 °C and 10*10⁶ Pa for 10 min. in two cycles. The combined extracts were concentrated
129 by rotary evaporation, dried under N₂ for weighing and further redissolved in 200 µL of CH₂Cl₂. An
130 aliquote of the total lipid extracts (TLE) was silylated with O-Bis(trimethylsilyl)trifluoroacetamide

131 (BSTFA) (ca. 10% of the CH₂Cl₂ volume) prior to GC-MS analyses for molecular identification. For lipid
132 specific isotope analyses, the TLE was fractionated using column chromatography on alumina (1 g for
133 10 mg of dried extract, Sigma-Aldrich 507C ~150 mesh). Apolar lipids were recovered after elution
134 with a solvent mixture (4 mL for 1 g of alumina) of heptane (C₇H₁₆):CH₂Cl₂ (99:1, V:V). The apolar
135 fraction was then concentrated by rotary evaporation and further dissolved in 3 mL of heptane.

136 The TLE was analyzed with an Agilent 6890N gas chromatograph (GC) coupled with an Agilent
137 5973N mass spectrometer (MS). The GC was fitted with a Restek RTX-5Sil-MS column (30 m × 0.25
138 mm i.d., 0.5 μm film thickness) under constant helium flow of 1 mL/min. Samples were injected (1
139 μL) in splitless mode with the injector temperature at 280°C. GC operating conditions were as
140 follows: initial temperature hold at 80°C for 30 s, then increased from 80 to 100°C at 10°C/min and
141 from 100 to 320°C at 4°C/min with a final isothermal hold at 320°C for 30 min. The temperature of
142 the transfer line to the MS was 320°C and the analytes were ionized by 70 eV electron impact at
143 220°C. The quadrupole was operated in scan mode (35-700 Da) at 120°C. Components were
144 identified according to retention times and mass spectra fragmentation patterns.

145 Compound-specific stable carbon isotope composition of apolar lipids were measured using
146 an online continuous flow gas chromatograph Trace-GC-Ultra coupled with an Isotopic Ratio Mass
147 Spectrometer Delta V Plus via a combustion furnace and a conflow IV interface from Thermo Fischer
148 Scientific. The GC was equipped with a split/splitless injector, kept at 320°C, and fitted with a 30 m
149 fused silica capillary column (TR-5MS, 0.25 mm i. d., 0.25 μm film thickness). The GC oven program
150 started at 80°C during 2 min., increased from 80 to 120°C at 10°C/min and from 120°C to 320°C at
151 5°C/min. Finally the oven was kept at 320°C during 5 min. Helium was used as carrier gas at constant
152 flow of 2mL/min. Accuracy and reproducibility of the GC-C-IRMS measurements were assessed with a
153 standard mixture of *n*-alkanes (*n*-C₂₀, *n*-C₂₅, *n*-C₃₀ and *n*-C₃₂) of known isotopic composition
154 (Schimmelmann, Indiana University). The standard mixture was injected three times at the beginning
155 of each run and then once every three samples. Analytical standard deviation for each run was

156 <0.5‰ (n=6). The $\delta^{13}\text{C}$ are expressed in ‰ against the international standard VPDB (Vienna Pee Dee
157 Belemnite).

158

159 **Results and discussion**

160 *Molecular composition of TLE*

161 Lipids account for less than 0.1 wt% of soil and cast samples while their content reaches
162 3.9 wt% and 17.2 wt% in root and shoot residues, respectively (Table 1). These lipid yields fall in the
163 range of literature, although higher than that previously reported for *L. multiflorum* (Nguyen Tu et al.
164 2003, Göcke et al. 2013, Müller et al. 2013). Five major families of compounds are identified in both
165 root and shoot residues: *n*-alkanes, *n*-alkanoic acids, *n*-alkanedioic acids, *n*-alkanols and sterols
166 (Figure 1). These compounds are typical plant-derived lipids (van Bergen et al. 1998; Bull et al. 2000;
167 Wiesenberg et al. 2010). Additionally, root TLE exhibits some lignin-derived compounds with *p*-
168 coumaryl, guaiacyl and syringyl structures, as well as phenol. The main differences between root and
169 shoot lipids stand in the presence of these aromatic compounds, as well as the higher relative
170 abundance of long chain *n*-alkanoic acids (> C₂₀) and sterols in root residues, while shoot residues
171 presented a higher relative abundance of C₂₆ *n*-alkanol (Fig.1).

172 The reference soil TLE (Soil-NE-control; Fig. 2.a) is dominated by C₁₂ to C₃₂ *n*-alkanols
173 maximizing at C₂₆, with an even-over-odd carbon-number predominance. Long chain *n*-alkanols (>
174 C₂₀) typically derive from shoot waxes of terrestrial plants (van Bergen et al. 1998; Bull et al. 2000;
175 Quénéa et al. 2004; Nierop et al. 2006; Quénéa et al. 2006) and are dominant constituents (C₂₆) of
176 shoot residue extract (Fig. 1). *n*-Alkanols can represent an important contribution to the OM
177 stabilized in soils (Mueller et al. 2012) and are essential indicators of past plant types (Jansen et al.
178 2008). For example, Nierop et al. (2006) found C₂₆ *n*-alkanol to be, by far, the dominant compound of
179 lipid extracted from a pine forest soil. As pine biomass did not contain this *n*-alkanol, the authors
180 suggested that the soil was probably covered by grass vegetation before pine trees were planted. *n*-
181 Alkanoic acids also occur in substantial amounts in the reference soil, ranging from C₉ to C₃₂, with a

182 strong even-over-odd carbon-number predominance and a maximum at C₁₆, as frequently reported
183 (Jandl et al. 2005; Wiesenberg et al. 2010). While C₁₆ and C₁₈ *n*-alkanoic acids are ubiquitous in living
184 organisms, C₂₀-C₂₆ *n*-alkanoic acids are related to vegetal biomass in soils (Wiesenberg et al. 2004).
185 The presence of trace amounts of iso- and anteiso-branched C₁₅ *n*-alkanoic acids points to a bacterial
186 contribution to soil lipids (Wannigama et al. 1981; Quénéa et al. 2012). C₁₆ to C₃₃ *n*-alkanes,
187 maximizing at C₂₉ are identified in the soil, with a strong odd-over-even preference, as commonly
188 observed (Lichtfouse et al. 1998; Bull et al. 2000; Wiesenberg et al. 2010). C₂₂, C₂₄ and C₂₆
189 ω -hydroxyalkanoic acids are also identified in the soil and can originate from the *in situ*
190 decomposition of cutin and suberin polymers (Quénéa et al. 2012). Taken together, the above data
191 show that most compounds identified in the reference soil TLE derive from plant biomass and more
192 specifically from grass plant. This is in agreement with the > 50 year permanent pasture land use of
193 the soil collected for the experiment (Vidal et al. 2016b).

194 The cast control extract is almost identical to the soil control extract (Fig. 2.b), with the
195 exception of two isomers of a long chain unsaturated *n*-alkanoic acid: the arachidonic acid (C_{20:4}) (Fig.
196 2). Arachidonic acid is a polyunsaturated *n*-alkanoic acid derived from eukaryotic organisms
197 (Harwood and Russel 1984). Studies illustrating the link between this acid and earthworms are
198 scarce. However, Schaefer et al. (2005) reported its significant increase in the presence of
199 earthworms in an oil-contaminated soil. It was also identified in earthworm tissues (Albro et al. 1992)
200 or in its gut content (Sampedro & Whalen 2007). Thus, in the present study, the arachidonic acid
201 identified in earthworm casts probably originates from (1) the earthworms themselves (i.e. their
202 tissue or gut content) and/or (2) microorganisms stimulated by earthworms.

203 Differences observed in TLE of roots and shoots (Fig. 1) are no longer visible in cast and soil
204 samples after 54 weeks of experiment (data not shown). Important differences are not necessarily
205 observed in the lipid composition of soils underlying different plant species and organs (Nierop et al.
206 2006; Mueller et al. 2012). The distortion between plant and soil lipid composition could be
207 explained by the strong legacy from other plant inputs in the soil (Nierop et al. 2006) and diverging

208 affinity of lipids from different origins with soil mineral phase (Lutzow et al. 2006). Moreover, lipids
209 might also be transformed in other compound classes over time. For example, *n*-alkanols might be
210 oxidized into *n*-alkanoic acids during decomposition (Amblès et al. 1994). The reference soil and cast
211 chromatograms highlighted a strong contribution from plant-derived compounds in the total lipid
212 extracts. After 54 weeks of experiment, the legacy from past plant inputs seems to hide the potential
213 differences in free lipids between the samples collected from mesocosms containing different
214 residue type. Detailed molecular and isotopic investigations of apolar lipids were thus achieved to
215 better document the fate of plant lipids in the presence or absence of earthworm.

216

217 *Apolar lipid distribution*

218 After one year of incubation, apolar lipids of all samples are dominated by *n*-alkanes that
219 correspond to ca. 2% and 10% of TLE for plant residue and soil samples, respectively (Table 1). These
220 bulk proportions are in agreement with those generally reported for plants and soils (Quénéa et al.
221 2004; Nguyen Tu et al. 2011; Srivastava & Wiesenberg 2018). The distribution of apolar lipids is
222 shown as histograms in Fig. 3. The reference soil without plant residue and without earthworm (soil-
223 NE-Control) shows a bimodal distribution of C₁₆-C₃₁ alkanes maximizing at C₂₉ and submaximizing at
224 C₂₀ (Fig. 3a). While long chain (> C₂₃) homologues with odd carbon number predominance mainly
225 correspond to plant contribution, shorter chain homologues with no carbon number predominance
226 are generally attributed to mixed sources including soil microbes, such as bacteria and fungi
227 (Kolattukudy et al. 1976; Jansen et al. 2006; Li et al. 2017). In addition, two pentacyclic triterpenes
228 are identified at the end of the chromatogram (Online Resource 1): a hopene [hop-22(29)-ene] and a
229 triterpenone [amyrenone]; they originate from bacteria and plants (Angiosperm), respectively
230 (Ourisson et al. 1979; Otto et al. 2005; Peters et al. 2005). However, these two compounds are not
231 detected in the apolar lipids of plant residues, which are exclusively composed of *n*-alkanes. The
232 triterpenone (amyrenone) is also a by-product the degradation/oxidation of the corresponding
233 triterpenol (amyrin). Its absence from initial plant residues is likely related to the undegraded stage

234 of this plant material. The absence of bacterial biomarker from plant lipids is likely due to the
235 absence of significant bacterial population on the plant residues. While shoot alkanes exhibit a
236 unimodal distribution markedly dominated by the C₂₉ homologue (Fig. 3c), that of root is bimodal
237 (maximum C₂₇, submaximum C₂₁; Fig. 3f). Difference in alkane distribution is a common pattern for
238 roots and shoots from a given species (Jansen et al. 2006; Huang et al. 2011), including *L. multiflorum*
239 (Gocke et al. 2013). Alkanes extracted from shoots of italian ryegrass were previously reported to
240 maximize at C₃₁ (Gocke et al. 2013) or C₂₉ (Malossini et al. 1990), this difference being probably
241 related to variations in developmental and/or environmental parameters (El-Otmani & Coggins 1985;
242 Gölz et al. 1991; Li et al. 2016).

243 Alkanes in the soils incubated in the presence of earthworms and/or plant residues (Fig. 3b,
244 3d, 3e, 3g and 3h) all exhibit a bimodal distribution as in the reference soil (Fig. 3a), although some
245 differences can be noted in the relative abundance of the two modes. In contrast, cast *n*-alkanes are
246 all dominated by odd chain long alkanes maximizing at C₂₉ (Fig. 3b, 3e and 3h), even in the mesocosm
247 without plant addition at the beginning of the experiment (cast-control). This distribution is typical of
248 plant alkanes and consistent with the feeding habits of earthworms that preferentially ingest plant
249 OM. The high abundance of the C₃₁ homologue in cast-root is unexpected given its low abundance in
250 both initial soil and root residue. In the cast-control, the plant signature displays that earthworms
251 select soil fractions rich in plant OM (Curry & Schmidt, 2007; Vidal et al. 2016b). In the presence of
252 plant residue (cast-root, cast-shoot), the plant signature of alkanes and the occurrence of
253 triterpenone reflect the ingestion of both OM initially present in soil, and plant residues deposited on
254 top of the mesocosms at the beginning of the experiment. In the same way, the hopene detected in
255 the casts of these mesocosms (cast-root, cast-shoot) may correspond to both hopene initially present
256 in the soil and that synthesized by earthworm gut microbiota. Indeed, previous analyses of faeces
257 from millipedes grown exclusively in the presence of plant leaves revealed the presence of hopene
258 while it was absent from the leaves, thus evidencing bacterial activity associated with gut transit
259 (Rawlins et al. 2006).

260 As indicative of plant material degradation, the odd-over-even predominance (OEP) was
261 calculated for *n*-alkanes (Hoefs et al. 2002):
262 $OEP = [n-C_{27} + n-C_{29} + n-C_{31} + n-C_{33}] / [n-C_{26} + n-C_{28} + n-C_{30} + n-C_{32}]$.
263 Root and shoot residues exhibit OEP slightly higher than 5 (Table 1), as expected for undegraded
264 plant material (Zech et al. 2009; Schaefer et al. 2016). In soils without earthworm, OEP is typical of
265 topsoils (<5; Table 1), the relatively low value for soil-NE-shoot (4.0) being likely related to a priming
266 effect due to the deposition of nutrient-rich shoot residue at the beginning of the experiment. The
267 priming effect consists of enhanced microbial degradation of native soil OM, induced by an input of
268 fresh OM (Fontaine et al. 2004; Kuzyakov 2010). In the mesocosms with earthworms and with shoot
269 residues or without plant residues, soils and casts display OEP <4 and >5, respectively (Table 1).
270 According to literature (Buggle et al. 2010; Zech et al. 2009), these OEP suggest alkanes underwent
271 some degradation in soils while they are less degraded in casts. This finding may be related to both
272 higher incorporation and better preservation of plant residues in casts. Soil and cast from the
273 mesocosm with root residues and earthworms exhibit very high OEP values (>>5) that are higher
274 than the corresponding sample in shoot mesocosm (Table 1). These high OEP may be related to (1)
275 the unexpectedly high abundance of the C₃₁ homologues in these samples, combined with (2) lower
276 decomposition of root alkanes when compared with shoot alkanes.

277

278 *Lipid specific isotope composition*

279 The specific isotope composition of apolar lipids is shown in Fig. 4 and detailed in Online
280 Resource 2. Lipids of the reference soil without plant residue and without earthworm (soil-NE-
281 control) exhibit $\delta^{13}C$ values varying between -36‰ and -28‰ (Fig. 4a), in agreement with literature
282 on soil lipids (Huang et al. 1996; Chikaraishi & Naraoka 2006; Mendez-Millan et al. 2014; Feakins et
283 al. 2018). While apolar lipids of the casts from the mesocosm with earthworm but without plant
284 residue (cast-control) display specific isotope compositions similar to the reference soil (-36‰ to -
285 33‰, soil-NE-control, Fig. 4b), that of the underlying soil (soil-E-control) appear ¹³C-enriched with

286 respect to the reference soil (-34‰ to -9‰, Fig. 4b). This ¹³C-enrichment is the highest for the C₂₁-C₂₇
287 alkanes (+11‰ to +19‰ with respect to the reference soil). As no ¹³C-labelled residue was applied to
288 this mesocosm, this pattern is probably related to earthworm activity, based on the following two
289 facts. First, the ¹³C-enrichment of soil alkanes may result, at least partially, from the preferential
290 ingestion of soil fractions rich in plant OM that is naturally ¹³C-depleted with respect to bulk soil, thus
291 leading to apparent ¹³C-enrichment of the remaining alkanes. Although, undetectable at bulk OM
292 level (Vidal et al. 2017), such enrichment, would be especially noticeable at specific alkane level, as
293 plant lipids are the main source of long-chain alkanes in soil. Secondly, the ¹³C-enrichment of soil
294 alkane in Soil-E-Control when compared with Soil-NE-Control may be indirectly related to the
295 production of carbonate granules in earthworm specialized glands (Dotterweich 1933; Versteegh et
296 al. 2014). Indeed, the carbon of the granules comes partly from atmospheric CO₂ that is ¹³C-enriched
297 with respect to OM (Canti 2009), and C₁₃-C₃₈ *n*-alkanes were previously reported in *L. terrestris* lipids
298 (Nooner et al. 1973; Stefanovic & Djurdjic 1976).

299 In undegraded plant residues, alkanes displayed very different $\delta^{13}\text{C}$ values according to chain
300 length: specific alkane isotope compositions range between +21‰ and +1502‰ in shoots, and
301 between +679‰ and +1672‰ in roots (Fig. 4c and 4f). As previously reported at the bulk scale for
302 ¹³C-labelled leaves (Nguyen Tu et al. 2013), this $\delta^{13}\text{C}$ range is much wider than that generally reported
303 for natural plant alkanes (ca. $\leq 10\%$). Such a wide range probably results from large variations in the
304 ¹³C-content of the growth chamber, which are common during plant labeling experiments (Nguyen
305 Tu et al. 2013; Soong et al. 2014). In addition, this variability was also probably driven by the natural
306 variability of isotope fractionation processes within a given plant, such as that due to organ position
307 (Waring & Silvester 1994; Lockheart et al. 1997), and/or by the heterogeneity of stomatal aperture
308 distribution naturally occurring in leaves (i.e. stomatal patchiness; Pospíšilová & Šantrůček 1994;
309 Beyschag & Eckstein 1997). Accordingly, the following discussion is mainly based on comparisons of
310 isotope compositions of a given homologue for a mesocosm and that of the relevant undegraded
311 plant residue.

312 Soil alkanes from the mesocosms with plant residue but without earthworm exhibit specific
313 isotope composition varying between -33‰ and +25‰ with shoots, and between -31‰ and +85‰
314 with roots (Fig. 4d and 4g). Alkane specific isotope compositions thus clearly point to incorporation,
315 in soil, of the carbon derived from labelled plant residue. The hopene and the triterpenone also
316 depict some incorporation of labelled carbon with specific $\delta^{13}\text{C}$ values ranging from +25‰ to +230‰
317 (Fig. 4d and 4g). Incorporation of ^{13}C in the soil triterpenone is likely due to the degradation of ^{13}C -
318 labelled plant triterpenol and subsequent contribution of its degradation product to the soil
319 triterpenone pool. In both mesocosms with labelled plant residue and without earthworm, hopene is
320 the most labelled lipid, showing that carbon from degrading plants is assimilated by soil bacteria.

321 Soil lipids from the mesocosms with labelled plant residues and earthworms display ^{13}C -
322 enrichment similar to that of mesocosms without earthworms: their specific isotope compositions
323 vary between -37‰ and +10‰ for the shoot mesocosm, and between -32‰ and +72‰ for the root
324 mesocosm (Fig. 4e and 4h). Here again, compound specific isotope measurements point to
325 incorporation of the labelled carbon in soils. Cast lipids appear even more ^{13}C -labelled than the
326 corresponding soil with specific $\delta^{13}\text{C}$ values ranging from -31‰ to +426‰ for the shoot mesocosm,
327 and from -18‰ to +501‰ for the root mesocosm (Fig. 4e and 4h). These results thus provide isotope
328 evidence for the preferential incorporation of fresh plant residues in earthworm casts and for their
329 persistence after one year of incubation.

330

331 *Carbon derived from the labelled plant residues*

332 Differences in the ^{13}C -content of shoot and root residues precludes advanced conclusions
333 based on direct comparison of $\delta^{13}\text{C}$ values of the considered mesocosms. Hence, the proportion of C
334 derived from the labelled plant residues in the soil or casts lipids (C_{lab}), was estimated, as a first
335 approximation, according to the simplified equation (Balesdent & Mariotti 1996):

$$336 \quad C_{\text{lab}}(\%) = [(\delta_s - \delta_c) / (\delta_r - \delta_c)] \times 100;$$

337 where δ_s is the $\delta^{13}\text{C}$ value of soil or cast samples with labelled residue, δ_c is the $\delta^{13}\text{C}$ value of the

338 control soil or cast sample without plant residue and δ_i is the $\delta^{13}\text{C}$ value of the labelled residue. As
339 the pentacyclic triterpenes are absent from the plant residue, the denominator was approximated
340 to:

341 - the mean alkane denominator for the triterpenone that is a typical plant lipid, similarly to medium-
342 and long-chain alkanes;

343 - the bulk sample denominator for the hopene, as this bacterial biomarker is synthesized from non-
344 linear molecules.

345 C_{lab} appears variable among apolar lipids, varying from -6% to +30% for a mean value of +7%
346 (Fig. 5 and Online Resource 2 for detailed data of alkanes). The few unusual negative values
347 correspond to the less abundant alkanes (C_{23} and C_{25}) of the soil of the mesocosm with earthworms
348 and shoot residue (soil-E-shoot). They are likely due, to the relative ^{13}C -enrichment of the soil-E-
349 control with respect to soil-NE-control (denominator of the equation). In addition, the negative C_{lab}
350 values also probably reflect extensive degradation of these alkanes, especially those from the ^{13}C -
351 labelled residues, as shoot biomass degrades relatively quickly (Rasse et al. 2005). Most of the C_{lab}
352 values calculated for apolar lipids are positive, evidencing incorporation of carbon derived from the
353 labelled plant residues in the lipid pool. They fall in the range of that previously found on bulk
354 samples from the same experiment (1-13%, Vidal et al. 2017). At first glance, this suggests that
355 apolar lipids behave similarly to bulk OM, in spite of the relative recalcitrance of their chemical
356 structure (i.e. due to aliphaticity, polycyclic nature or no or few functional groups prone to degradation,
357 such as hydroxyl and carboxyl groups). However, lipid C_{lab} varies in a wider range than that of bulk
358 samples, with different pattern according to their relative abundance and chemical structure, as well
359 as the presence of earthworm and/or plant residue in mesocosms.

360 Whatever plant residue type and apolar lipid constituent, the proportion of C derived from
361 the labelled residue tends to follow the same pattern according to sample type (Fig. 5):
362 $C_{\text{lab}}(\text{cast}) \geq C_{\text{lab}}(\text{soil without earthworm}) \geq C_{\text{lab}}(\text{soil with earthworm})$. According to the feeding ecology
363 of *L. terrestris*, more labelled carbon is incorporated in its casts than in the underlying soil. The

364 proportion of C derived from the labelled residue results from the balance between residue
365 incorporation and its degradation in the sample. Previous chemical and microscopic characterizations
366 of earthworm casts from the same experiment showed that they underwent two distinct
367 transformation phases (Vidal et al. 2016a; 2019): (1) a phase dominated by plant residue
368 incorporation during the first two months and then (2) a phase marked by decomposition of plant
369 OM and formation of organomineral associations. Additionally, above OEP calculation showed that
370 alkanes were less degraded in casts than in the surrounding soils. Taken together, these results
371 suggest that besides higher incorporation of plant residues, earthworms may also induce a better
372 preservation of plant alkanes in their casts than in soils at the longer term. In the same way, the
373 intermediate ^{13}C -incorporation level of apolar lipids in the soils without earthworm may suggest
374 lower decomposition of ^{13}C -residues when compared with the mesocosm with earthworm, in
375 agreement with previous conclusions on bulk OM (Vidal et al. 2017).

376 In the soils without earthworm, apolar lipid C_{lab} is higher for the mesocosm with root
377 residues than for that with shoot residues (Fig. 5). This is also the case in soils with earthworms,
378 although to a lesser extent. Combined with higher OEP for root vs shoot mesocosms, these findings
379 may further suggest that root lipids are less degraded than shoot lipids, as previously found on bulk
380 OM from the same experiment (Vidal et al. 2017). The difference, in the molecular structure of lipids,
381 between root and shoot residues is too small to account for such contrasted C_{lab} . More likely, such a
382 better preservation for root lipids may be due to the better preservation of bulk root tissues, in
383 agreement with the higher recalcitrance of root tissues vs shoot tissues, often mentioned in
384 literature (Kögel-Knabner 2002; Shi et al. 2013). Additionally, it may be due to the lower palatability
385 of roots vs shoots, the latter being generally favored by earthworms for feeding (Bouché and
386 Kretzschmar 1974; Cortez 1998; Curry and Schmidt 2007). In any case, it should be noted that in the
387 present study, the intrinsic properties of plant residues is probably the main responsible for this
388 difference, and not their availability/accessibility as often mentioned to explain root vs. shoot
389 preservation difference. Indeed, both plant residues were introduced in the mesocosms in the same

390 way, i.e. deposited on top. Conversely, no marked difference in cast C_{lab} can be evidenced between
391 root and shoot mesocosms suggesting that plant OM assimilation by earthworms smooths the
392 difference between both residue types, as previously found on bulk OM from the same experiment
393 (Vidal et al. 2017).

394 Within a given sample, C_{lab} is maximal for either C_{29} alkane or hopene, and minimal for the
395 less abundant alkane measured (Online Resource 3). Proportionality between alkane abundance and
396 C_{lab} is likely related to mass balance. High C_{lab} for hopene confirms intense assimilation of ^{13}C -labelled
397 carbon by soil bacteria, which activity was probably stimulated by the deposition of undegraded ^{13}C -
398 residues onto the mesocosms at the beginning of the experiment. These results obtained from 20 g
399 of soil or cast samples, also substantiate observations by nanoSIMS of ^{13}C -labelled bacteria on few μg
400 of samples from same mesocosms (Vidal et al. 2016b, 2019). High incorporation of ^{13}C -labelled
401 carbon in this bacterial biomarker further provides molecular evidence that microorganisms are key
402 recyclers of plant OM in soils. As a result, this one-year experiment support bacteria and other micro-
403 organisms as substantial source of OM in soil as acknowledged for a few years (Schaeffer et al. 2015;
404 Kögel-Knabner 2017). Hopene corresponds, at least partly, to the degradation product of more
405 functionalized hopanoids (Innes et al. 1997; Peters et al. 2005) and several bacterial remains were
406 identified through microscope examination of the present samples (Vidal et al. 2019). Hence, hopene
407 is probably typical of both bacterial living biomass and necromass, at least for the present
408 mesocosms. As OM degradation is the dominant process in the mesocosms after one-year incubation
409 (Vidal et al. 2016a, 2019), higher C_{lab} for hopene than alkanes further suggests less degradation and
410 better preservation of bacterial living biomass and necromass than initial plant OM. In other words,
411 these results tend to support that on the year-scale, organic carbon may be efficiently sequestered
412 in soils as bacterial necromass (Liang and Balsler 2011; Miltner et al. 2012).

413

414 **Conclusion**

415 In the present study, we investigated the impact of earthworms on soil lipid composition, in
416 the presence of root or shoot residues, after one year of incubation. In soils and casts, total lipid
417 extracts were dominated by markers from past plant inputs, hiding the signal from recently
418 incorporated OM. Except for the presence of arachidonic acid in casts while it was absent in soil, we
419 depicted no impact of earthworms and plant residue type on soil total lipid extracts. However, the
420 use of the molecular and compound-specific isotope composition of apolar lipids revealed
421 differences between samples. Higher odd-over-even predominance and proportion of carbon
422 derived from the labelled residues in cast alkanes with respect to soil suggested higher incorporation
423 of plant residues and/or enhanced preservation of plant apolar lipids in earthworm casts.

424 The high level of plant-derived carbon in casts was associated with a high ^{13}C -incorporation level for
425 the bacterial biomarker hopene, confirming higher bacterial presence in casts than in soils. Hopene
426 being a marker for both living biomass and bacterial necromass, we can suggest the importance of
427 both living and dead microorganism in casts. Further efforts should be achieved to depict the
428 contribution of microbial necromass in organic carbon sequestration in soil and casts.

429 In this one year experiment, molecular and isotope features of soil lipids pointed towards a
430 higher contribution of root-derived lipids compared with shoot-derived lipids, likely reflecting the
431 lower decomposition rate of root lipids. This difference was lowered in casts suggesting that
432 although substantially incorporated and preserved in casts, apolar lipids undergo some degradation
433 that probably smoothed the difference between root and shoot residues. In soils, apolar lipids
434 roughly followed decomposition trends similar to that of bulk OM, which was not always the case in
435 casts, likely reflecting the influence of both molecular properties of OM and soil (micro)structure on
436 OM decomposition. Finally, these first results obtained from a one year experiment suggest that, at
437 larger time scale, it is can be important to differentiate the influence of earthworms on soils from
438 that on casts. Indeed, casts represent a specific microenvironment where both plant accumulation
439 and preservation, as well as microbial activity are enhanced.

440

441 **Acknowledgements**

442 The study was funded by an EC2CO (CNRS-INSU) grant that was greatly appreciated. We are
443 grateful to Patrick Dumont of Sorbonne Université greenhouse facilities for access to the
444 experimental chamber and technical support. Elise Canolle and Anne-Sophie Permal are thanked for
445 their help in preparing the lipids during their internship at METIS. We are indebted to Christelle
446 Anquetil and Véronique Vaury for GC-MS and EA-IRMS analyses, respectively. CSIA were obtained
447 from the ALYSES facility (IRD-SU) that was supported by grants from Région Ile-de-France. Thanks are
448 also due to the two anonymous referees who provided constructive reviews of the manuscript.

449

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709 **Table 1.** Bulk geochemical characteristics of the sample studied: undegraded residues, soils and casts
 710 after one year of incubation.

Sample name	Figs. 3 & 4 ^a	Residue	Earthworms	$\delta^{13}\text{C}_{\text{bulk}}$ ^b	Lipid content ^c	Alkane content ^d	Alkane OEP ^e
undeg-Shoot	c	Shoot	-	+1632.3	17.24	2.2	5.5
undeg-Root	f	Root	-	+1323.5	3.88	1.3	5.1
soil-NE-Control	a	No	No	-28.1	0.03	12.9	4.8
soil-NE-Shoot	d	Shoot	No	+23.3	0.03	13.4	4.0
soil-NE-Root	g	Root	No	+125.7	0.03	12.5	4.9
soil-E-Control	b	No	Yes	-28.1	0.05	10.4	3.9
cast-Control	b	No	Yes	-28.6	0.05	7.8	5.4
soil-E-Shoot	e	Shoot	Yes	-12.1	0.04	13.5	3.3
cast-Shoot	e	Shoot	Yes	+167.6	0.06	9.8	6.6
soil-E-Root	h	Root	Yes	+25.3	0.09	11.7	5.8
cast-Root	h	Root	Yes	+126.6	0.08	10.1	7.8

711

712 ^a associated graph on Figs. 3 and 4;

713 ^b ‰, modified from Vidal et al. (2017);

714 ^c weight % of dried sample;

715 ^d estimated alkane content assessed from area % of the summed alkanes with respect to the
 716 summed components of the total lipid chromatogram;

717 ^e odd-over-even carbon number predominance of alkanes.

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720 **Figure captions**

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727

728 **Figure 2.** TIC trace for lipid extracts of **a**, reference soil (soil-NE-control) and **b**, cast (cast-control),
729 after 54 weeks of experiment. 4-methoxybenzoic acid (★); *n*-alkanes (⊙); *n*-alkanoic acids (◆); ω -
730 hydroxyalkanoic acids (↘); *n*-alkanols (♥); **sterols:** Ch, cholesterol (▲); St, stigmasterol (▲); Si,
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734 **Figure 3.** Molecular distribution of the apolar lipids extracted from undegraded residues, soils and
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738 Relative abundances were normalized to the most abundant compound.

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744 Root (yellow). See table 1 for sample details. Exact isotope values are available in Online Resource 2

745 **Figure 5.** Proportion of carbon derived from the labelled residues (C_{lab}) in the soils and casts
746 incubated in the presence of earthworms. Alkane C_{lab} corresponds to the weighted mean of C_{21} , C_{23} ,
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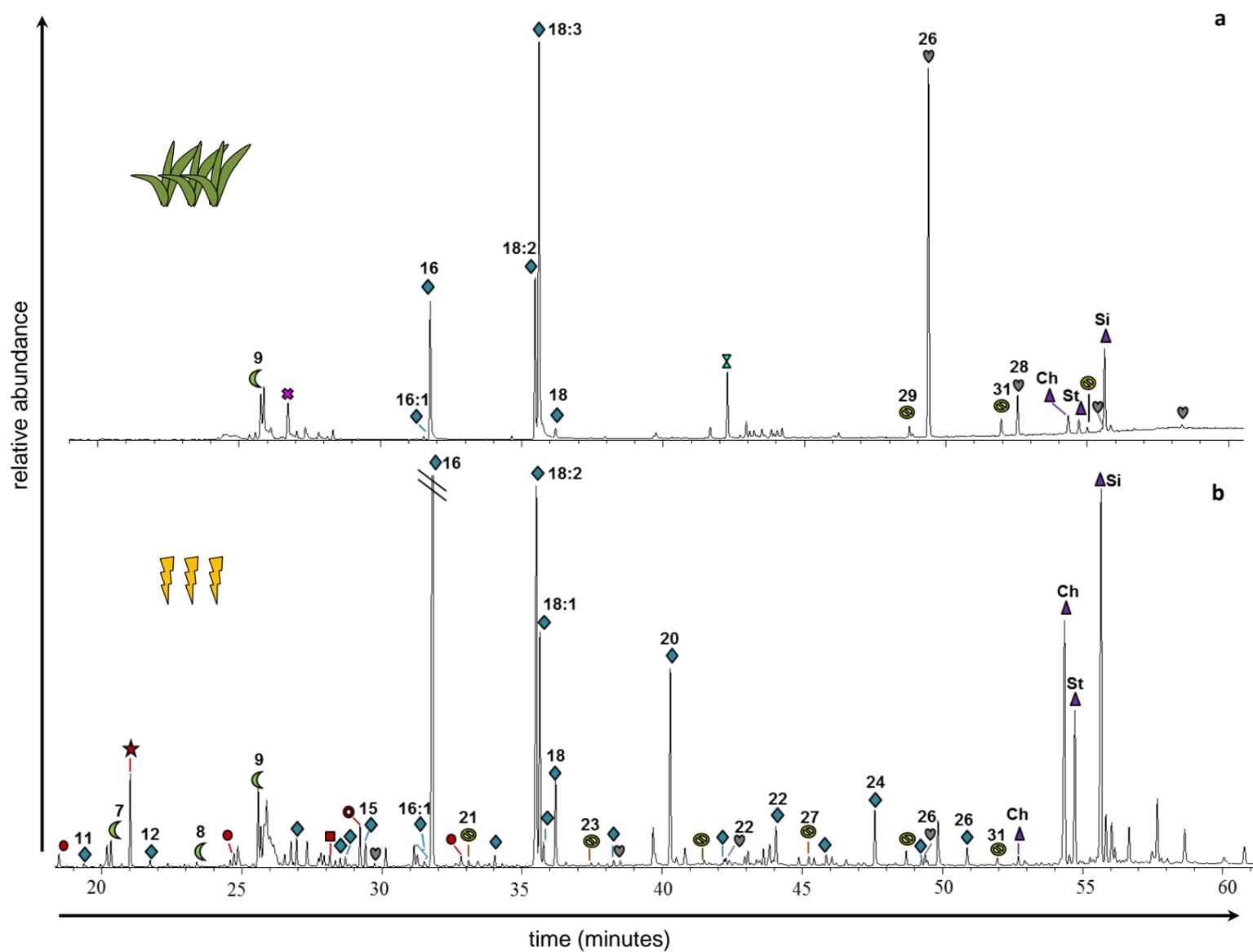


Figure 2

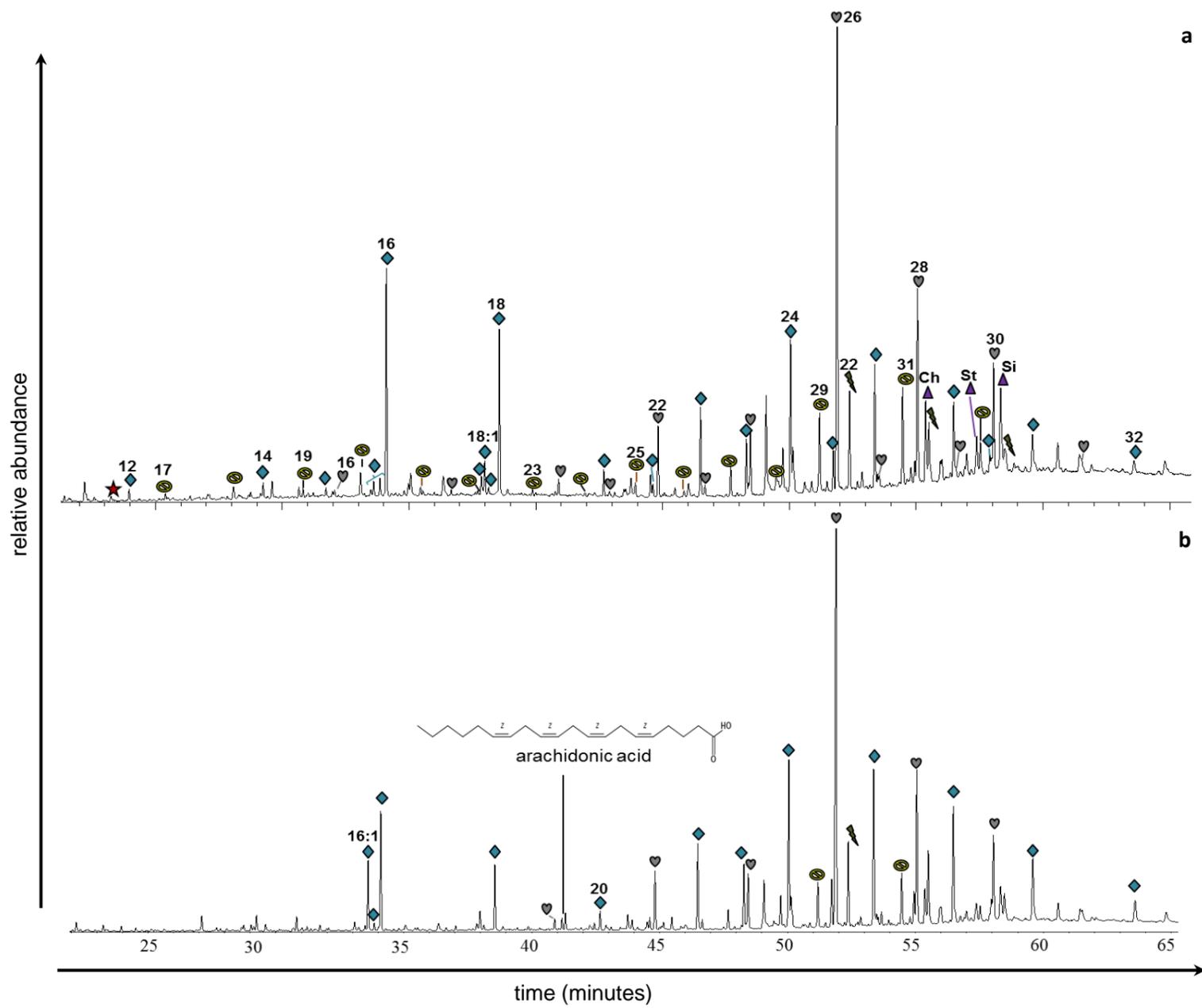


Figure 3

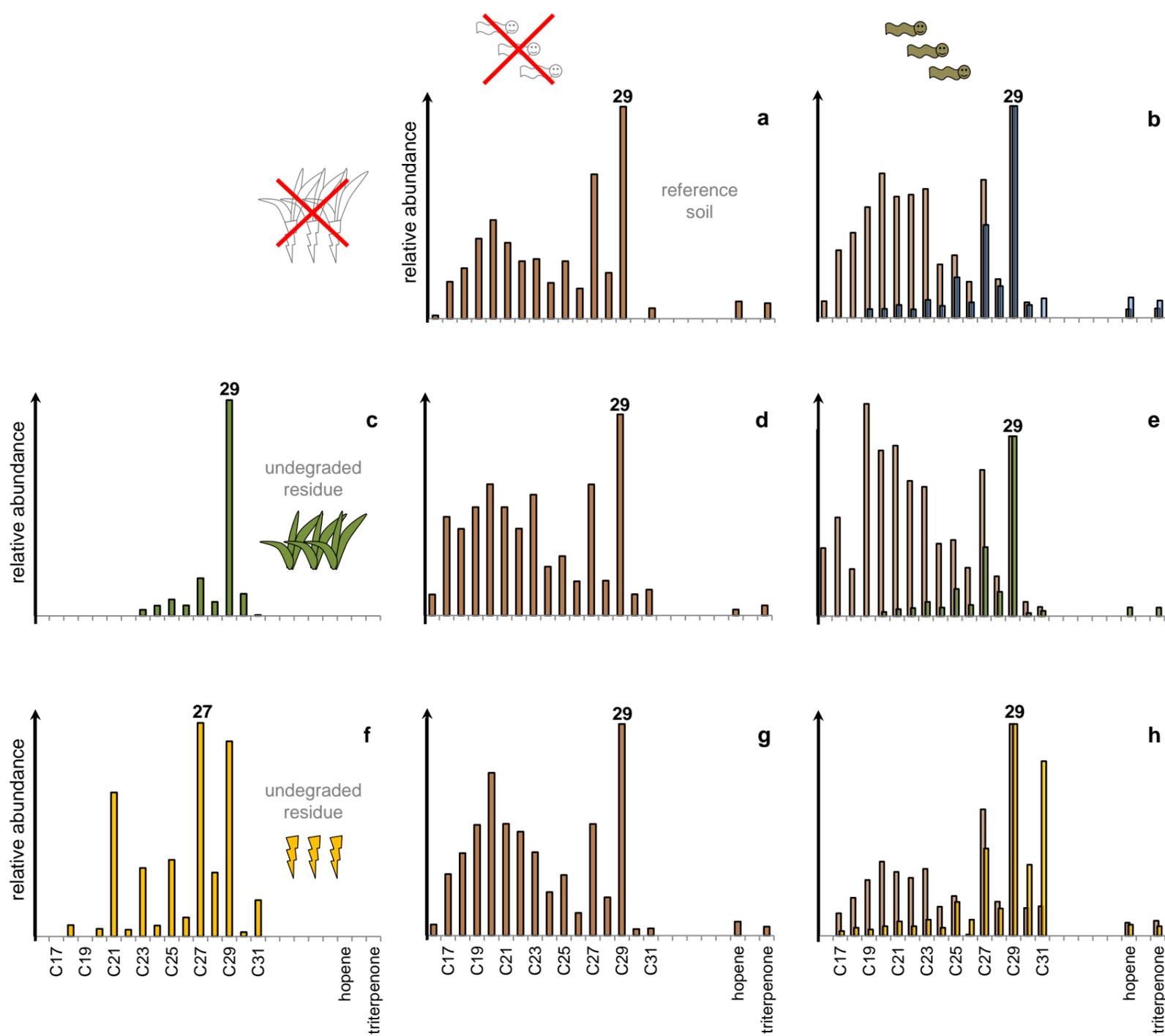


Figure 4

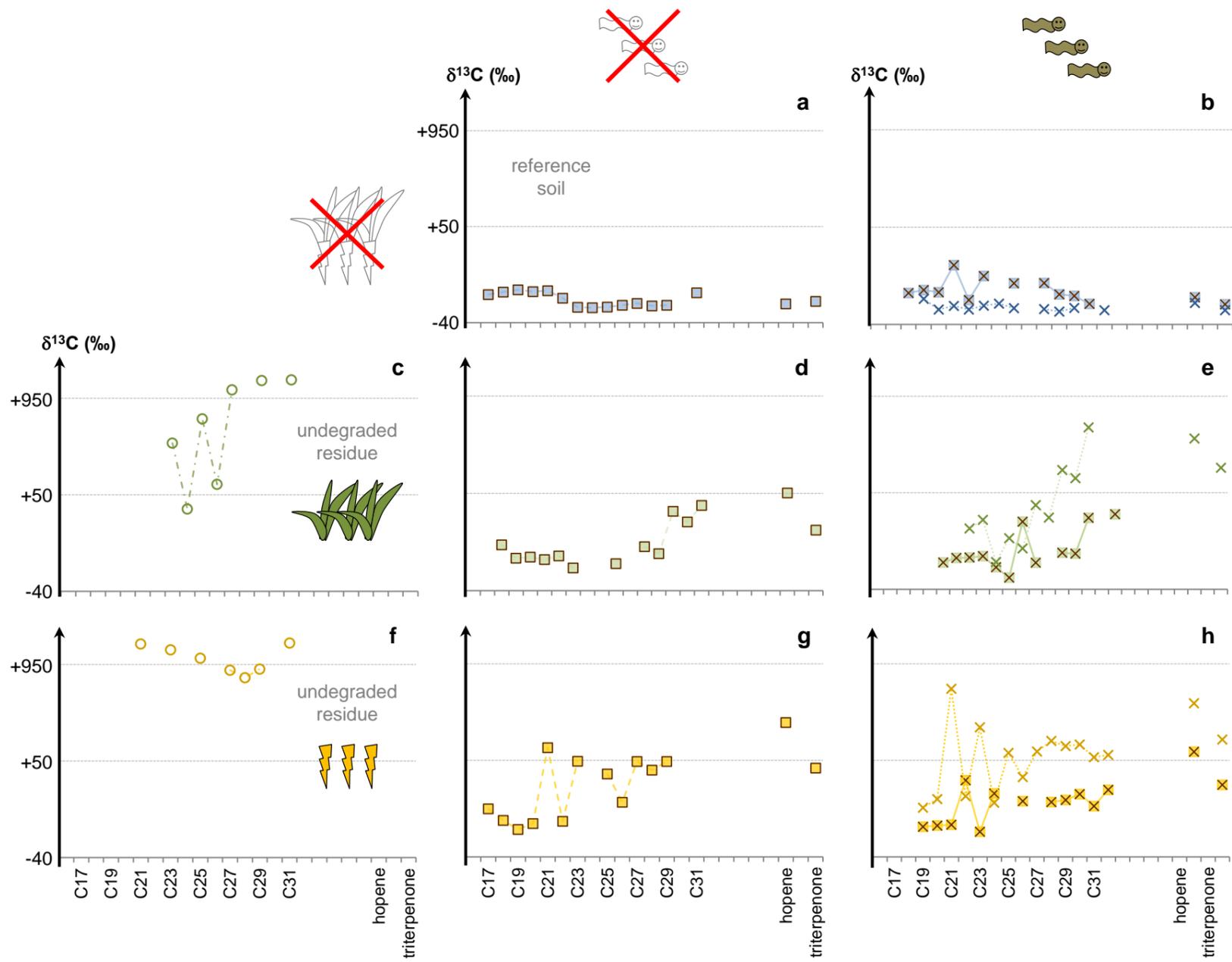
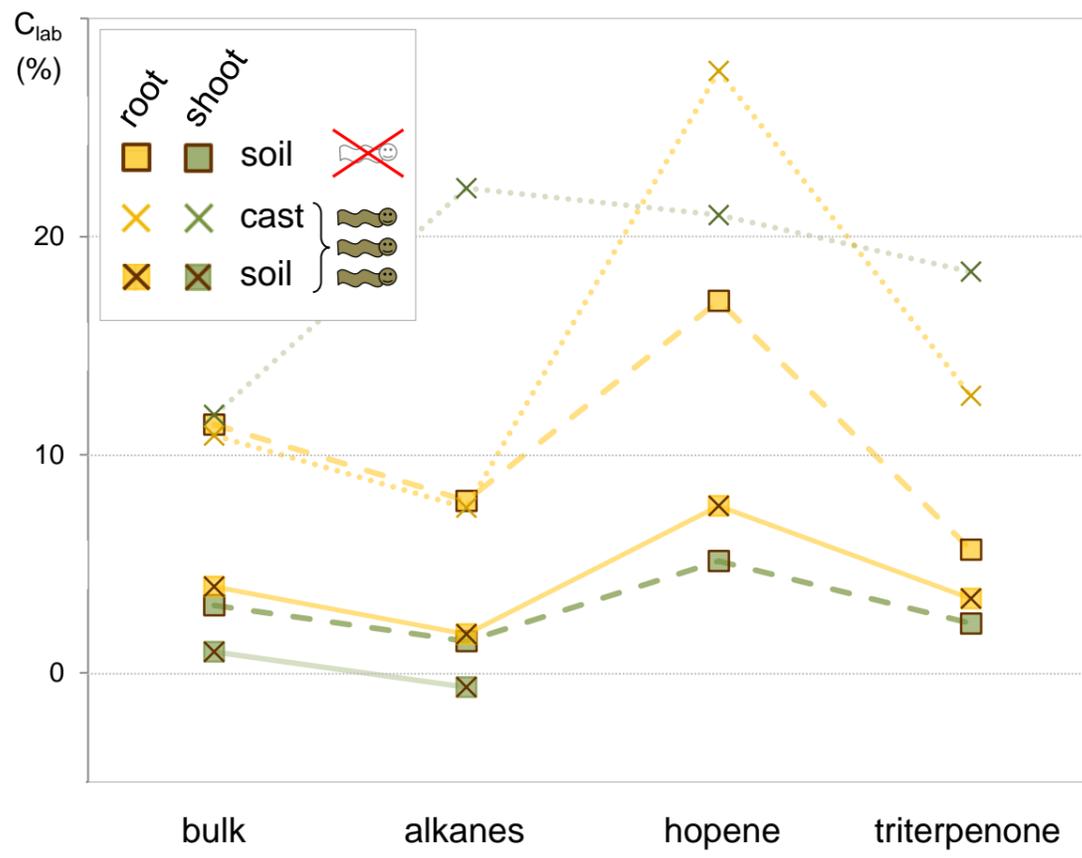


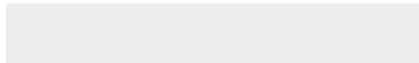
Figure 5





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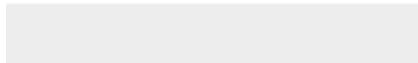
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