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Abundance of lipids in differently sized aggregates depends on their chemical composition

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Abstract Evidence for a vital role of soil mineral matrix interactions in lipid preservation is steadily increasing. However, it remains unclear whether solvent-extractable ('free') or hydrolyzable ('bound') lipids, including molecular proxies, e.g., for cutin and suberin, are similarly affected by different stabilization mechanisms in soil (i.e., aggregation or organo-mineral association). To provide insights into the effect of these stabilization mechanisms on lipid composition and preservation, we investigated free and bound lipids in particulate and mineral soil fractions, deriving from sand- and silt-/clay-sized aggregates from a forest subsoil. While free lipids accumulated in sand-sized aggregates, the more complex bound lipids accumulated in silt- and clay-sized aggregates, particularly in the respective mineral fractions < 6.3 µm (fine silt and clay). The presence of both, cutin and suberin markers indicated input of leaf- and root-derived organic matter to the subsoil. Yet, our cutin marker (9,10,ω-trihydroxyoctadecanoic acid) was not extracted from the mineral aggregate compartments < 6.3 µm, perhaps due to its chemical structure (i.e., cross-linking via several hydroxy groups, and thus higher 'stability', in macromolecular structures). Combined, these results suggest that the chemical composition of lipids (and likely also that of other soil organic matter compounds) governs interaction with their environment, such as accumulation in aggregates or association with mineral soil compartments, and thus indirectly influences their persistence in soil.

Keywords Cutin · Suberin · *Alnus glutinosa* L. · Organo-mineral association · Particulate organic matter · Subsoil

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Introduction

Lipids are frequently used as molecular proxies to reconstruct vegetation patterns or to investigate turnover and origin of soil organic matter (SOM) (e.g., Lichtfouse et al. 1995; Feng et al. 2010; Wiesenberg

et al. 2010b). Depending on whether they are part of polymerized organic structures or not, lipids are often separated into a solvent-extractable ('free') and a hydrolyzable ('bound') compartment. While free lipids in soil/sediment indicate the vegetation they derive from (Bull et al. 2000; Diefendorf et al. 2011; Freimuth et al. 2017), bound lipids may be diagnostic for the above- or belowground origin of SOM, depending on their occurrence in the aliphatic macromolecule cutin, exclusive to cuticles aboveground (leaves, fruits, flowers), or the aliphatic/aromatic biopolymer suberin, exclusive to periderms (roots, bark, tubers, stolons; Kolattukudy 1980; Kögel-Knabner 2002).

In soil science, the application of these and other molecular proxies has exponentially increased during the last decade (Jansen and Wiesenberg 2017), of which most studies rely on the (often un-replicated) extraction of free and bound lipids from bulk soil (e.g., Otto and Simpson 2006; Gocke et al. 2013; Angst et al. 2016b). This approach, however, entails potential biases (e.g., with respect to estimation of vegetation chronology, SOM turnover, or leaf versus root contribution to SOM) because lipid preservation may vary within different soil compartments (Poirier et al. 2005; Wiesenberg et al. 2010a; Van der Voort et al. 2017). Lipids seem to quickly decompose within the non-protected, 'light' particulate organic matter (POM) fractions (Griepentrog et al. 2015; Angst et al. 2016a), while interaction with soil mineral compartments enhances their preservation (Wiesenberg et al. 2010a; Lin and Simpson 2016). For example, Lin and Simpson (2016) observed drastically increasing yields of cutin and suberin markers (81–98%) after bulk soils were treated with hydrofluoric acid (HF). For free lipids, Li et al. (2017) obtained similar results. Lipid concentrations in soil may thus be largely governed by either organo-mineral association (molecular interactions between organic matter and inorganic soil components) or aggregation (physical exclusion of decomposers/enzymes from organic matter occluded within the aggregate; von Lützow et al. 2006).

Despite these recent advances in our understanding of lipid preservation in soil, it remains unclear whether aggregation and organo-mineral association operate similarly across different lipid classes (e.g., free or bound) or whether individual lipids within these classes (e.g., cutin- or suberin-derived) show different behavior with respect to physico-chemical stabilization. Gaining a more thorough understanding of the relevance of these processes will not only improve our capacity to interpret lipid patterns in soil, which in turn enhances the understanding of allocation and stabilization mechanisms of SOM, but also help to confine constraining factors in the use of molecular proxies, such as cutin or suberin markers.

Many studies investigate SOM stabilization mechanisms (i.e., aggregation and organo-mineral association) by separating bulk soil by physical means into soil compartments with different characteristics (e.g., turnover time or chemical composition; Sollins et al. 2009; Bimüller et al. 2014; Mueller et al. 2014). Involved in these treatments are combinations of density (e.g., using sodium polytungstate solution), disruption (e.g., slaking, ultrasound) and sieving/sedimentation steps to yield different POM, particle-size, or aggregate fractions, according to the specific requirements. Fractionation protocols vary across studies but specifically aggregate fractionations often focus on sand-sized 'macro-'and 'microaggregates' (Six et al. 2002; Bossuyt et al. 2005; Yavitt et al. 2015). Concentrating on these aggregate-sizes, however, may underestimate the relevance smaller-sized aggregates (i.e, of clay- and silt-size; Chenu and Plante 2006) have in stabilizing SOM (Kölbl et al. 2005; Virto et al. 2008) and lipids in particular (Angst et al. 2017b).

To provide insights into the role individual SOM stabilization mechanisms play in stabilizing free and bound lipids, including cutin- and suberin-derived compounds, and potential differences between sand- and silt-/clay-sized aggregates, we investigated the lipid composition of SOM fractions deriving from a deciduous forest subsoil. We chose subsoil for our analyses because here, effects of physico-chemical SOM stabilization should be better perceivable than in the upper soil layers, where fresh, unprotected SOM often more significantly contributes to total soil organic carbon (SOC) stocks (Poeplau and Don 2013; Angst et al. 2016c). We particularly aimed at isolating POM and mineral compartments from aggregates of different size (sand- vs. silt-/clay-size) to elucidate the impact of individual stabilization mechanisms (physical separation and organo-mineral association) and potential interplay effects on lipid composition in each aggregate class. Because free and bound lipids show different turnover times (Feng et al.

2010) and lipids abundant in roots but not in leaves often predominate in soil (Mueller et al. 2013), we expected lipids to distinctly associate with our soil fractions based on their mode of occurrence (free or bound) and whether deriving from cutin or suberin.

Materials and methods

Soil sampling and sample pre-treatment

Composite soil samples were taken in the B horizon from the profile walls of a Haplic Fluvisol (at 40 cm depth; pH 5.8; A horizon to ~ 10 cm depth) located south of České Budějovice (48°54′10″N 14°28′37″E) in a monocultural alder (*Alnus glutinosa* (L.) Gaertn.) stand, a tree species common to most parts of Europe, the north-eastern USA, and Canada (Funk 1990). The study site was under the same vegetation for at least 20 years (State Administration of Land Surveying and Cadastre 2018). Clay mineralogy, determined on the clay fraction (see below) with a Bruker D8 ADVANCE X-ray diffractometer, was characterized by the presence of kaolinite and illite, including mixed layers of likely vermiculite and illite. Three replicate soil samples were taken at a distance of ~ 1.50 m to the next tree and at a distance of ~ 35 cm from each other to cover potential spatial heterogeneities. Coarse and fine roots and freshly fallen leaves were sampled at the same site. In the laboratory, roots were cleaned from adhering soil by washing with de-ionized water. Subsequently, all plant material was air-dried and aliquots were finely ground using a cutting mill (SM 100; Retsch GmbH, Haan, Germany). The soil samples were air-dried and passed through a 2 mm sieve.

Physical fractionation and organic carbon analysis

To separate aggregates larger and smaller than 63 µm (sand- and silt-/clay-sized) and associated organic matter, aliquots of the air-dried and sieved soils (30 g) were subjected to a combined density and particle-/aggregate-size fractionation (similar to the approach used by Puget et al. 2000; Fig. 1). We chose a cutoff of 63 µm for our aggregate classes because the often used cutoffs of 53 and 250 µm (e.g., Six et al. 2002) set the focus on sand-sized aggregates but neglect smaller aggregates that have recently been shown to be highly relevant to lipid and SOM stabilization (Virto et al. 2008; Angst et al. 2017b). Samples were submerged in 150 ml of a sodium-polytungstate solution (SPT; TC-Tungsten compounds, Grub am Forst, Germany) with a density of 1.8 g cm^{-3}. Shortly after submersion, slaking—the breakdown of aggregates by the build-up of air pressure (Blankinship et al. 2016)—occurred, releasing POM occluded within unstable aggregates (Six et al. 2002). This POM, together with the POM freely occurring in the soil (i.e., not occluded), was removed from the solution as a floating "fPOM" fraction. The fPOM fraction was washed with de-ionized water until the conductivity of the eluted water was below 5 µS, dried (at 40 °C), and stored until further analysis.

The remaining soil residue, containing slaking-resistant aggregates (from now on termed "stable aggregates") and primary mineral particles, was wet-sieved through a 63 µm sieve to separate stable sand-sized from stable silt- and clay-sized aggregates and 'primary particles' (see also Chenu and Plante 2006). The fraction remaining on the 63 µm sieve (containing sand and sand-sized stable aggregates) was washed with de-ionized water until the electrical conductivity of the eluted water was below 50 µS and dried at 40 °C. The dried fraction > 63 µm was then dispensed in SPT solution (1.8 g cm^{-3}) at a solid:liquid ratio of 1:5 and sonicated (440 J ml^{-1}; Kaiser and Berhe 2014) using a calibrated ultrasonic probe to release POM occluded within stable aggregates > 63 µm ($oPOM_{large}$). The soil-SPT slurry was centrifuged and $oPOM_{large}$ was removed analogous to the fPOM fraction. The slurry was shaken, centrifuged again, and floating $oPOM_{large}$ removed. By repeating this step three to five times (depending on the amount of $oPOM_{large}$ present), all $oPOM_{large}$ was removed from the SPT. The $oPOM_{large}$ was washed with de-ionized water until the electrical conductivity of the eluted water was below 5 µS, dried at 40 °C, and stored until further analysis. The remaining mineral residue was washed with de-ionized water until the electrical conductivity was below 50 µS and the combined fine-silt and clay fraction was separated from the medium/large silt and sand fraction by sedimentation and subsequent sieving. All fractions were dried at 40 °C and stored in glass vials until further analysis.

Corresponding fractions of the soil that passed the 63 µm sieve used in the first sieving step were recovered analogously (using sonication at 500 instead of 440 J ml^{-1}; Kaiser and Berhe 2014) and

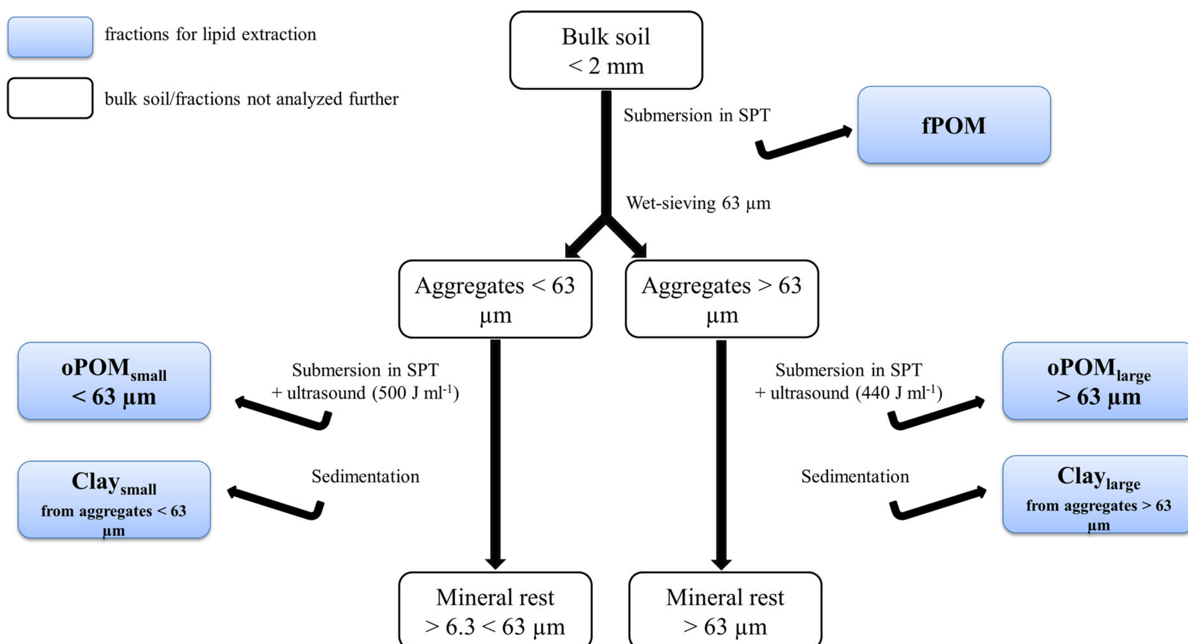

Fig. 1 Overview of the physical fractionation approach

collectively yielded a fPOM fraction, four soil fractions from the soil remaining on the 63 µm sieve (sand, medium/large silt from aggregates > 63 µm, the combined fine-silt and clay fraction from aggregates > 63 µm, termed $clay_{large}$ hereafter, and $oPOM_{large}$), and four soil fractions from the soil passing the 63 µm sieve (medium/large silt from aggregates < 63 µm, the combined fine-silt and clay fraction from aggregates < 63 µm, termed $clay_{small}$ hereafter, and POM occluded within aggregates < 63 µm, termed $oPOM_{small}$ hereafter). We acknowledge that fractionation may partly change the chemical composition of SOM within the fractions by using water or SPT (e.g., Crow et al. 2007). However, C loss during fractionation is likely minor (Plaza et al. 2012) and, due to the poor water solubility of the target compounds, we believe that potential effects of our fractionation on lipids are negligible.

For determination of total organic C contents, the bulk soil and all fractions were measured on a Fisons Instruments NCS NA 1500 analyser. Fractions larger than 63 µm were finely ground using pestle and mortar (POM fractions) or a ball mill (bulk soil, mineral fractions) prior to analysis. Due to sand and medium/large silt fractions containing small amounts of C (Table 1), only the fPOM, $oPOM_{large}$, $oPOM_{small}$, $clay_{large}$, and $clay_{small}$ fractions were considered for further chemical analyses.

Lipid extraction and measurement

Aliquots of the leaf and root material and the fPOM, $oPOM_{large}$, $oPOM_{small}$, $clay_{large}$, and $clay_{small}$ fractions containing at least 5 mg C were treated with a dichloromethane:methanol (DCM:MeOH, 9:1, v:v) solution to remove solvent-extractable lipids. To this end, the POM fractions were weighed into centrifuge tubes (several hundred mg), 4 ml of DCM:MeOH was added, the samples were sonicated in an ultrasonic bath for 10 min, subsequently centrifuged, and the supernatant transferred to glass vials. Fresh DCM:MeOH was added to the residual POM and the procedure repeated five times. All DCM-MeOH extracts were combined and dried under nitrogen. Due to higher available amounts (several g) and lower C contents of the clay fractions, solvent-extractable lipids were Soxhlet extracted from these fractions for 24 h using DCM:MeOH (Naafs et al. 2004a; Wiesenberg et al. 2010a; Pisani et al. 2015). We used a DCM:MeOH mixture with a ratio of 7.5:1 (v:v) in the solvent reservoir, corresponding to a ratio of approximately 9:1 (v:v) during the extraction;

Table 1 Weight of fractions in mg per g bulk soil and C contents of bulk soil, leaves, roots, and fractions

	Weight of fraction (mg g^{-1} bulk soil)	C (mg g^{-1})
Bulk soil	–	3.3
Leaves	–	403.9
Roots	–	479.2
fPOM	0.6 ± 0.07d	319.6 ± 15.7a
$oPOM_{large}$	1.1 ± 0.5d	299.5 ± 65.4a
$oPOM_{small}$	0.8 ± 0.3d	371.0 ± 22.9a
Sand	648.0 ± 13.6a	0.4 ± 0.01b
$Silt_{large}$	84.2 ± 11.6c	1.7 ± 0.1b
$Silt_{small}$	116.1 ± 5.3b	1.8 ± 0.05b
$Clay_{large}$	11.5 ± 3.0d	17.7 ± 1.8b
$Clay_{small}$	137.8 ± 15.8b	9.9 ± 0.2b

Significant differences ($p < 0.05$) are indicated by lower case letters

hence an extraction similar to the POM fractions. The extracts were dried by a gentle stream of nitrogen.

The solvent-extractable lipids and the residual POM fractions were saponified with 1 M KOH in MeOH (96%) for 3 h at 70 °C in 4 ml vials. The residual clay fractions were saponified by refluxing in 1 M KOH in MeOH (96%) for 3 h (similar to the approach used by Nierop et al. (2003), Otto and Simpson (2006), or Angst et al. (2017a)). The reaction mixtures were allowed to cool, and subsequently acidified to pH 1 using a 2 M HCl solution and washed with DCM at least three times or until the DCM phase was colorless (Angst et al. 2016a, 2017a). The combined DCM phases were filtered over Na_2SO_4 and dried under nitrogen. Known amounts of squalane were added to the extracts as an internal standard and the extracts were subsequently methylated (using diazomethane) and silylated (using *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA; Sigma) in pyridine) prior to GC analyses. From now on, lipids released by solvent extraction and subsequent saponification of these extracts are referred to as 'free lipids' and lipids extracted by the hydrolysis of solid residues after solvent extraction are referred to as 'bound lipids' ('free/bound lipid extracts' analogously).

For quantitation of the analytes, all samples were measured on a HP 6890 series GC (CP-Sil 5 CB column; 30 m length, 320 µm diameter, 0.1 µm film thickness) coupled to a flame ionization detector (FID; detector temperature 330 °C) and operated at a constant pressure of 100 kPa. The concentration of each compound was normalized to the C content of each sample (expressed as mg per g C). For qualification of the analytes (aided by a mass spectral library and previously published spectra; Nierop and Verstraten 2004), selected samples were measured on an Agilent 7890B series GC (CP-Sil 5 CB column; 25 m length, 320 µm diameter, 0.12 µm film thickness) at a constant He flow of 1.6 ml/min coupled to an Agilent 5977B MSD mass spectrometer. The transfer line temperature was 320 °C, the source temperature 250 °C, and the scanning mass range *m/z* 50–800. Samples were dissolved in ethyl acetate and injected cold on-column. The temperature program was the same for both GCs: The initial oven temperature was 70 °C, ramped at 20 °C/min to 130 °C, then at a rate of 4 °C/min to 320 °C and held at that temperature for 20 min. We particularly focused on alcohols and fatty acids because these compounds, due to their functional hydroxy- and carboxy-groups, are likely more prone to interactions with the soil mineral phase than e.g., alkanes (Kleber et al. 2005).

We evaluated the extraction efficiency of our approach for the POM fractions by measuring selected samples using pyrolysis-GC/MS. Pyrolysis was carried out on a Horizon Instruments Curie-Point pyrolyser. Samples (typically 1–2 mg) were pressed onto Ni/Fe Curie point wires and subsequently heated for 5 s at 600 °C. The pyrolysis unit was directly connected to a Carlo Erba GC8060 gas chromatograph and the products were separated by a fused silica column (Varian, 25 m, 0.32 mm i.d.) coated with CP-Sil5 (film thickness 0.40 µm). Helium was used as carrier gas. The GC column was directly connected to the pyrolysis unit through a splitless injector set at 280 °C. The oven was initially kept at 40 °C for 1 min, next it was heated at a rate of 7 °C/min to

320 °C and maintained at that temperature for 15 min. The column was coupled to a Fisons MD800 mass spectrometer (mass range *m/z* 45–650, ionization energy 70 eV, cycle time 0.7 s). The chromatograms indicated only a trace of dihydroxyhexadecanoic acids and the absence of monohydroxy and diacids in the lipid region, suggesting a thorough extraction.

Hydrofluoric acid treatment

We treated selected samples of the $clay_{large}$ and $clay_{small}$ fractions with HF to remove the mineral phase and test whether some lipids had been so strongly bound to these fractions that they were not extractable by our sequential lipid extraction procedure (cf. Hernes et al. 2013 or Lin and Simpson 2016). Briefly, 0.3–1.2 g of the samples were weighed into plastic beakers (depending on the amount available), saturated with 15 ml of 10% HF, and shaken for 2 h. The HF was decanted and the beakers filled with concentrated HCl. De-ionized water was then added to the beakers, they were shaken, centrifuged, and the supernatant water decanted. This step was repeated until the pH of the supernatant water was $\sim$ 6 to 7. Samples were then dried. The dry residues were saponified again and measured on the GC analogously to the non-HF treated samples (cf. Section "Lipid extraction and measurement").

To assess potential losses of SOM, we also treated two bulk soil samples with HF: one non-extracted sample and one after extraction with DCM:MeOH (9:1, v:v). After the HF treatment, both samples were extracted with DCM:MeOH again and all extracts were qualified and quantified via GC–MS and GC-FID. The summed lipid concentrations of the pre-extracted sample (before and after HF) were compared to the lipid concentrations of the non-extracted sample (after HF only). We regarded the difference between these two samples as an approximate estimate of lipid loss due to the HF treatment.

Statistics and calculations

To assess statistically significant differences between individual and summed lipids of each type among fractions, we performed one-way analysis of variance (ANOVA) or the Kruskal–Wallis test after having tested the data for normality and homoscedasticity, using Shapiro–Wilk and Bartlett test, respectively. The Tukey HSD (following ANOVA) and Dunn's test (following Kruskal–Wallis) were used as post hoc tests. Differences were regarded as significant ($p < 0.05$), marginally significant ($p < 0.1$), or non-significant ($p > 0.1$). All analyses were performed using the statistical software R for Windows (R Development Core Team 2016).

Results

Soil fractions and C contents

Our fractionation approach yielded eight different fractions, i.e., three POM fractions and five mineral fractions. The amount of the POM fractions did not differ between whether the POM was recovered as fPOM, POM occluded within aggregates > 63 µm ($oPOM_{large}$), or POM occluded within aggregates < 63 µm ($oPOM_{small}$). Although these fractions contributed only minor amounts of weight to the bulk soil (0.6–1.1 mg g^{-1} bulk soil; Table 1), their C contents exceeded those of the mineral fractions by a factor of 20–100 ($p < 0.05$; Table 1).

The mineral fractions were dominated by the sand fraction, while the yield of the silt fraction was 6–8 times lower ($p < 0.05$). Because these mineral fractions contained minor amounts of C (C contents of 0.4–1.8 mg g^{-1}) and have been shown to be less relevant to the stabilization of SOM as compared to the fractions < 6.3 µm (Mueller et al. 2009; Angst et al. 2016c), we decided to focus our analyses and interpretations on the $clay_{large}$ and $clay_{small}$ (cf. Section "Physical fractionation and organic carbon analysis"). The yield of these fractions differed by a factor of $\sim$ 10 ($p < 0.05$) based on whether they were recovered from aggregates > 63 µm ($clay_{large}$) or occurred as primary particles and/or within aggregates < 63 µm ($clay_{small}$). Although $clay_{small}$ tended to have a smaller C content than $clay_{large}$, C contents between these fractions did not differ statistically (Table 1).

Lipids in plant material and soil fractions

We divided the compounds released by the solvent extraction and saponification into *n*-alcohols, *n*-carboxylic acids ('*n*-acids'), α,ω-dicarboxylic acids ('diacids'), ω-hydroxycarboxylic acids ('ω-hydroxy

acids'), and mid-chain substituted hydroxycarboxylic acids ('mid-chain acids'). In the following Sections, the lipids are presented according to their mode of occurrence, i.e., free or bound.

Free lipids

The total amount of free lipids extracted from the leaves and roots was 20.6 and 10.6 mg g C^{-1}, respectively (Table 2). These lipid yields were mainly caused by high concentrations of *n*-alcohols, and saturated and unsaturated C_{16} and C_{18} *n*-acids (Table 2), together accounting for 75.7 (leaves) and 77.9% (roots) of total free lipids. Concentrations of ω-hydroxy acids with a similar or higher chain length (C_{16}–C_{26}) did not exceed 0.7 mg g C^{-1} and the only free diacid released was α,ω-octadec-9-enedioic acid ($C_{18:1}$DA). Free mid-chain acids were not detected in the plant material.

In the soil fractions, the sum of free lipids ranged from as little as 1.5 mg g C^{-1} in fPOM to as much as 21.6 mg g C^{-1} in $clay_{large}$ (Fig. 2a). The large amount of lipids in the latter fraction was mainly due to very high concentrations (up to 13 times higher as compared to the other fractions) of *n*-alcohols and *n*-acids. Their chain lengths ranged from C_{14} to C_{30} for *n*-acids (C_{16} and C_{18} were most abundant; Online Resource 1a) and from C_{12} to C_{30} for *n*-alcohols (C_{18} was most abundant; Online Resource 2a). In contrast to the *n*-alcohols and *n*-acids, concentrations of the ω-hydroxy acids, ranging from C_{12} to C_{26} in chain length (Online Resource 1a), did not differ among fractions (Fig. 2a). Diacids and mid-chain acids were not detected in the solvent extracts.

Bound lipids

The concentrations of total bound lipids from leaves and roots were 11.3 mg g C^{-1} and 8.6 mg g C^{-1}, respectively (Table 2). Although *n*-alcohols and acids with various chain lengths and functional groups were released from both plant materials, more than 50% of the total amount of bound lipids were represented by only a small group of acids. For leaves, these were the x,ω-dihydroxyhexadecanoic acids (x,ω-C_{16}; x = 8, 9, or 10) and for roots, these comprised the ω-hydroxy acids (ω-C_{16}–ω-C_{26}; Table 2). While concentrations of these and other acids between leaves and roots varied 3–25 fold, most of the extracted acids were present in both plant materials. The only acid exclusively extracted from leaves was the 9,10,ω-trihydroxyoctadecanoic acid (9,10,ω-C_{18}) and the acids exclusively extracted from roots comprised α,ω-docosanedioic acid (C_{22}DA) and C_{20}, C_{24}, and C_{26} ω-hydroxy acids (ω-C_{20}, ω-C_{24}, ω-C_{26}).

In the soil fractions, the sum of bound lipids was lowest in oPOM (large and small) and $clay_{large}$, tended to be higher in fPOM, and was highest in $clay_{small}$ (Fig. 2b). This latter fraction had very high *n*-alcohol concentrations (up to fold 50 fold higher as compared to the other fractions), but also *n*-acids and ω-hydroxy acids contributed to the high amounts of bound lipids in $clay_{small}$ (Fig. 2b). The slightly higher concentrations of total bound lipids in fPOM as compared to oPOM (large and small) and $clay_{large}$ was mainly due to higher concentrations of the ω-hydroxy and, partly, mid-chain acids in this fraction (Fig. 2b). Among the fractions, these acids decreased (ω-hydroxy acids) or tended to decrease (mid-chain acids) from fPOM to $oPOM_{large}$, $oPOM_{small}$, and $clay_{large}$, while this pattern was not observed for *n*-alcohols or *n*-acids, and only partly for diacids ($p > 0.1$; Fig. 2b).

After HF treatment, the chromatograms of the re-saponified clay fractions were barren (Online Resource 3). Our 'test approach' on bulk soil (cf. chapter 2.4) indicated that approximately 28% of lipids were lost during the HF treatments. Assuming that all organic components are affected similarly by the HF treatment (Schmidt et al. 1997; Gonçalves et al. 2003), this percentage is in the range of C loss values reported in the literature (Gonçalves et al. 2003; Rumpel et al. 2006). Even if taking these losses into account, saponification of $clay_{large}$ and $clay_{small}$ removed the major part of bound lipids from these fractions (Online Resource 3).

Discussion

Quantity and sources of extracted lipids and identification of cutin and suberin markers

The quantity of extracted free and bound lipids from our plant material and soil was partly in the range of lipid concentrations found in plant tissues of other tree species or soil types (e.g., Nierop et al. 2005; Mueller et al. 2012; Angst et al. 2016b), but sometimes, especially for the plant material, deviated from these

Table 2 Free and bound lipids extracted from alder leaves and roots in mg g C^{-1}, organized according to functional groups (*n*-alcohols, *n*-acids, diacids, ω-hydroxy acids, mid-chain acids)

	Lipids (mg g C^{-1})			
	Free		Bound	
	Leaves	Roots	Leaves	Roots
n-Alcohols				
Octadecanol (C_{18})			0.15	
Eicosanol (C_{20})	0.03			
Docosanol (C_{22})	1.35	0.65	0.14	0.64
Tetracosanol (C_{24})	0.99	0.45	0.07	0.14
Pentacosanol (C_{25})	0.06			
Hexacosanol (C_{26})	0.83	0.37	0.05	
Octacosanol (C_{28})	1.37		0.10	
Sum of *n*-alcohols	4.62	1.46	0.37	0.78
n-Acids				
Tetradecanoic acid (C_{14})	0.11		0.06	0.04
Hexadecenoic acid ($C_{16:1}$)	0.16	1.73		
Hexadecanoic acid (C_{16})	2.79	1.97	1.60	0.55
Octadecenoic acid ($C_{18:1}$)	7.47	2.87	0.26	0.82
Octadecanoic acid (C_{18})	0.58	0.22	0.28	0.03
Eicosanoic acid (C_{20})	1.13	0.14	0.02	0.02
Heneicosanoic acid (C_{21})	0.07			
Docosanoic acid (C_{22})	1.04	0.16	0.11	0.06
Tricosanoic acid (C_{23})		0.16		
Tetracosanoic acid (C_{24})	0.40	0.17	0.10	0.06
Pentacosanoic acid (C_{25})	0.07	0.10		
Hexacosanoic acid (C_{26})	0.21	0.49	0.10	0.03
Octacosanoic acid (C_{28})	0.26	0.38	0.07	0.02
Sum of *n*-acids	14.30	8.40	2.60	1.63
Diacids				
α,ω-Hexadecanedioic acid (C_{16}DA)			0.06	0.36
α,ω-Octadecenedioic acid ($C_{18:1}$DA)	0.89		1.00	1.18
α,ω-Docosanedioic acid (C_{22}DA)				0.49
Sum of diacids	0.89	0.00	1.06	2.03
ω-Hydroxy acids				
ω-Hydroxyhexadecanoic acid (ω-C_{16})		0.13	0.66	0.86
ω-Hydroxyoctadecenoic acid (ω-$C_{18:1}$)	0.43		0.71	0.17
ω-Hydroxyeicosanoic acid (ω-C_{20})				0.30
ω-Hydroxydocosanoic acid (ω-C_{22})	0.05	0.39	0.10	1.77
ω-Hydroxyetradecanoic acid (ω-C_{24})		0.20		0.67
ω-Hydroxyhexadecanoic acid (ω-C_{26})				0.11
Sum of ω-hydroxy acids	0.48	0.72	0.15	3.90
Mid-chain acids				
x,ω-Dihydroxyhexadecanoic acid (x,ω-C_{16})			5.76	0.25
9,10-Dihydroxyoctadecanoic acid (9,10-C_{18})	0.34	0.01		
9,10,ω-Trihydroxyoctadecanoic acid (9,10,ω-C_{18})			0.10	
Sum of mid-chain acids	0.34	0.01	5.86	0.25
Sum of extracted lipids	20.61	10.60	11.34	8.58

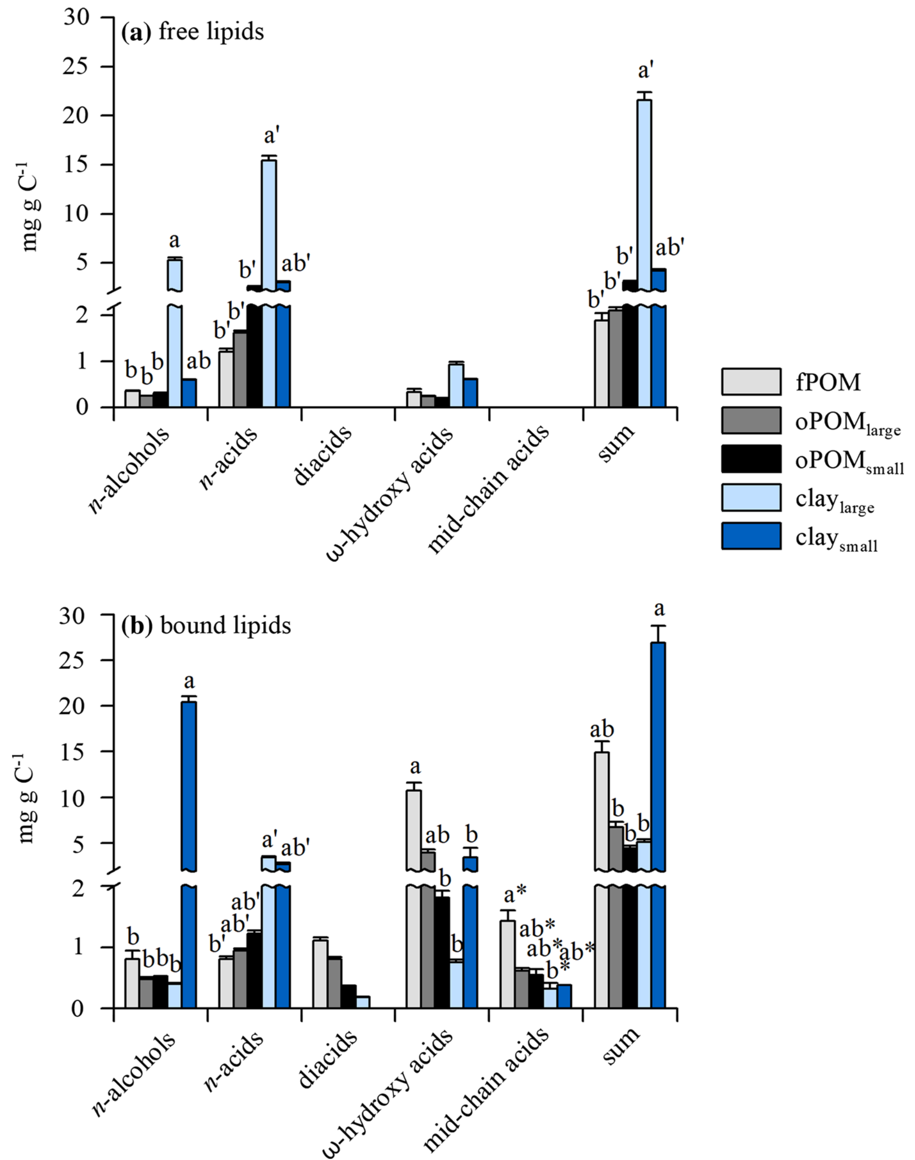

Fig. 2 Free (**a**) and bound lipids (**b**) extracted from the different soil fractions depicted as total concentrations (sum) and separated into compounds with different functional groups. Lower case letters not followed by an asterisk or a quotation mark indicate significant differences at $p < 0.05$, lower case letters followed by an asterisk indicate marginal significant differences at $p < 0.06$, and lower case letters followed by a quotation mark indicate marginal significant differences at $p < 0.07$. Non-significant differences are not labelled. Error bars display standard errors

by several factors (from − 2 to 11). This deviation most likely occurred because lipid composition in plants and the underlying soil may vary with environmental conditions and specific plant genetics and traits (even within the same plant species; Mueller et al. 2012; Freimuth et al. 2017).

The free lipid extracts from the alder leaves and roots were dominated by even *n*-alcohols (C_{12}–C_{30}) with a maximum at C_{20} (together with C_{28} for leaves) and even *n*-acids (C_{14}–C_{30}) maximizing at C_{16} and C_{18} (Table 2). The minor amount of ω-hydroxy acids appearing in the free lipid extracts were derived from cutin and/or suberin (Kolattukudy 1980; Naafs et al. 2004a). The lipid patterns of roots were well reflected in the soil fractions in as much as *n*-alcohols, *n*-acids, and ω-hydroxy acids were present in each fraction in similar relative amounts as found in the alder roots (*n*-acids:alcohols:ω-hydroxy acids = 11:5:1 in roots and 9:5:1 in soil fractions; Fig. 2a). However, the chain lengths dominating in plants were only in part resembled by the lipid patterns in the fractions (C_{16} and $C_{18:1}$ *n*-acids; Online Resources 1a and 2a), and in soil, *n*-alcohols and *n*-acids < C_{20} may also derive from microorganisms (Lichtfouse et al. 1995; Naafs

et al. 2004b; Jansen et al. 2006). Because microorganisms, fungi in particular, have been shown to be capable of producing lipids with higher chain lengths under acid conditions ($> C_{20}$; Jambu et al. 1978), some of the *n*-alcohols and *n*-acids $> C_{20}$, commonly dominating in plant tissues, probably also derive from microorganisms in our (moderately acidic) soil samples. This may be particularly true for the subsoil we sampled, because here plant inputs are commonly reduced and, as compared to the topsoil, microbial-derived compounds may be more relevant contributors to SOM (Liang and Balser 2008; Wallander et al. 2009).

Some of the free lipids in our soil fractions could also derive from previous vegetation; cutin/suberin-derived ω-hydroxydodecanoic (ω-C_{12}) and ω-hydroxytetradecanoic (ω-C_{14}) acids were extracted from $clay_{small}$, but were not present in the recent vegetation (Table 1; Online Resource 1a). These hydroxy acids derive from Pinaceae (Nierop and Verstraten 2004; Feng et al. 2010; Pisani et al. 2013), and were likely protected from decomposition via organo-mineral association. Similarly, the presence of free C_{30} *n*-alcohol in $clay_{large}$ but its absence from alder leaves and roots points to a contribution from sources different from alder.

When aiming at investigating the contribution of genuine plant-derived lipids to subsoil organic matter, the bound lipids may be more diagnostic than the free lipids (Nierop et al. 2006). Apart from minor amounts of *n*-alcohols and *n*-acids (as compared to the free lipid extracts), the bound leaf and root extracts contained diacids, ω-hydroxy acids, and mid-chain acids that indicate alder as a source of SOM. Depending on their species specific occurrence in leaves and roots, these acids may further distinguish cutin- (leaf) from suberin- (root) derived SOM. Monomers exclusively occurring in the alder roots and corresponding to previously suggested suberin markers of other species were α,ω-docosanedioic acid (C_{22}DA) and ω-hydroxy acids with chain lengths of C_{20}, C_{24}, and C_{26} (Andreetta et al. 2013, Angst et al. 2016b). The only plant-derived acid distinct to alder leaves was 9,10,ω-trihydroxyoctadecanoic acid (9,10,ω-C_{18}). Surprisingly, x,ω-dihydroxyhexadecanoic acids (x,ω-C_{16}), often used as cutin markers in forest soils (Riederer et al. 1993; Filley et al. 2008; Feng et al. 2010), were present also in alder roots, though lower by a factor of 23. Despite the large concentration differences, we did not use these acids as cutin markers because subsoil likely receives its main organic matter input from roots (Rasse et al. 2005; Jackson et al. 2017; Nierop 1998), making them the most probable source of x,ω-C_{16} acids in our soil fractions.

Consequently, while the combination of C_{22}DA, ω-C_{20}, ω-C_{24}, ω-C_{26} acids from the bound lipids and ω-C_{24} acid from the free lipids as suberin markers for each soil fraction (Table 1, Fig. 3) enables a relatively sound interpretation of root-derived SOM, inferences about leaf-derived SOM based only on 9,10,ω-C_{18} acid as sole cutin marker may be complicated due to its comparatively low concentrations in leaves and a potential contribution of lipids from previous vegetation at the site (see above). However, the fact that concentrations of 9,10,ω-C_{18} acid in fPOM were high (Fig. 3), a fraction that has turnover times of < 10 to ~ 20 years (Gaudinski et al. 2000; von Lützow et al. 2007; Herold et al. 2014) and is (apart from commencing decomposition processes) chemically most similar to the plant input (Grandy and Neff 2008), implies that at least part of this acid in our fractions is derived from alder cutin. We thus assigned 9,10,ω-C_{18} acid to being cutin-derived but cautiously interpret its patterns in the soil fractions. These 'cutin' and suberin markers, but also less specific monomers (occurring in both cutin and suberin), such as C_{16}DA or ω-C_{16}, were detected in the soil fractions (Fig. 3 and Online Resources 1b and 2b), indicating recent alder leaf and root inputs to the subsoil investigated. The bound *n*-alcohols and *n*-acids in the fractions may derive from various sources, either from

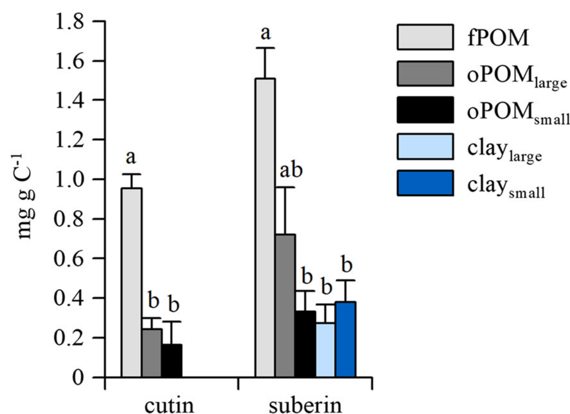

Fig. 3 Cutin and suberin markers in the different soil fractions. Lower case letters indicate significant differences at $p < 0.05$. Error bars display standard errors

microorganisms or plants (such as cutin/suberin; Otto and Simpson 2006).

Distribution of lipids across SOM fractions

The most striking pattern in the free lipid extracts was the preferential accumulation of *n*-alcohols and *n*-acids in the < 6.3 µm fraction from sand-sized aggregates ($clay_{large}$; Fig. 2a), which are likely dominated by relatively fresh or partly decomposed plant inputs (Oades 1984; Baisden et al. 2002; Six et al. 2004). Due to their chemical characteristics (i.e., not part of a larger macromolecule) free lipids may quickly dissociate from these inputs (Bridson 1985) and attach with reactive mineral surfaces in $clay_{large}$ together with short-chain lipids from associated microbes (Online Resources 1a and 2a; Naafs et al. 2004a). When the free lipid concentrations in $clay_{large}$ are combined with those in $oPOM_{large}$ (these fractions containing major amounts of C in aggregates > 63 µm; Table 1), 72.2% of free lipids were contained in sand-sized aggregates. These results indicate that the persistence of free lipids (particularly *n*-alcohols and *n*-acids) in soil may at least partly depend on the degree of (macro-)aggregation (cf. Angst et al. 2017b).

However, only a minor amount of free lipids (22.1%) was contained within silt- and clay-sized aggregates ($oPOM_{small}$ + $clay_{small}$; Fig. 2a). These aggregates contain more microbial-derived and increasingly processed SOM (John et al. 2005; Wagai et al. 2009; Trivedi et al. 2015), where other compounds than free lipids may be more relevant to total SOM. This was probably also the reason why $clay_{small}$ (as compared to $clay_{large}$) was enriched in the more complex bound lipids (Cranwell 1981; Meyers and Ishiwatari 1993), from which cross-links need to be cleaved before these compounds may dissociate from macromolecular structures and associate with the soil mineral matrix. Particularly, bound *n*-alcohols (C_{16}–C_{28}) were highly abundant in $clay_{small}$ (Fig. 2b), exceeding the concentrations in the plant material by a factor of ~ 26 to 56 and potentially reflecting a major microbial contribution (Naafs et al. 2005). However, the high abundance of bound *n*-alcohols may also indicate accumulation of cutin/suberin compounds in $clay_{small}$, being in line with high concentrations of plant-derived ω-hydroxy acids (and, partly, mid-chain acids) in this fraction (Fig. 2b). Due to their mode of occurrence (bound), these lipids probably remain in macromolecular structures for a longer period of time; thus their abundance gradually decreases with increasing decomposition of the POM fractions (Fig. 2b), and they finally accumulate in silt-/clay-sized aggregates, particularly in $clay_{small}$. When combining the total bound lipid concentrations of the latter fraction with those of $oPOM_{small}$ (these fractions contributing the major amount of C in aggregates < 63 µm; Table 1), 53.9% of bound lipids were contained in aggregates < 63 µm (20.4% in sand-sized aggregates and 25.7% in fPOM). These results indicate a major role of silt- and clay-sized aggregates for the stability of bound lipids, including a distinct contribution of plant-derived lipids, which competes with previous findings of small microaggregates containing little or no plant material (Oades and Waters 1991; Monreal et al. 1997).

Based on the lipid patterns and as far as we can infer aggregate formation from lipid distributions, our data support models of aggregate formation where organic matter inputs, such as POM, form the nucleus of macroaggregates (here represented by $oPOM_{large}$ and $clay_{large}$) in which smaller aggregates (represented by $oPOM_{small}$ and $clay_{small}$) then develop as a consequence of decomposition, fragmentation, and organo-mineral encrustation processes (e.g., Oades 1984). Our data further imply that inherent chemical composition, although having minor relevance for long-term stability (Mikutta et al. 2006; Marschner et al. 2008; Lehmann and Kleber 2015), may play a role in the allocation of lipids (free and bound), and perhaps other SOM components, to different aggregate fractions in which they may subsequently be stabilized.

Cutin and Suberin markers

The distribution of biomarkers across the SOM fractions (Fig. 3) resembled that of all bound ω-hydroxy and mid-chain acids (decreasing from fPOM to $oPOM_{large}$ and $oPOM_{small}$, staying constant in the clay fractions). The large decrease of these acids from the free to the occluded POM fractions indicates that the previously observed rapid decay of cutin and suberin markers in the forest floor (Angst et al. 2016a) also proceeds in the mineral soil as long as the biomarkers are not physico-chemically protected (within oPOM or organo-mineral associations) and soil pH is only moderately acidic (> 5) at which lipids

degrade more rapidly than under more acid soil conditions (van Bergen et al. 1998). While our suberin markers could be detected in all fractions, 9,10,ω-C_{18} acid (our cutin marker) was only extracted from fPOM and both oPOM fractions (Fig. 3). These patterns indicate that leaf input to the subsoil occurred likely via bioturbation (Nierop and Verstraten 2004), but our marker did not seem to associate with the mineral phase. The 9,10,ω-C_{18} acid may be relatively stable within, and still part of, (intact) macromolecular structures, due to several functional hydroxy groups potentially involved in cross-linking (Kolattukudy 1980; Nierop et al. 2003; Angst et al. 2016a). This inference may be supported by the fact that the number of hydroxy groups had a significant positive effect on the concentration of lipids remaining during a short-term decomposition experiment of leaves and roots from coniferous and deciduous forest sites (Angst et al. 2016a). Moreover, less hydrophobic (i.e., having several hydroxy functions and short chain-lengths, such as 9,10,ω-C_{18} acid) versus more hydrophobic lipids (i.e., having less hydroxy functions and longer chain lengths, such as suberin-derived ω-hydroxy acids $> C_{20}$) may have had an effect on lipids entering organo-mineral associations, similar to mechanisms described for sorption of dissolved organic matter compounds to soil and pedogenic oxides (Kaiser and Zech 1997; Guo and Chorover 2003). Apart from these compositional considerations, 9,10,ω-C_{18} acid and other lipids may be so strongly protected by the mineral phase that currently available methods may fail to extract these compounds entirely (Hernes et al. 2013). For example Lin and Simpson (2016) observed a by 81% higher yield of hydrolyzable lipids from a forest soil after it had been treated with HF. In contrast, the bound lipid extracts from the HF treated clay fractions in the current study did not contain any additional cutin or suberin monomers, perhaps because we disrupted aggregate structures by our fractionation method (unlike Lin and Simpson 2016), making SOM within these aggregates more susceptible to the subsequent lipid extractions.

Combined, these interpretations reinforce the notion that aggregates protect cutin- and suberin-derived lipids from decomposition (Angst et al. 2017b) and lipid chemical structure may govern interaction with their environment (e.g., the soil mineral phase; cf. Section "Distribution of lipids across SOM fractions"; Mueller et al. 2013) and thus indirectly influences their persistence in soil.

Conclusions

The distinct enrichment of free and bound lipids in different SOM fractions revealed a potential effect of the lipids' mode of occurrence (free or bound) on their allocation to aggregates of different size (sand-sized aggregates enriched in free lipids; silt-/clay-sized aggregates enriched in bound lipids). Combined with our cutin marker not occurring in mineral soil compartments, potentially due to its chemical properties, our results support the notion that chemical composition governs interaction of SOM compounds, such as (leaf- and root-derived) lipids, with their environment (e.g., differently sized aggregates or the soil mineral phase) and thus indirectly influences their persistence in soil.

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