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ABSTRACT

The fate of leaf lipids upon early diagenesis was monitored in a two year litterbag experiment in a soil and at the water-sediment interface of an adjacent pond. The biomarker content of degrading leaves exhibited substantial variability among litterbags, even for a given time step within a given environmental condition, likely reflecting natural microenvironmental variability. Due to this variability and the oxic conditions in the pond, no substantial difference between the soil and the pond could be evidenced in the biomarker degradation pattern. An occasional increase in the abundance of several biomarkers (β-sitosterol, oleanolic acid, $C_{16}$ phytyl ester, $C_{27}$ *n*-alkane) was also noted during the experiment, which was attributed to release of bound compounds and/or an external contribution. Nevertheless, absolute quantification showed that the concentration of all lipid constituents was reduced, but they exhibited different decay profiles: (i) rapid extensive degradation (phytyl ester), (ii) exponential-like decrease (fatty lipids) and (iii) variable degradation profile (polycyclic triterpenoids). However, all the main constituents initially present in the senescent leaves were still detected after two years of degradation in both environments. Fatty lipid abundance generally decreased to < 10% of the initial content but their main distribution features (carbon number maximum and predominance) remained unchanged. The results thus tend to validate their use as proxy for source and environment in ancient organic matter. They also suggest that, on a mid-term basis, a plant biomarker signature is not substantially affected by differential degradation in soil and at the water-sediment interface, at least for a qualitative approach.

**Keywords:** diagenesis, biomarker, litterbag, leaf lipids, soil, surface sediment, GC-MS

## 1. Introduction

Lipid biomarkers constitute efficient tools for tracing the sources of organic matter (OM) in marine (Harada et al., 1995; Aquilina et al., 2010), fluvial (Jaffé et al., 1995; Hemingway et al., 2016) or lacustrine environments (Rieley et al., 1991; Jacob et al., 2007). Quantifying biomarker variation through sedimentary sequences facilitates reconstruction of past environmental variability over various timescales, from centuries (McCaffrey et al., 1991; Ortiz et al., 2016) to millions of years (Pearson and Obaje, 1999; Tzortzaki et al., 2013). However, the diagenetic fate of biomarkers must be assessed before their distribution and abundance can be used as quantitative proxies. From their structures, it would be anticipated that different biomarkers would exhibit varying degrees of susceptibility to degradation, which would ultimately affect their preservation in sediments (Meyers and Ishiwatari, 1993). Several stability scales have been established for lipids in sediments; *n*-alkanes are generally considered as the most stable components, while functionalized compounds such as pigments or *n*-alkanols as the most readily degraded (Cranwell, 1981; Colombo et al., 1997). Sterols and fatty acids (FAs) exhibit intermediate stability that varies according to functionality and degree of unsaturation (Cranwell, 1981; Meyers and Ishiwarati, 1993; Colombo et al., 1997). Environmental parameters in the water column, at the water-sediment interface and within the sediment also influence lipid degradation/preservation (Meyers, 1997; Rontani and Volkman, 2003).

Vascular plants are major contributors to sedimentary OM and plant biomarkers often serve as proxies for continental OM in marine sediments (Volkman, 1986; Hedges and Oades, 1997). The plant biomarker signature in sediments can be biased by differences in productivity among taxa, and biomarker solubility or transport mode (Diefendorf et al., 2014). Among polycyclic terpenoids, Giri et al. (2015) showed that these pre-sedimentation processes tend to favor diterpenoids at the expense of triterpenoids, leading to an overestimation of conifer input vs. angiosperm input in paleovegetation reconstruction. Before deposition in marine sediments, plant detritus may remain for varying extents of time in soil and/or in fresh water. The contrasting degradation conditions in these

environments may in turn affect the plant biomarker signature. Surface degradation of a given plant on land was shown to modify bulk leaf chemistry to a different extent than degradation in buried conditions (Webster and Benfield, 1986; Steart et al., 2009). Although the fate of molecular biomarkers was independently studied in several environments via various approaches (e.g. soil litterbags, soil/sediment profiles/sections, laboratory incubations; Cranwell, 1981; Meyers and Ishiwarati, 1993; Rieley et al., 1998; Zech et al., 2011, Wiesenberg et al. 2012; Wang et al. 2014), comparative studies addressing the fate of biomarkers in diverse natural environments are rare. However, the plant biomarkers recovered from sedimentary OM may have undergone different pre-burial histories that may have influenced their robustness as a quantitative proxy.

As the comparative fate of plant lipids in different environments is not fully documented, the aim of the present study was to compare the degradation of lipids from a given plant species in two differing environments: soil and the water-sediment interface. The investigation focused on leaf lipids, as epicuticular wax is the main source of the fatty lipids commonly used as continental biomarkers in marine sediments. The study was based on a litterbag experiment, in order to (i) allow the recovery of the small leaf debris generally obtained after several months of degradation and (ii) precisely monitor the changes undergone in the leaves initially introduced into the bags, without potential bias due to contamination by other plant material. European beech (*Fagus sylvatica* L.*)* was chosen because it commonly occurs in temperate forests. The lipid composition of the leaves from two litterbag experiments conducted simultaneously in a soil and in a nearby pond was investigated to test whether these different environments might lead to different biomarker signatures. The key questions were: Do different lipid classes degrade similarly in these two environments? Is the distribution of fatty lipids modified similarly in the two environments?

## 2. Material and methods

### *2.1. Experimental site*

The experiment was set up in the "Breuil-Chenue" experimental forest site, in Burgundy, France (47°18'10"N, 4°4'44"E). The forest is part of the "Parc Naturel Régional du Morvan", a protected area where urban pollution is minimal. The experimental area is on a shelf 650 m above sea level and exhibits a slight northwest facing slope. Mean annual temperature and precipitation at the site are 9 °C and 1,280 mm, respectively. The bedrock is the alkaline granite of "La Pierre qui Vire", covered by a thin layer of eolian silt. The incubations took place in a 30 yr old European beech (*Fagus sylvatica* L) stand planted after a clear-cut in 1976, and in a ca. 300 $m^2$ adjacent pond, respectively. The soil is classified as a dystric cambisol (WRB, 2006) with moder-type humus. It is acidic with pH 4-4.5 and has a poorly saturated cation exchange complex. More information on the site is available elsewhere (Moukoumi et al., 2006; Andrianarisoa et al., 2010). The pond had a maximum depth of 1.5 m. The main characteristics were determined using a CTD (conductivity – temperature – depth) probe equipped with additional sensors and external fluorometers (ASD sensors). During the course of the study the water column displayed nearly constant temperature, electrical conductivity and pH with water depth (Supplementary Table A.1). The water had a slightly acid pH (ca. 6.1), low dissolved ion load (low electrical conductivity, ca. 25.7 µS/cm), low turbidity (FTU < 20), high CDOM content (chromolenic dissolved OM, > 200 µg/l) and saturated or close to saturation dissolved $O_2$ concentration (ca. 98%). Overall, this is overall typical of surface and subsurface waters of forests soils on granitic substratum (Beaucaire and Michard, 1982). Thermal stratification only occurs during summer, when photosynthetic activity is enhanced (high chlorophyll-*a* concentration and oversaturation for dissolved $O_2$; Supplementary Table A.1 and Fig. A.1).

*2.2. Litterbag incubations*

At the end of October 2004, senescent leaves were collected from the beech stand where the soil incubation took place. The soil litterbag experiment has been described elsewhere (Nguyen Tu et al., 2011). The leaves were collected from the same height (ca.1.5 m) from the branches of several different trees in order to provide an averaged composition and to minimize bias linked to intrapopulation variability. After harvesting, the leaves were rinsed with distilled water to remove extraneous particles and dried at 45 °C to constant weight. Each batch was composed of 10-15 leaves and corresponded to ca. 1 g OM. Batches were assembled using leaves from as many different trees as possible to provide equivalent batches.

Litterbags (9.5 × 14.5 cm) were made of Al wire; mesh size was 1.4 × 1.8 mm, i.e. sufficient to retain small leaf litter debris but sufficiently large to permit free entry of mesofauna (main functional group at the site) and microbial activity (bacteria and fungi). Pond litterbags were ballasted with glass sticks to maintain them at the bottom of the pond. Before being enclosed in litterbags, leaves were weighed and then re-moistened with distilled water to recreate natural conditions. The litterbags were placed in the field in December 2004. Litterbags (in duplicate) were then regularly retrieved over 2.5 yr: after 5, 10, 15, 20, 30, 40, 52, 79 and 129 weeks for the soil, and 9, 27, 66 and 100 weeks for the pond. The soil litterbags correspond to the unlabeled leaves analyzed before for their specific alkane isotope composition (Nguyen Tu et al. (2011).

*2.3. Lipid extraction and fractionation*

The recovered material was first rinsed with distilled water to remove obvious extraneous particles such as moss, roots or arthropods. Leaves were then dried at 45 °C to constant weight and weighed. Samples were stored dark in Al foil at 5 °C until analysis. All the leaves for each litterbag were combined and ground fine enough to pass through a 500 μm mesh. Leaf powder was ultrasonically extracted for 20 min with $CH_2Cl_2$/MeOH (2/1; v/v). The mixture was centrifuged (10 min, 4,000 rpm) and the lipids were recovered in the

supernatant. The extraction procedure was repeated 6× with the centrifugation residue. The combined extracts were concentrated via rotary evaporation and dried under $N_2$. The extract was fractionated using column chromatography on alumina (Sigma-Aldrich 507C, ca. 150 mesh) deactivated to Brockmann grade IV by adding 0.1 wt % distilled water. The non-polar fraction (1) was recovered with heptane. The slightly polar fraction (2) was recovered with toluene. The medium polarity fraction (3) was recovered with $CH_2Cl_2$ and $CH_2Cl_2$/MeOH (9/1; v/v). Each fraction was concentrated via rotary evaporation and dissolved in ca. 3 ml heptane, toluene, and $CH_2Cl_2$, respectively. The main components of the three fractions were quantified using internal standards: tetratriacontane for fraction 1 and tricosan-1-ol for fractions 2 and 3.

*2.4. Analysis*

The hydrocarbon fraction was analyzed as such, while the other two fractions were converted to trimethylsilyl derivatives prior to analysis. Aliquots (30 µl) of the fractions were dried under $N_2$ and then silylated with 30 µl N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) for 1 h at 80 °C. After cooling, the aliquot was dried under a gentle stream of $N_2$ and dissolved in 30 µl of appropriate solvent (toluene for fraction 2 and $CH_2Cl_2$ for fraction 3). Assignment and quantification of lipids were carried out using gas chromatography coupled-mass spectrometry (GC-MS) and GC with flame ionization detection (FID, 350 °C), respectively. Separation was with an Agilent 6890N gas chromatograph equipped with a split/splitless injector (350 °C) and fitted with a fused silica column, coated with VF5-MS (50 m × 0.32 mm i.d., 0.12 µm film thickness). The GC conditions were: 80 °C to 100 °C at 10 °C/min, and then to 325 °C (held 30min) at 4 °C/min. He was the carrier gas at a constant 2 ml/min. For GC-MS an Agilent 5973N electron impact mass spectrometer (70eV, scan range *m/z* 35-800) was used. Components were assigned according to library and published mass spectra. Quantification was achieved from the response factor in GC-FID of the measured components and comparison of GC-FID peak areas with those of the internal standards, introduced to the fractions in known

amount. Response factors were obtained from calibration curves of the standard and the component, or compound with similar structure, polarity and molecular weight, if no reference compound was available. Coelution in GC-FID occasionally prevented quantification of some lipid components. Biomarker content was quantified as µg/g dry biomass and further expressed in % relative to initial content in Fig. 1 for comparison purposes.

## 3. Results and discussion

### *3.1. Biomarker behaviour*

The composition of lipids from senescent leaves of *Fagus sylvatica* has been described by Nguyen Tu et al. (2007). The lipids were dominated by sterols, the most abundant being β-sitosterol, and to a lesser extent, by acyclic isoprenoids, including phytadienes and phytyl esters ($C_6$-$C_{20}$, max. $C_{16}$). Oleanolic triterpenoids, mainly oleanolic acid, and fatty lipids were also abundant. The latter comprise an homologous series with even chain length predominance: mainly *n*-alkan-1-ols ($C_{14}$-$C_{30}$, max. $C_{28}$), *n*-acids ($C_8$-$C_{28}$, max. $C_{16}$, $C_{28}$) and, to a lesser extent, *n*-aldehydes ($C_{20}$-$C_{32}$, max. $C_{28}$). Predominantly odd numbered series also occurred in substantial amounts: mainly *n*-alkanes ($C_{12}$-$C_{33}$, max. $C_{27}$), and to a lesser extent *n*-alkan-2-ols ($C_{23}$-$C_{29}$, max. $C_{27}$) and *n*-alkan-2-ones ($C_{25}$-$C_{29}$, max. $C_{27}$). The lipid fingerprint of degrading beech leaves revealed no major qualitative changes during 2 yr of diagenesis in the soil or at the water-sediment interface. Most of the initial components were still present at the end of the experiment and no degradation products were evidenced. Quantification of the main components of each lipid class for each degradation stage showed that the concentration of all the biomarkers studied was (i) very variable but (ii) substantially reduced at the end of the experiment, although to varying extent (Fig. 1).

*3.2. Variability in biomarker concentration*

In this 2 yr experiment, the biomarker content of degrading leaves exhibited substantial variability among litterbags, even for a given time step within a given environmental condition (Fig. 1). Therefore, we address the potential origin of such variability before discussing degradation patterns. The difference in biomarker content between replicate litterbags maximized in soil at 70% and 100% of initial leaf content, for oleanolic acid and $C_{16}$ phytyl ester, respectively. Other quantified compounds (β-Sitosterol, $C_{16}$ acid, $C_{28}$ alcohol and $C_{27}$ alkane) exhibited maximum differences between replicate litter bags of around 40%. Such variability may relate to (i) initial variability of leaf lipid content and/or to (ii) variability in microenvironmental conditions. The concentration of leaf lipids is known to vary substantially between species, within species, within individuals and even within a given leaf (Herbin and Robins, 1969; Gülz et al., 1991; Gao et al., 2015). In agreement with the lower variability of in vitro experiments vs. field studies, the variability in lipid decay is higher here than reported for beech lipids in a laboratory experiment (Rieley et al., 1998). Soils, and especially forest soils, are recognized as highly heterogeneous environments (Hedges and Oades, 1997; Bélanger and Van Rees, 2008; Lehmann et al., 2008). A number of biogeochemical characteristics of soils commonly vary within a few decimeters, so that different decay profiles between litterbags separated by ca. 20 cm is not unexpected (Witkamp and Olson, 1963; Murphy et al., 1998; Suchewaboripont et al., 2011). Additionally, the main drivers of litter decomposition in Breuil-Chenue experimental forest are saprophytic fungi, which are represented mainly by three species (*Mycena inclinata, Mycena galopus* and *Phallus impudicus*) that generally exhibit a heterogeneous spatial distribution (Zeller et al., 2007). Decay conditions within sediments are believed to exhibit smaller spatial variability, although leaf degradation at the water-sediment interface under a substantial water column has seldom been investigated in the field. Nevertheless, leaf decomposition in seawater and stream water was shown to be variable over a few months monitoring and/or within small areas (Suberkropp et al., 1976; Rublee and Roman, 1982; Webster and Benfield, 1986; Boulton and Boon, 1991; Machás et

al., 2006; Moretti et al., 2007). Hydrocarbon and FA content of deciduous leaves degrading in a blackwater stream were shown to vary substantially over three months (Mills et al., 2001).

*3.3. Occasional increase in biomarker concentration*

The lipids occasionally exhibited a higher content in degrading leaves than in initial senescent leaves. That was the case for 4 of the 6 classes investigated: β-sitosterol reached 112% of its initial content in pond leaves after 27 weeks of degradation, oleanolic acid had a content of between 114% and 141% for some litterbags in the soil and in the pond between 9 and 40 weeks of degradation, 123% of the initial $C_{16}$ phytyl ester was detected after 5 weeks in a soil litterbag, and the *n*-$C_{27}$ alkane reached 124% and 103% of its initial content in pond litterbags after 27 and 103 weeks, respectively. Laboratory incubation of beech leaves by an estuarine microbial community (Rieley et al., 1998) also showed an initial sterol increase that was attributed to the release of free compounds from a bound state. Grass degradation in soil litterbags similarly exhibited an occasional increase in *n*-alkane content over 10 months, reaching up to 230% of the initial content (Wang et al., 2014). Initial increase in alkane content was also noted in maple, ash and beech leaves during a 2 year litterbag degradation, and their deuterium content suggested a microbial contribution (Zech et al., 2011). Isotope characterization ($^{13}C$) of the alkanes from the soil litterbags of the present study showed that microbial alkanes also contributed to the alkane pool in degrading leaves (Nguyen Tu et al., 2011). The occasional increase in some of the biomarkers may thus reflect either an external contribution, especially for rather ubiquitous compounds such as fatty lipids, and/or moiety release from bound structures.

*3.4. Biomarker degradation profiles*

Degradation at the water-sediment interface would be expected to be less extensive than in a soil. However, in the present experiment the high variability in biomarker concentration precludes such a conclusion (Fig. 1). Indeed, this high variability prevented

calculation of representative decay rates for most biomarkers. Consequently, no difference in degradation rates could be evidenced between the soil and the pond litterbags. This lack of slower degradation in the pond than in the soil may also be partly due to the oxic conditions at the bottom of the pond (Section 2.1), which are known to be more favorable for degradation than anoxic ones. The variability of the present results calls for caution when comparing decomposition patterns, but the quantified lipids seemed to degrade according to three main profiles, at least during the first 6 months (Fig. 1).

- Phytyl esters were rapidly extensively degraded, as shown by the phytyl hexadecanoate content, which dropped to ≤ 10% after 15 weeks in the soil, as well as in the pond.

- Fatty lipids (*n*-alkanes, *n*-acids and *n*-alcohols) underwent exponential-like decrease, reaching ca. 7% of the initial content at the end of the experiment. Mass loss of bulk leaves is often approximated by an exponential decay model in soils and streams, as the rate of mass loss is assumed to be a constant fraction of the amount of material remaining (Olson, 1963; Webster and Benfield, 1986). The present results may thus suggest similar degradation kinetics for fatty lipids.

- Polycyclic triterpenoids exhibited a variable degradation pattern and remained at ≥ 15% of the initial content after 2 yr, as shown by the β-sitosterol and oleanolic acid profiles (Fig. 1).

These results mainly match those reported for a three month laboratory incubation of beech leaves, although the mentioned laboratory experiment was more favorable for phytyl ester preservation and less for alkanes (Rieley et al., 1998). The present results also differ slightly from the stability scales often deduced from sediment studies since *n*-alkanes are generally considered as the most stable lipid components and functionalized polycyclic terpenoids as being moderately stable (Cranwell, 1981; Colombo et al., 1997). Our results thus emphasize the complexity and variability of lipid degradation in natural environments.

*3.5. Distribution pattern of fatty lipids*

Despite the substantial degradation of fatty lipids, their main distribution pattern at the end of the experiment was similar to the starting one (Table 1, Fig. 2): they remained markedly dominated by a single homologue (*n*-$C_{28}$ for alcohols, *n*-$C_{16}$ for acids, and *n*-$C_{27}$ for alkanes), with a slight narrowing of the distribution range. Chain length distribution indexes remained similar to the initial values (Table 1): the average chain length (ACL) centered around the dominant homologue and the carbon preference index (CPI) close to zero for acids and alcohols, and markedly >1 for alkanes. The only noticeable change in lipid series distribution concerns FAs, for which the long chain homologue (*n*-$C_{28}$) is rapidly degraded (i.e. no longer detectable after 5 weeks). However, such degradation did not modify chain length distribution indexes (Table 1). On the contrary, several studies have reported ACL and CPI modifications through degradation (Meyers and Ishiwarati, 1993; Huang et al., 1996; Celerier et al., 2009; Zech et al., 2011; Wiesenberg et al., 2012), which were attributed to various causes, including contribution from microbial lipids, root lipids or else anthropogenic pollution. Nevertheless, *n*-alkanes from several grass species in soil litterbags showed variable degradation profiles but no change in ACL and CPI (Wang et al., 2014), similarly to the present study. Taken together, the results suggest that, in spite of degradation and potential contribution from external lipids, the original plant lipids can remain the main source of lipids in degraded plant debris and can typically retain their chain length characteristics. The results thus tend to validate the use of chain length characteristics of aliphatic series for source and paleoenvironment reconstruction, at least on the mid-term scale (i.e. a few years).

## 4. Conclusions

Comparative degradation of leaf lipids in a soil and at the water-sediment interface of a well oxygenated pond revealed that most of the biomarkers exhibited highly variable content, likely reflecting natural microenvironmental variability. Due to this variability and the oxic conditions in the pond, no substantial difference between the soil and the pond

could be detected in biomarker degradation patterns during the 2 yr litterbag decay. All the constituents initially present in the senescent leaves were degraded, although to variable extent. Phytyl esters were the most extensively degraded, while polycyclic triterpenoids appeared relatively better preserved, with fatty lipids exhibiting intermediate degradation patterns. Nevertheless, the main lipid constituents of initial senescent leaves were still detected after two 2 yr degradation in both environments. During the experiment, an occasional increase in the abundance of several biomarkers was noted, implying moiety release from bound structures and/or an external contribution. Although fatty lipids concentrations were markedly reduced, the distribution patterns were not substantially affected, validating their use as source and environmental biomarkers in ancient OM. The results suggest that, for the mid-term, a plant biomarker signature is not substantially affected by differential degradation in soil and at the water-sediment interface under oxic conditions, at least for a qualitative approach.

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## Appendix A. Supplementary data

Geochemical characteristics of the water column are given: the range of values measured in the water column (Table A.1) and examples of typical depth profiles for environmental parameters (Fig. A.1).

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## Figure captions

**Fig. 1**. *Fagus sylvatica* leaf lipid degradation profiles. Concentration (% relative to initial concentration) of major lipid components vs. time: ■, soil; ◇, pond water-sediment interface.

**Fig. 2.** Distribution of *n*-alkanes, *n*-acids and *n*-alcohols in senescent leaves and after 2 yr degradation in a soil and at the water-sediment interface of a pond. Arrows indicate when a homologue was detected but was below quantification level.

**Figure 1**

Nguyen Tu et al. Figure 1

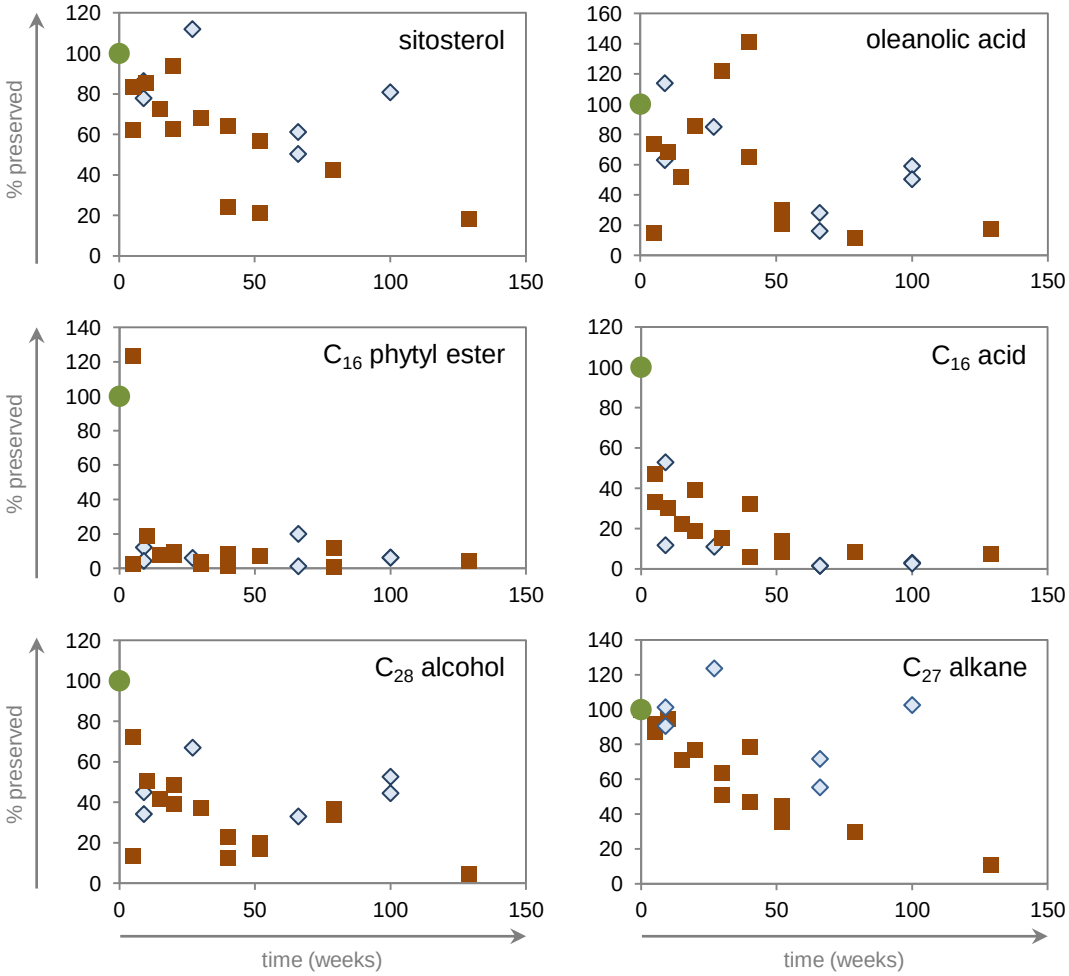

**Figure 2**

Nguyen Tu et al. Figure 2

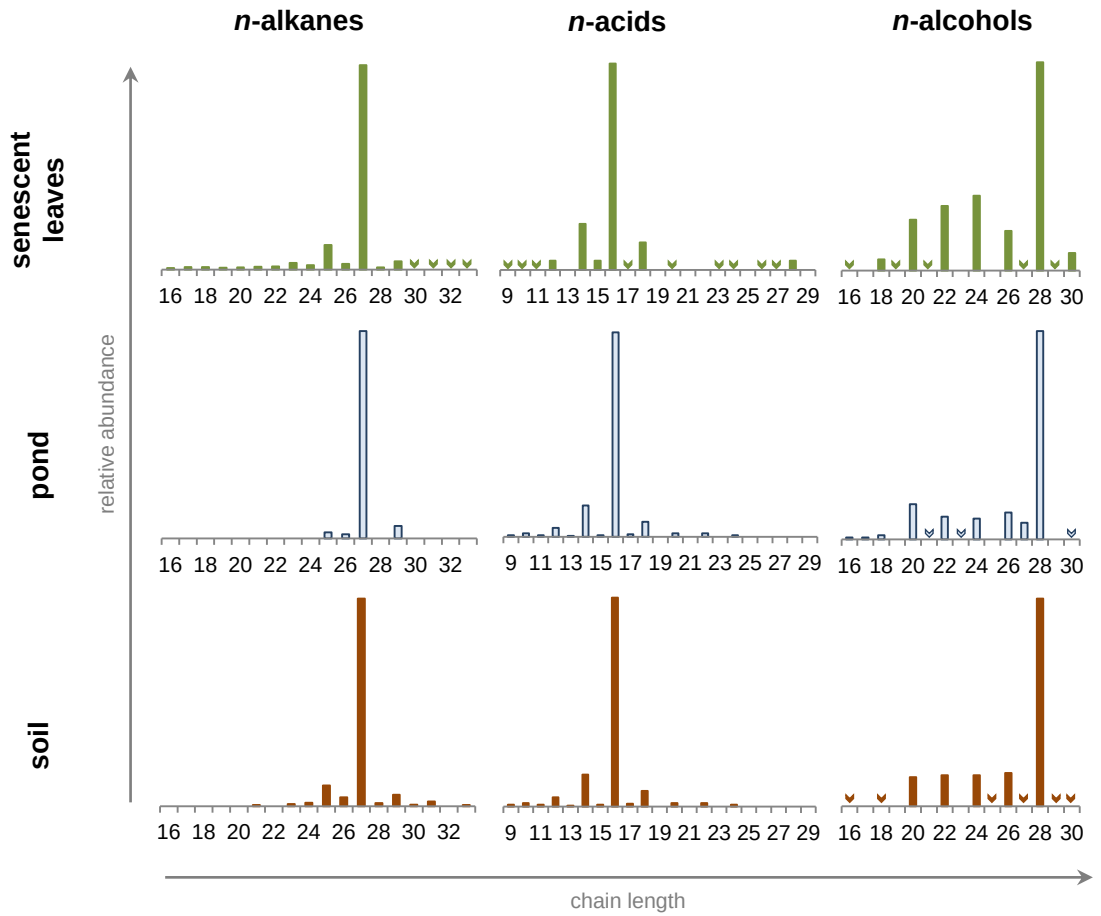

**Table 1**

Distribution pattern of main fatty lipid series in *F. sylvatica* leaves.

| | *n*-Alkanes | | | *n*-Acids | | | *n*-Alcohols | | |
|---|---|---|---|---|---|---|---|---|---|
| | $C_{range}$ ($C_{\mathbf{max}}$) | ACL[a] | CPI[b] | $C_{range}$ ($C_{\mathbf{max}}$ $C_{submax}$) | ACL[a] | CPI[b] | $C_{range}$ ($C_{\mathbf{max}}$) | ACL[a] | CPI[b] |
| Initial leaves | $C_{12}$-$C_{33}$ ($C_{\mathbf{27}}$) | 26.4 | 9.9 | $C_9$-$C_{28}$ ($C_{\mathbf{16}}$, $C_{28}$) | 16.1 | 0.0 | $C_{14}$-$C_{30}$ ($C_{\mathbf{28}}$) | 25.3 | 0.0 |
| Pond | $C_{25}$-$C_{29}$ ($C_{\mathbf{27}}$) | 27.0 | 54.5 | $C_9$-$C_{24}$ ($C_{\mathbf{16}}$) | 15.8 | 0.0 | $C_{16}$-$C_{30}$ ($C_{\mathbf{28}}$) | 26.4 | 0.1 |
| Soil | $C_{12}$-$C_{33}$ ($C_{27}$) | 26.9 | 14.7 | $C_9$-$C_{24}$ ($C_{\mathbf{16}}$) | 16.6 | 0.1 | $C_{16}$-$C_{30}$ ($C_{\mathbf{28}}$) | 26.2 | 0.0 |

[a] $\Sigma(n\times[C_n]) / \Sigma[C_n]$

[b] $[C_{17+19+21+23+25+27+29+31+33}]/[C_{16+18+20+22+24+26+28+30+32}]$

**Highlights**

•Biomarker content varied substantially in soil and at water-sediment interface. •Almost all lipids still present after 2 yr degradation but had different decay profiles. •Although fatty lipid content decreased to <10% after 2yr, main distribution pattern unchanged.

**Graphical Abstract**

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