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Thanh Thuy Nguyen Tu, Alix Vidal, Katell Quenea, Mercedes Mendez-Millan, Sylvie Derenne

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T.T. NGUYEN TU[a,*], A. VIDAL[b], K. QUENEA[a], M. MENDEZ-MILLAN[c] & S. DERENNE[a]

[a] Sorbonne Université, CNRS, EPHE, UMR 7619 Metis, F-75005, Paris, France

[b] Lehrstuhl für Bodenkunde, TU München, Emil-Ramann-Strasse 2, 85356 Freising, Germany

[c] IRD, Sorbonne Université, CNRS, MNHN, IPSL, LOCEAN, Bondy, France

* Corresponding author at: Sorbonne Université, Tour 56-66, 4 place Jussieu, 75252 Paris, France.

E-mail address: Thanh-thuy.Nguyen_tu@upmc.fr (T.T. Nguyen Tu).

Abstract

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

Keywords

Earthworms, Organic matter, Lipids, ^{13}C-labelling, Soil, Cast

Introduction

Soil organic matter (OM) is a key pool in the biogeochemical cycle of carbon. In the current global change context, precisely documenting the processes leading to OM decomposition and sequestration in soils is crucial as it may help in mitigating atmospheric CO_2 levels (Jobbágy and Jackson 2000; Walthall et al. 2012). Soil ecosystem engineers, including earthworms, not only drive soil fertility and structure, but also OM decomposition (Bossuyt et al. 2005). They can enhance OM decomposition in soil but may also incorporate OM into their dejections (i.e. casts) that leads to its stabilization (Lavelle & Martin 1992; Zangerlé et al. 2011; Vidal et al. 2017). Casts correspond to a mixture of minerals and OM at different degradation stages, which are fragmented and complexed with intestinal mucus thanks to transit in the earthworm gut (Lee 1985; Six et al. 2004). Bacterial activity promoted by earthworms in casts favors OM decomposition at the short-time scale (Brown et al. 2000; Vidal et al. 2016a). Conversely, at the month or year scale OM may be stabilized in drying casts (Brown et al. 2000; Martin 1991). Mechanisms behind OM decomposition in the presence of earthworms have often been often explored at the short-term, while factors controlling OM decomposition and stabilization at the larger time scale remain unclear. Indeed, recent studies have highlighted the lack of experiments at larger time scales (> 200 days) to depict the impact of earthworm of OM decomposition and carbon cycling (Lubbers et al. 2013; Angst et al. 2017).

So far, the influence of earthworms on OM decomposition in soils was mainly studied at the bulk OM scale (Lavelle et al. 1998; Jégou et al. 2000; Six et al. 2004; Fonte et al. 2012; Stromberger et al. 2012; Fahey et al. 2013). However, soil OM corresponds to a complex mixture of molecules with diverse chemical structures and contrasted decomposition rate (Alexander 1981; Kelleher & Simpson 2006). Few authors have investigated the chemical modifications induced by earthworms on soil and cast OM (Guggenberger et al. 1996; Filley et al. 2008; Crow et al. 2009; Hong et al. 2011; Frouz et al. 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

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

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

Material and methods

Experimental setup

The samples used in the present study were collected from the mesocosm experiment described in Vidal et al. (2017). Briefly, six containers were filled with approximatively 75 L of a 4 mm-sieved loamy-sand soil, collected from permanent grassland in North of France (Oise, France). Six *L. terrestris* earthworms per container were deposited onto the surface of three of the containers. The amount of earthworms and plant residues deposited on the soil, as well as mesocosm size were designed so as to optimize earthworm survival in condition closest to reality (Vidal et al. 2017). Plants of Italian Ryegrass (*Lolium multiflorum*) were artificially labelled with ^{13}C at the *Commissariat à l'Energie Atomique et aux Energies Alternatives* (CEA) in Cadarache (France). After three months of growth under $^{13}CO_2$ enriched atmosphere, the mean $\delta^{13}C$ values were 1324‰ (±43) and 1632‰ (±16) for the roots and shoots, respectively (Vidal et al. 2017). Shoots and roots were separated, dried and subsequently mixed and homogenized during 40 s. with a laboratory blender (Waring 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 (CH_2Cl_2):methanol (CH_3OH) (2:1, v:v) in an accelerated solvent extractor (ASE 100
128 Dionex) at 60 °C and $10*10^6$ Pa for 10 min. in two cycles. The combined extracts were concentrated
129 by rotary evaporation, dried under N_2 for weighing and further redissolved in 200 µL of CH_2Cl_2. An
130 aliquote of the total lipid extracts (TLE) was silylated with O-Bis(trimethylsilyl)trifluoroacetamide

(BSTFA) (ca. 10% of the CH_2Cl_2 volume) prior to GC-MS analyses for molecular identification. For lipid specific isotope analyses, the TLE was fractionated using column chromatography on alumina (1 g for 10 mg of dried extract, Sigma-Aldrich 507C ~150 mesh). Apolar lipids were recovered after elution with a solvent mixture (4 mL for 1 g of alumina) of heptane (C_7H_{16}):CH_2Cl_2 (99:1, V:V). The apolar fraction was then concentrated by rotary evaporation and further dissolved in 3 mL of heptane.

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

Compound-specific stable carbon isotope composition of apolar lipids were measured using an online continuous flow gas chromatograph Trace-GC-Ultra coupled with an Isotopic Ratio Mass Spectrometer Delta V Plus via a combustion furnace and a conflow IV interface from Thermo Fischer Scientific. The GC was equipped with a split/splitless injector, kept at 320°C, and fitted with a 30 m fused silica capillary column (TR-5MS, 0.25 mm i. d., 0.25 µm film thickness). The GC oven program 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 5°C/min. Finally the oven was kept at 320°C during 5 min. Helium was used as carrier gas at constant flow of 2mL/min. Accuracy and reproducibility of the GC-C-IRMS measurements were assessed with a standard mixture of *n*-alkanes (*n*-C_{20}, *n*-C_{25}, *n*-C_{30} and *n*-C_{32}) of known isotopic composition (Schimmelmann, Indiana University). The standard mixture was injected three times at the beginning of each run and then once every three samples. Analytical standard deviation for each run was

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

Results and discussion

Molecular composition of TLE

Lipids account for less than 0.1 wt% of soil and cast samples while their content reaches 3.9 wt% and 17.2 wt% in root and shoot residues, respectively (Table 1). These lipid yields fall in the range of literature, although higher than that previously reported for *L. multiflorum* (Nguyen Tu et al. 2003, Göcke et al. 2013, Müller et al. 2013). Five major families of compounds are identified in both root and shoot residues: *n*-alkanes, *n*-alkanoic acids, *n*-alkanedioic acids, *n*-alkanols and sterols (Figure 1). These compounds are typical plant-derived lipids (van Bergen et al. 1998; Bull et al. 2000; Wiesenberg et al. 2010). Additionally, root TLE exhibits some lignin-derived compounds with *p*-coumaryl, guaiacyl and syringyl structures, as well as phenol. The main differences between root and shoot lipids stand in the presence of these aromatic compounds, as well as the higher relative abundance of long chain *n*-alkanoic acids (> C_{20}) and sterols in root residues, while shoot residues presented a higher relative abundance of C_{26} *n*-alkanol (Fig.1).

The reference soil TLE (Soil-NE-control; Fig. 2.a) is dominated by C_{12} to C_{32} *n*-alkanols maximizing at C_{26}, with an even-over-odd carbon-number predominance. Long chain *n*-alkanols (> C_{20}) typically derive from shoot waxes of terrestrial plants (van Bergen et al. 1998; Bull et al. 2000; Quénéa et al. 2004; Nierop et al. 2006; Quénéa et al. 2006) and are dominant constituents (C_{26}) of shoot residue extract (Fig. 1). *n*-Alkanols can represent an important contribution to the OM stabilized in soils (Mueller et al. 2012) and are essential indicators of past plant types (Jansen et al. 2008). For example, Nierop et al. (2006) found C_{26} *n*-alkanol to be, by far, the dominant compound of lipid extracted from a pine forest soil. As pine biomass did not contain this *n*-alkanol, the authors suggested that the soil was probably covered by grass vegetation before pine trees were planted. *n*-Alkanoic acids also occur in substantial amounts in the reference soil, ranging from C_9 to C_{32}, with a

182 strong even-over-odd carbon-number predominance and a maximum at C_{16}, as frequently reported
183 (Jandl et al. 2005; Wiesenberg et al. 2010). While C_{16} and C_{18} *n*-alkanoic acids are ubiquitous in living
184 organisms, C_{20}-C_{26} *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 C_{15} *n*-alkanoic acids points to a bacterial
186 contribution to soil lipids (Wannigama et al. 1981; Quénéa et al. 2012). C_{16} to C_{33} *n*-alkanes,
187 maximizing at C_{29} 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). C_{22}, C_{24} and C_{26}
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

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

Apolar lipid distribution

After one year of incubation, apolar lipids of all samples are dominated by *n*-alkanes that correspond to ca. 2% and 10% of TLE for plant residue and soil samples, respectively (Table 1). These bulk proportions are in agreement with those generally reported for plants and soils (Quénéa et al. 2004; Nguyen Tu et al. 2011; Srivastava & Wiesenberg 2018). The distribution of apolar lipids is shown as histograms in Fig. 3. The reference soil without plant residue and without earthworm (soil-NE-Control) shows a bimodal distribution of C_{16}-C_{31} alkanes maximizing at C_{29} and submaximizing at C_{20} (Fig. 3a). While long chain (> C_{23}) homologues with odd carbon number predominance mainly correspond to plant contribution, shorter chain homologues with no carbon number predominance are generally attributed to mixed sources including soil microbes, such as bacteria and fungi (Kolattukudy et al. 1976; Jansen et al. 2006; Li et al. 2017). In addition, two pentacyclic triterpenes are identified at the end of the chromatogram (Online Resource 1): a hopene [hop-22(29)-ene] and a triterpenone [amyrenone]; they originate from bacteria and plants (Angiosperm), respectively (Ourisson et al. 1979; Otto et al. 2005; Peters et al. 2005). However, these two compounds are not detected in the apolar lipids of plant residues, which are exclusively composed of *n*-alkanes. The triterpenone (amyrenone) is also a by-product the degradation/oxidation of the corresponding 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 C_{29} homologue (Fig. 3c), that of root is bimodal
237 (maximum C_{27}, submaximum C_{21}; 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 C_{31} (Gocke et al. 2013) or C_{29} (Malossini et al. 1990), this difference being probably
241 related to variations in developmental and/or environmental parameters (El-Otmani & Coggins 1985;
242 Gülz 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 C_{29} (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 C_{31} 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).

As indicative of plant material degradation, the odd-over-even predominance (OEP) was calculated for *n*-alkanes (Hoefs et al. 2002):

$$OEP = [n\text{-}C_{27} + n\text{-}C_{29} + n\text{-}C_{31} + n\text{-}C_{33}]/[n\text{-}C_{26} + n\text{-}C_{28} + n\text{-}C_{30} + n\text{-}C_{32}].$$

Root and shoot residues exhibit OEP slightly higher than 5 (Table 1), as expected for undegraded plant material (Zech et al. 2009; Schaefer et al. 2016). In soils without earthworm, OEP is typical of topsoils (<5; Table 1), the relatively low value for soil-NE-shoot (4.0) being likely related to a priming effect due to the deposition of nutrient-rich shoot residue at the beginning of the experiment. The priming effect consists of enhanced microbial degradation of native soil OM, induced by an input of fresh OM (Fontaine et al. 2004; Kuzyakov 2010). In the mesocosms with earthworms and with shoot residues or without plant residues, soils and casts display OEP <4 and >5, respectively (Table 1). According to literature (Buggle et al. 2010; Zech et al. 2009), these OEP suggest alkanes underwent some degradation in soils while they are less degraded in casts. This finding may be related to both higher incorporation and better preservation of plant residues in casts. Soil and cast from the mesocosm with root residues and earthworms exhibit very high OEP values (>>5) that are higher than the corresponding sample in shoot mesocosm (Table 1). These high OEP may be related to (1) the unexpectedly high abundance of the C_{31} homologues in these samples, combined with (2) lower decomposition of root alkanes when compared with shoot alkanes.

Lipid specific isotope composition

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

respect to the reference soil (-34‰ to -9‰, Fig. 4b). This ^{13}C-enrichment is the highest for the C_{21}-C_{27} alkanes (+11‰ to +19‰ with respect to the reference soil). As no ^{13}C-labelled residue was applied to this mesocosm, this pattern is probably related to earthworm activity, based on the following two facts. First, the ^{13}C-enrichment of soil alkanes may result, at least partially, from the preferential ingestion of soil fractions rich in plant OM that is naturally ^{13}C-depleted with respect to bulk soil, thus leading to apparent ^{13}C-enrichment of the remaining alkanes. Although, undetectable at bulk OM level (Vidal et al. 2017), such enrichment, would be especially noticeable at specific alkane level, as plant lipids are the main source of long-chain alkanes in soil. Secondly, the ^{13}C-enrichment of soil alkane in Soil-E-Control when compared with Soil-NE-Control may be indirectly related to the production of carbonate granules in earthworm specialized glands (Dotterweich 1933; Versteegh et al. 2014). Indeed, the carbon of the granules comes partly from atmospheric CO_2 that is ^{13}C-enriched with respect to OM (Canti 2009), and C_{13}-C_{38} *n*-alkanes were previously reported in *L. terrestris* lipids (Nooner et al. 1973; Stefanovic & Djurdjic 1976).

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

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

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

Carbon derived from the labelled plant residues

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

$$C_{lab}(\%) = [(\delta_s - \delta_c)/(\delta_l - \delta_c)] \times 100;$$

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

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

- the mean alkane denominator for the triterpenone that is a typical plant lipid, similarly to medium- and long-chain alkanes;
- the bulk sample denominator for the hopene, as this bacterial biomarker is synthesized from non-linear molecules.

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

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

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

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

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

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

Conclusion

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

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

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

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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	$\delta^{13}C_{bulk}$[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

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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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Figure captions

Figure 1. Total Ion Chromatogram (TIC) trace for lipid extracts of **a**, initial root residue and **b**, initial shoot residue. **Aromatic compounds** (red symbols) including compounds with guiacyl structure (●), 3,4,5-trimethoxy benzaldehyde (■) and 3-(4-methoxyphenyl) 2-propenoic acid (◎), and 4-methoxybenzoic acid (★); **aliphatic compounds**: *n*-alkanes (⊘); *n*-alkanoic acids (◆); *n*-alkanedioic acids (☾); *n*-alkanols (♥); monopalmitin (⧗); phytadiene (✖); **sterols**: Ch, cholesterol (▲); St, stigmasterol (▲); Si, sitosterol (▲). Numbers indicate chain length.

Figure 2. TIC trace for lipid extracts of **a**, reference soil (soil-NE-control) and **b**, cast (cast-control), after 54 weeks of experiment. 4-methoxybenzoic acid (★); *n*-alkanes (⊘); *n*-alkanoic acids (◆); *ω*-hydroxyalkanoic acids (↯); *n*-alkanols (♥); **sterols**: Ch, cholesterol (▲); St, stigmasterol (▲); Si, sitosterol (▲). Numbers indicate chain length. A focus was made on the qualitative differences observed between Control-NE and Cast-Control. As both samples were very similar, only the dominant molecules are indicated on the chromatogram of Cast-Control.

Figure 3. Molecular distribution of the apolar lipids extracted from undegraded residues, soils and casts of the mesocosms. **a,** soil-NE-Control; **b,** soil-E-Control (brown) and cast-Control (blue); **c,** undeg-shoot; **d,** soil-NE-Shoot; **e,** soil-E-Shoot (brown) and cast-Shoot (green); **f,** undeg-Root; g, soil-NE-Root; **h**, soil-E-Root (brown) and cast-Root (yellow). See table 1 for sample details. Relative abundances were normalized to the most abundant compound.

Figure 4. Specific isotope composition of the apolar lipids extracted from undegraded residues, soils and casts of the mesocosms. Isotope compositions are shown under logarithmic scale for easier comparison of the mesocosms with and without labelled residues [Log_{10} ($\delta^{13}C+50$)]. **a,** soil-NE-Control; **b,** soil-E-Control (brown) and cast-Control (blue); **c,** undeg-shoot; **d,** soil-NE-Shoot; **e,** soil-E-Shoot (brown) and cast-Shoot (green); **f,** undeg-Root; g, soil-NE-Root; **h**, soil-E-Root (brown) and cast-Root (yellow). See table 1 for sample details. Exact isotope values are available in Online Resource 2

Figure 5. Proportion of carbon derived from the labelled residues (C_{lab}) in the soils and casts incubated in the presence of earthworms. Alkane C_{lab} corresponds to the weighted mean of C_{21}, C_{23}, C_{25}, C_{27}, C_{29} and C_{31} C_{labs}. Detailed alkane C_{lab} are displayed in Online Resource 3.

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Figure 1

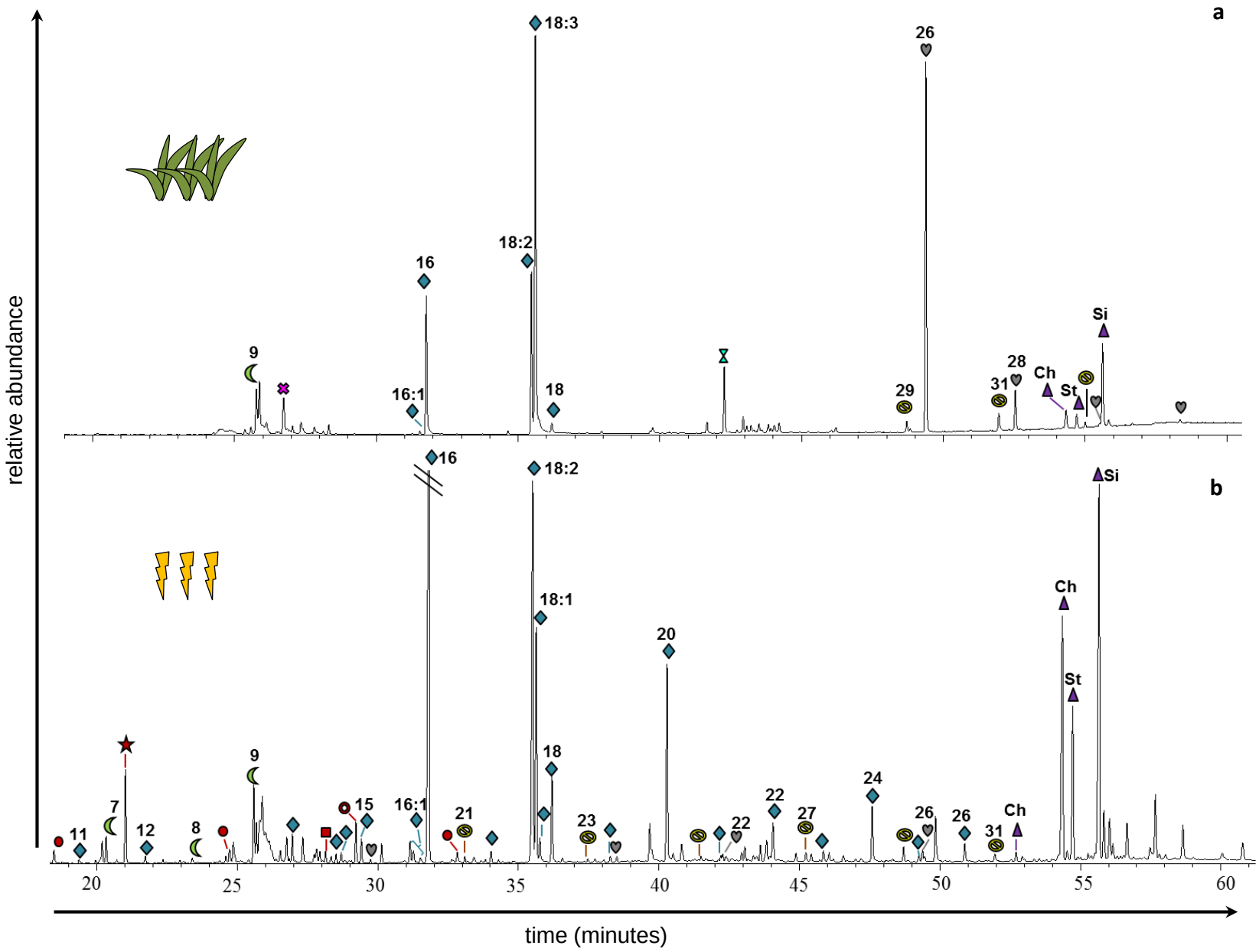


Figure 2

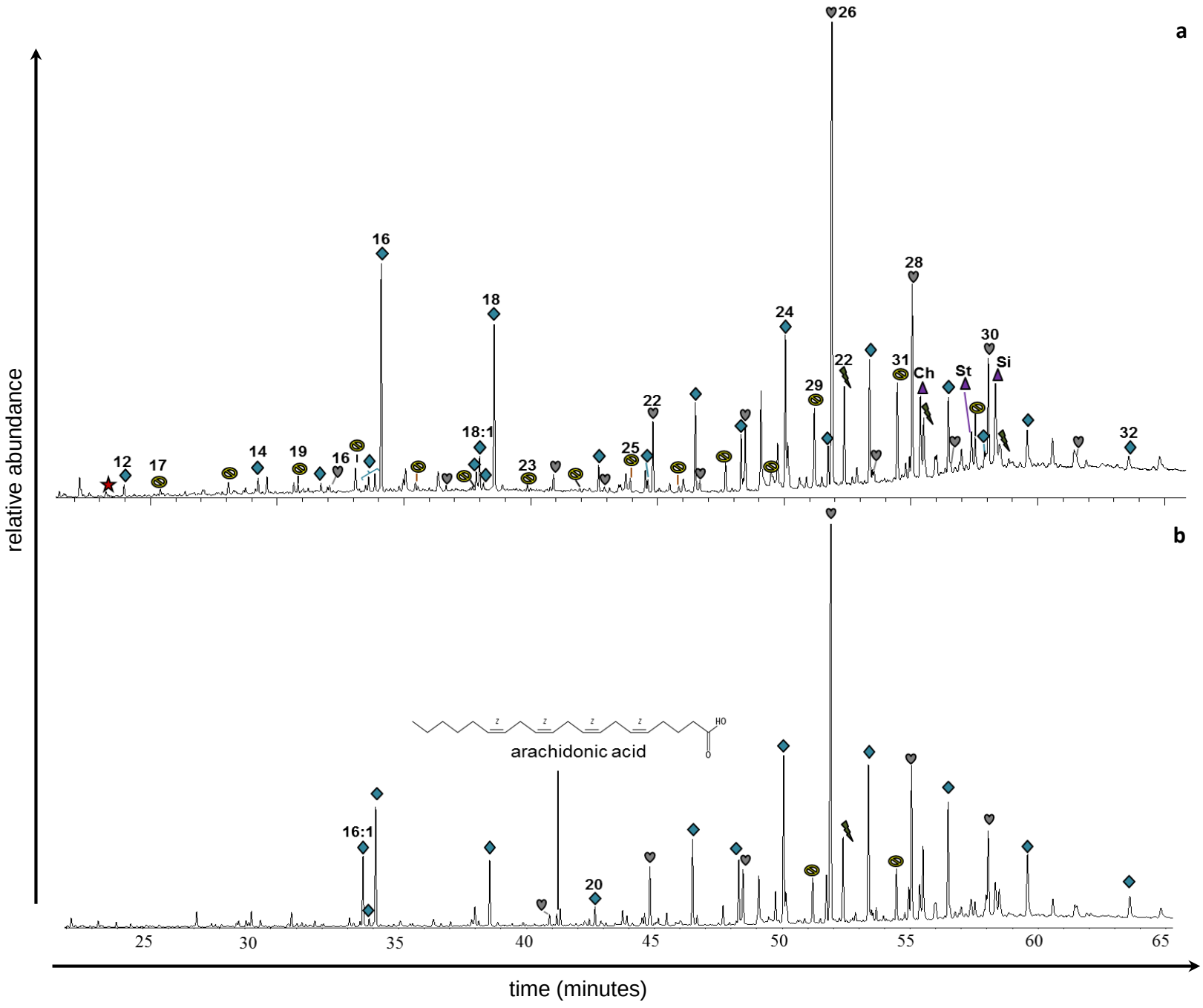
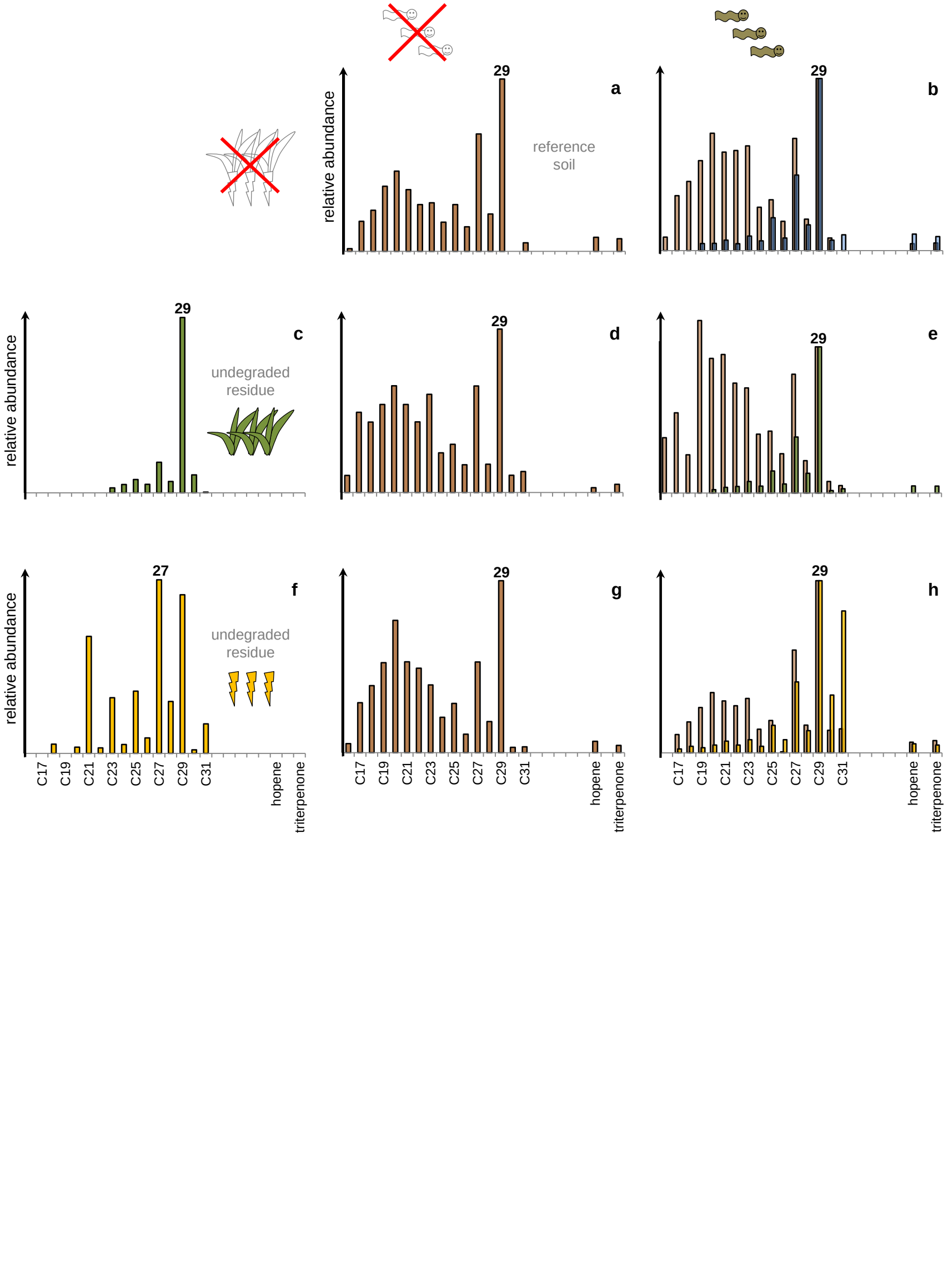


Figure 3



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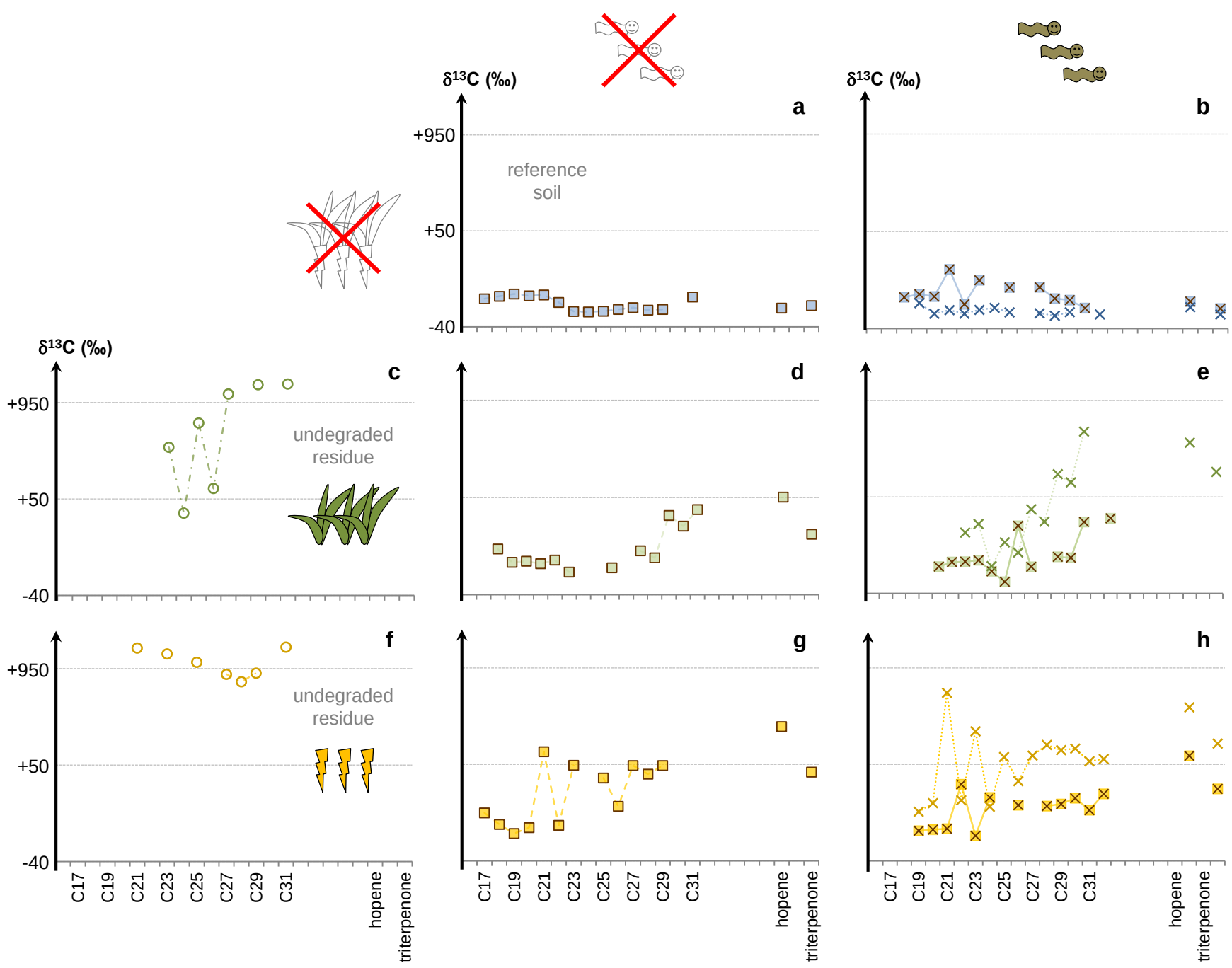

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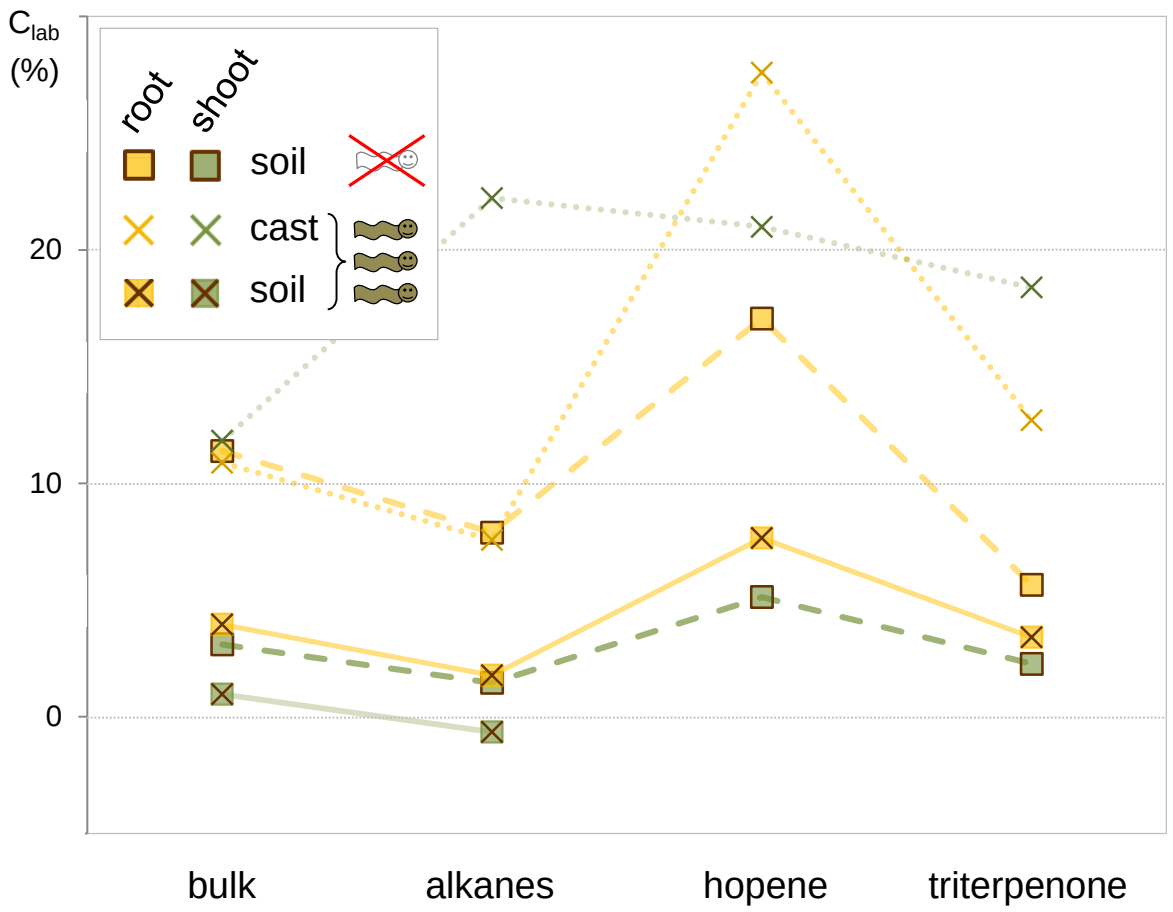

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