Soil organic carbon under lockdown: Fresh plant litter as the nucleus for persistent carbon

WITZGALL1*, KRISTINA; VIDAL1, ALIX; SCHUBERT2, DAVID I.; HÖSCHEN1, CARMEN; SCHWEIZER1, STEFFEN A.; BUEGGER, FRANZ3; POUTEAU4, VALERIE; CHENU4, CLAIRE; MUELLER1,5, CARSTEN W.

1 Chair of Soil Science, TUM School of Life Sciences (Weihenstephan), Technical University of Munich, Freising-Weihenstephan, Germany
2 Institute for Organic Farming, Soil and Resource Management, Bavarian State Research Center for Agriculture, Freising-Weihenstephan, Germany
3 Institute of Biochemical Plant Pathology, Helmholtz Zentrum München (GmbH), German Research Center for Environmental Health, Neuherberg, Germany
4 UMR Ecosys, AgroTechParis, Batiment EGER, Thiverval Grignon, France,
5 Department for Geoscience and Environmental Management, University of Copenhagen, Copenhagen, Denmark

*Kristina Witzgall
E-mail: kristina.witzgall@tum.de
Phone: +49 (0) 8161 71 4206
Abstract

The largest terrestrial organic carbon pool, carbon in soils, is regulated by the intricate connection between plant carbon inputs, microbial activity, and soil matrix. This is manifested by how microorganisms, the key players in transforming plant-derived carbon into soil organic carbon, are controlled by the physical arrangement of organic and inorganic soil particles. We studied the role of soil structure on the fate of litter-derived organic matter and we propose that the persistence of soil carbon pools is directly determined at plant–soil interfaces. We show that while microbial activity and fungal growth is controlled by soil structure, occlusion of organic matter into aggregates and formation of organo-mineral associations occur in concert on litter surfaces regardless of soil structure. These two mechanisms—the two most prominent processes contributing to the persistence of organic matter—occur directly at fresh litter that constitutes a key nucleus in the build-up of soil carbon persistence.
Introduction

Sustained by a continuous input of plant-derived carbon (C), soils comprise the largest terrestrial C pool; therefore, it has a decisive role in the global C cycle\(^1,2\). Microbial decomposition is a crucial process in transforming plant-derived organic matter (OM) and in fostering the formation of soil organic matter (SOM). Consequently, the abundance and activity of microorganisms determine the pathway of C from early-stage plant litter residues to persistent SOM\(^3,4\). In turn, the microbiome is controlled by the soil environment, where biological, chemical, and physical factors determine microbial growth and activity. One major factor for the biogeochemical functioning of soils is the 3D arrangement of solids. The physical soil structure defines the porous network, affecting the movement and bioavailability of gases (e.g., CO\(_2\) and O\(_2\)) and water\(^5\). Determined by pore size, the differences in soil water contents can shape ecological niches suitable for certain microbial taxonomic groups. The size of pores also controls the contact between microorganisms and their essential source of energy and nutrients—the litter\(^6\). The effect of soil structure on the functionality of the microbial community can be predicted, e.g., via oxygen availability, which regulates C turnover\(^7\).

Over the last decades, it has become more evident that inherent recalcitrance, \textit{i.e.}, the reduced decomposability due to the chemical composition of OM, is of less importance to SOM persistence compared to soil structure-driven mechanisms that rely on soil aggregation and accessibility of reactive mineral surfaces\(^8,9\). Soil C is mostly stored in the following two major pools: as particulate OM (POM; particulate organic residues mostly of plant origin) and mineral-associated OM (MAOM; OM adhering to mineral surfaces)\(^10,11\). Physical mechanisms, such as the potential of OM compounds to adhere to mineral surfaces\(^12\), or the accessibility of substrates for microorganisms\(^3\), are now paving the way for a better understanding of OM cycling and persistence in soils. This persistence of soil C is regulated in microscale hot spots at which microorganisms transform plant-derived OM into SOM. The functioning of biogeochemical interfaces between plant litter substrates, microorganisms, and soil mineral surfaces requires chemical, physical and biological factors to be considered in consortium.

We applied a systemic approach by investigating how physical soil texture governs the pathway of litter-derived C compounds from initial plant litter into more persistent SOM pools via microbial
transformation in a relevant process scale (µm–mm). To disentangle mineral-microorganism interactions that regulate these processes, we incubated two differently textured soils together with $^{13}$C-labeled litter in a 95-day microcosm experiment. Aside from monitoring CO$_2$ production and litter-derived $^{13}$CO$_2$ release, we followed the alterations in the chemical composition of SOM in POM and MAOM, and the microbial communities and their uptake of litter-derived $^{13}$C into phospholipid fatty acids (PLFA). The intact biogeochemical interfaces between plant residues, microorganisms, and soil minerals were, for the first time, directly studied using nano-scale secondary ion mass spectrometry (NanoSIMS). Our objective was to quantify the interactions between microbial litter decay and the parallel formation of more persistent soil C pools in regard to aggregate formation, and the association of microbial C with mineral surfaces controlled by soil texture.
Results

Litter decomposition and native soil carbon priming. We measured how different soil textures and litter addition (enriched in $^{13}$C, $\delta^{13}$C = 2129 ± 82 ‰ V-PDB) affected the soil heterotrophic respiration by monitoring the soil-CO$_2$ emissions. By analyzing $^{13}$CO$_2$, we were able to differentiate CO$_2$ derived from native soil organic C from CO$_2$ derived from the added litter. We report the CO$_2$-derived C per amount C in incubated samples to directly showcase the mechanistic process level. In the coarse-textured soil, the total native respiration (105.6 mg CO$_2$-C g$^{-1}$ C$_{bulk}$) and the net litter-derived CO$_2$ (47.7 mg CO$_2$-C g$^{-1}$ C$_{bulk}$) were significantly higher than in the fine-textured soil (65.3 mg CO$_2$-C g$^{-1}$ C$_{bulk}$ and 29.0 mg CO$_2$-C g$^{-1}$ C$_{bulk}$, $p < 0.001$, $t = -7.512$ and $t = -6.593$ respectively; df = 8 for both, Fig. 1).

While the litter-derived CO$_2$ accounted for around 30% of the total respiration in both soil textures, the litter addition induced a higher total priming effect in the coarse-textured soil (Fig. 1 b and d), accounting for a net release of 27.5 mg CO$_2$-C g$^{-1}$ C$_{bulk}$ from the native soil organic C in the coarse-textured soil compared to 12.8 mg CO$_2$-C g$^{-1}$ C$_{bulk}$ in the fine-textured soil ($p < 0.001$, $t = -7.686$, df = 8).

Figure 1 | Cumulative heterotrophic respiration in fine- and coarse-textured soil. a Respired CO$_2$-C g$^{-1}$ C$_{bulk}$ during the 95-day incubation in a, b coarse-textured, and c, d fine-textured soil. The total respired CO$_2$-C in soil with b coarser and d finer texture is displayed on the right (means, SDs displayed with errors bars, n = 5), together with the total priming effect. Asterisks represent significant differences between the textures ($***p < 0.001$).
Fate of litter-derived carbon in particulate and mineral-associated OM fractions. We assessed the contribution of OC derived from the decaying litter to the formation of differently stabilized OM pools in two soils with contrasting textures divided into three depths (top, center, and bottom) by soil fractionation according to density and size. The soil-derived C in mg g$^{-1}$ C$_{bulk}$ was similarly distributed across OM fractions for both differently structured soils. The MAOM fraction dominated the C storage in both soils (Fig. 2 a). In the coarse-textured soil, we found a significantly higher litter-derived C content occluded within aggregates (oPOM) (71.1 mg C g$^{-1}$ C$_{bulk}$ compared to 36.8 mg C g$^{-1}$ C$_{bulk}$ in the fine-textured soil, $p = 0.007$, $t = -5.03$, df = 4) and a slightly higher content in the MAOM fraction (101.3 mg C g$^{-1}$ C$_{bulk}$ in the soil with coarser texture compared to 48.8 mg C g$^{-1}$ C$_{bulk}$ in the soil with finer texture, $p = 0.08$, $W = 0$) (Fig. 2 b). Although not statistically significant, a tendency of a higher contribution of litter-derived C recovered as oPOM and MAOM in the coarse-textured soil further extended down with soil depth to the center layer of the microcosms ($p = 0.06$ in both cases, $t = -2.66$ and $-2.60$, respectively; df = 4 for both).

![Figure 2](image-url)  
**Figure 2** | Allocation of soil- and litter-derived C to OM fractions. Content of free POM (fPOM), occluded POM (oPOM, oPOM$_{small}$) and mineral-associated OM (MAOM) in mg C g$^{-1}$ C$_{bulk}$ of a soil and b litter origin in three depths of coarse- and fine-textured soil (means, n = 3). Asterisks represent significant differences between the textures (*$p < 0.05$).

Fresh litter incorporated into soil aggregate structures. The chemical composition of OM fractions was analyzed using $^{13}$C solid-state nuclear magnetic resonance spectroscopy (NMR). Carbohydrates (O/N alkyl C) clearly dominated the NMR spectra of all fPOM fractions (Fig. 3), and a
similar chemical composition was also detected in oPOM fractions of both textures with litter. In oPOM fractions, the added litter had induced an increase in relative intensity from around 50% to 70% in the O/N-alkyl C region, demonstrating the dominance of polysaccharides (mainly cellulose and hemicellulose). According to the molecular mixing model results, this was further supported by the relative increase in carbohydrates (26% in the coarse- and 20% in the fine-textured soil) accompanied by a relative decrease in lignin (−12% in the coarse- and −20% in the fine-textured soil) in oPOM fractions. The incorporation of litter-derived OC into soil aggregate structures was also demonstrated by the decrease in aliphaticity (alkyl:O/N alkyl ratio) in oPOM compared to control samples.

**Figure 3 | Differences in the chemical composition of particulate OM fractions.** Solid-state $^{13}$C-PMAS NMR spectra displaying the chemical compositions of free POM (FPOM) and occluded POM (oPOM, oPOM$_{small}$) in coarse- and fine-textured soil (control samples in black). The chemical shift regions represent the following functional groups: 0-45 ppm (alkyl C), 45-110 ppm (O/N alkyl C), 110-160 ppm (aromatic C), and 160-220 ppm (carboxyl C). n = 3 for the following fractions: FPOM with litter (both textures), oPOM with litter (both textures), oPOM$_{small}$ with litter in the finer texture. For the rest of the samples, n = 1.
**Fungi respond the strongest to litter addition.** The changes caused by litter addition in microbial community structures between the textures were captured via the measurement of microbial-derived PLFA. The litter amendment led to a slight increase in the total PLFA content in the top layers of both the coarse- (61 nmol g$^{-1}$ mg soil C$^{-1}$, $p = 0.07$, $t = -2.40$, df = 4) and fine-textured soil (76 nmol g$^{-1}$ mg soil C$^{-1}$, $p = 0.1$, $t = -2.15$, df = 4), whereas the total PLFA contents in the center and bottom layers were similar to those of the controls (Fig. 4 a and b). While the differences in soil texture had no effect on the overall community structure, a strong response to litter addition was detected in fungal biomarkers. The increase in fungal markers was particularly pronounced in the top layer of the coarse-textured soil where fungal abundance increased by a factor of 5.4 ($p = 0.01$, $t = -4.11$, df = 4) compared to 2.6 in the fine-textured soil ($p = 0.15$, $t = -1.75$, df = 4). As opposed to the other observed microbial groups, the increase in fungal markers also extended into the center layer of microcosms with coarse-textured soil (2.1 nmol g$^{-1}$ mg soil C$^{-1}$ compared to 0.4 nmol g$^{-1}$ mg soil C$^{-1}$ in the control, $p = 0.02$, $t = -3.81$, df = 4) (Fig. 4 c), while there was no corresponding increase in the respective layers in the finer-textured soil (Fig. 4 d).
Figure 4 | Community structures and functionality of microorganisms. The total abundance of phospholipid fatty acids (PLFA) normalized for bulk C in nmol C-FA g⁻¹ mg⁻¹ Cbulk (means, SDs displayed with errors bars, n = 3) in soil with a coarser and b finer texture, divided into four microbial subgroups in c coarse- and d fine-textured soil. Significance levels indicated by dots and asterisks (*p < 0.1, **p < 0.05) represent the differences between the litter treatment and controls, and lowercase letters represent the significant (p < 0.05) differences between the layers. Control samples are displayed as hatched.

When considering the proportion of fatty acids with incorporated litter-derived ¹³C within the observed groups in relation to the total amount of enriched FAs in the sample, neither texture nor depth had an effect (Fig. 5 a). This corroborated the consistent community structures detected during the PLFA analysis. When considering the proportions of ¹³C-enriched FAs to unlabeled FAs within each microbial group, the proportion was by far the highest in the fungal markers (92% in the coarser- and 82% in finer-textured soil, p = 0.11 between textures, t = −2.04, df = 4, Fig. 5 b). This distinction of fungi compared to other microbial groups was significant in top layers of both textures, as well as in the center layer of the coarse-textured soil (over 42% of FAs were enriched compared to 21% in the fine-textured soil). Furthermore, the proportion of enriched gram-negative markers were significantly higher in all layers...
of the coarser-textured soil ($p = 0.004$, $t = -5.79$, $p = 0.019$, $t = -3.83$, and $p = 0.0007$, $t = -9.54$; df = 4 for all) compared to the fine-textured soil.

Formation of MAOM fostered by microbial activity on decaying POM surface. We gained a direct insight on the biogeochemical interface between decaying plant residues (POM), mineral particles, and microorganisms at the microscale using scanning electron microscopy (SEM) and NanoSIMS. Large areas of litter-derived POM particles were covered in $^{13}$C-enriched microbial-derived extracellular polymeric substances (EPS), forming a biofilm-like structure that was intertwined with fungal hyphae and unicellular microorganisms (presumably bacteria). Clay-sized minerals were directly enclosed into the biofilm on the POM surface (Fig. 6 a and b). The microorganisms and EPS were significantly enriched in N compared to the underlying POM, with a higher $^{12}$C:$^{14}$N:$^{13}$C ratio obtained for EPS, followed by hyphae and bacteria (Fig. 6 d). The $^{13}$C:$^{12}$C$^{-}$ ratio for fungal hyphae, bacteria, and EPS (3.0, 2.3 and 3.0 atom % $^{13}$C, respectively) were well over the natural abundance level (1.1 atom % $^{13}$C), and the hyphae showed a significantly higher enrichment compared to bacteria and POM ($p < 0.05$, df = 3, Fig. 6 c).
Figure 6 | High $^{13}$C enrichment detected in fungal hyphae and EPS based on NanoSIMS imaging. Scanning electron microscopy (SEM) images of $^{13}$C-enriched maize litter incubated in microcosms and isolated as particulate organic matter (POM) in a coarse- and b fine-textured soil. a1/b1 SEM micrographs of measurement spots in (a1) coarse- and (b1) fine-textured soil, which were later analyzed by nano-scale secondary ion mass spectrometry (NanoSIMS). a2/b2 NanoSIMS composite images displayed as RGB (Red = $^{12}$C$^-$, Green = $^{12}$C$^{14}$N$^-$ and Blue = $^{16}$O$^-$). a3/b3 NanoSIMS hue-saturation intensity (HIS) images displaying the $^{13}$C : ($^{12}$C$^-$ + $^{13}$C$^-$) isotope ratios of POM, fungal hyphae and extracellular polymeric substances (EPS) in the coarse- and fine-textured soil. Here the enrichment level is displayed as HIS images with a color scale ranging from natural abundance (0.011) in blue to high enrichment in purple (0.065). Scale bars represent 10 µm. c Boxplots of $^{13}$C : ($^{12}$C$^-$ + $^{13}$C$^-$) isotope ratios and d $^{12}$C$^{14}$N$^- :$^{12}$C$^-$ ratios of hyphae (n = 8), EPS (n = 16), bacteria (n = 10), and POM (n = 13) in both textures obtained by NanoSIMS (medians, error bars denote data ranging between the 5th and 95th percentiles). The natural abundance of $^{13}$C is indicated by the hatched line. The regions of interest were selected manually on continuous fragments of hyphae, individual bacteria, patches of EPS and exposed POM surfaces. Significant differences ($p < 0.05$) between the four groups are indicated by lowercase letters.


**Discussion**

Soil texture, and thus the 3D structure of soils, controls overall microbial activity; the coarser soil texture entailed both higher decomposition of litter-derived OM, and an increased priming effect, fostering the mineralization of native soil C (Fig. 1 b and d). Plant litter fragments located in larger soil pores of coarse-textured soils are more easily accessible; therefore, litter decomposition is enhanced\textsuperscript{16-18}. In the coarse-textured soil, the increased accessibility and, hence, increased bioavailability of litter-derived C was further demonstrated by consistently higher proportions of labeled PLFAs in gram-negative bacteria across all soil depths (Fig. 5 b); bacteria that are specialized in the processing of labile plant C sources\textsuperscript{19,20}.

We show that the coarse-textured soil offered a more favorable habitat for fungi in a micro-environment rich in bioavailable substrates formed by fresh unprotected litter. Fungal abundance increased by more than five-fold following the litter addition in the coarse-textured soil. Furthermore, a substantial part (92 % in the coarse-textured soil) of the fungal biomass was directly derived from the added plant litter, as demonstrated by the PLFA profiles (labeled PLFA profiles; Fig. 5 b). This highlights the key role of the fungal community for rapid litter decomposition, particularly in coarse-textured soils. Soil structure with a distinct soil pore network determines the abundance and community structure of microbiota\textsuperscript{21}. Fungi are mainly found in macropore spaces (> 10 µm) that are noticeably larger than the hyphae itself\textsuperscript{16,22,23}. The filamentous growth of the mycelium enables fungi to bridge air-filled pore spaces, supporting them to overcome capillary boundaries between wet and dry soil, and to adapt to heterogeneous pore networks\textsuperscript{24-26}. Consequently, under the physical conditions of coarse-textured soils, fungi have a clear advantage over other microorganisms to reach OM in hard-to-access soil compartments that are not connected via water nor biofilms\textsuperscript{21}. We stress that in sandy soils, fungi are key to sustain crucial soil functions such as C and nutrient cycling by the transformation of litter-derived OM into SOM.

In the coarse-textured soil, fungal activity extended away from the litter source, thereby promoting a downward transfer of litter-derived C into deeper soil layers (PLFA depth profiles; Fig. 4). This pattern can partly be attributed to the apical properties of the fungal mycelium, enabling the translocation of C sources throughout the fungal colony\textsuperscript{27-29}. The expansion of hyphal networks facilitates the incorporation
The stabilization of aggregated soil structures can be ascribed to the exudation of EPS (e.g., polysaccharides, Fig. 6 a and b) from the hypha. We propose that the expansion of fungal hyphae, together with its interactions with mineral particles, results in the build-up of litter-derived oPOM in the deeper soil layers away from the litter source (Fig. 2 b). This intricate interaction between fungal hyphae, plant residues, and mineral particles adhering to microbial-derived EPS was underlined by spectromicroscopic imaging (Fig. 6). With the direct measurement of intact plant-fungi interfaces, we emphasize the key role of fungi in the translocation of litter-derived C within soils, as well as in the formation of aggregates and mineral-associated OM–a process which ultimately drives the stabilization of litter-derived C compounds in soils.

We were able to demonstrate the incorporation of plant C into microbial biomass directly in the interface of plant residues and soil minerals. This was quantified with high levels of $^{13}$C enrichment in fungal hyphae and microbial EPS on the POM surface (Fig. 6). The direct contact between minerals ($^{16}$O$^{-}$ distribution; Fig. 6) and microbial biomass ($^{12}$C$^{14}$N$^{-}$; Fig. 6), together with the enmeshment of fresh litter (free POM) with fungal hyphae and microbial-derived EPS (Fig. 6 a and b), promotes the gluing of fine-sized soil minerals. This agglomeration of fine mineral particles, driven by microbial activity and regulated by the bioavailability of litter-derived C, drives aggregate formation and soil structure development directly at the plant–soil interface. In addition, the chemical composition of the litter-derived OM that got entrapped in soil aggregates (oPOM) by this soil structure formation resembled the undecomposed litter (Fig. 3). Thus, particulate OM acts as an important precursor for aggregate formation and parallel occlusion of litter-derived POM into aggregated soil structures (Fig. 7).
Figure 7 | Aggregate and mineral-associated organic matter formation in soils of different textures driven by interactions between litter, microorganisms and soil matrix. Fresh litter surfaces serve as hotspots of microbial activity driving the formation of organo-mineral associations in concert with comprising a nucleus for aggregate formation. (A) Coarse soil texture fosters higher mineralization of native and litter-derived organic matter resulting in higher CO$_2$ emissions compared to the fine-textured soil. (B) Fungal hyphae in coarse-textured soils promote the translocation of litter-derived C away from the litter source. (C) Regardless of texture, gluing of fine-sized minerals, driven by microbial products (EPS) on the fresh litter surface lead to (D) the formation of soil aggregates directly at the plant–soil interface.

Regardless of soil texture, fresh litter surfaces serve as hotspots of microbial activity driving the formation of organo-mineral associations in concert with comprising a nucleus for aggregate formation. Thus, the biogeochemical interfaces of decaying plant litter determine—via promoted microbial activity—the two most prominent mechanisms which increase the persistence of OC in soils; the (i) occlusion of POM in soil aggregates and (ii) the association of OM with mineral surfaces as simultaneous universal processes across soils of different structure (Fig. 7). These two mechanisms strongly rely on the spatial proximity of particulate litter and its surfaces, microbial residues, and fine-sized mineral particles. Thus, the formation of persistent POM and MAOM, both constituting soil organic C pools with low turnover times$^4,10$, is directly fueled by the decomposition of POM and controlled by microbial activity.
Methods

Study site and soil sampling

Soil was collected in December 2017 at 5–20 cm (Ap horizon) from an agricultural field located in Southern Germany (Freising, Bavaria, 48°23'53.8"N, 11°38'39.7"E). The sampling area is situated within the lower Bavarian upland, and characterized by a mean annual temperature of 7.8 °C and mean annual precipitation of 786 mm. The soil type is a Cambisol (silty clay loam; 32% clay, 53% silt, and 14% sand) with a considerable amount of loess mixed with underlying Neogene sandy sediments. The collected soil was oven-dried (2 days, 40 °C), sieved (< 2 mm), and visible plant remains were manually removed using tweezers.

Experimental setup

The experimental design involved four treatments; soils of two textures, either with or without $^{13}$C-labeled maize stalks. In order to obtain a coarse-textured soil (sandy clay loam; 24% clay, 15% silt, and 60% sand), half of the initial soil was mixed with quartz sand (Quarzwerke, Frechen, Germany). Approximately 120 g (for coarser texture) and 90 g (for finer texture) of soil was filled homogeneously and gently packed (bulk density 0.9–1.3 g cm$^{-3}$) into microcosms (polymethylene; height: 5 cm, internal diameter: 5 cm, total volume: 98.2 cm$^3$). While the control microcosms were filled entirely with soil, 330 mg of air-dried and grounded $^{13}$C-labeled maize stalks (2–3 mm, $\delta^{13}$C = 2129 ± 82 ‰ V-PDB; Agroscope, Zurich, Switzerland) were mixed into the upper 1.67 cm of the soil within the other microcosms to create a quasi-natural gradient, with aboveground litter addition from the top. Each of the four treatments was replicated five times. The microcosms were sealed from below with polyester gauzes (37-µm mesh) and placed into Ball Mason Jars (475 mL) on top of metal grids to ensure downward gas diffusion.

Heterotrophic respiration

After making all containers gas-tight and rinsing them with synthetic air (Westfalen AG, Münster, Germany), 12 mL of gas samples (IVA Analysentechnik, Meerbusch, Germany) were collected from the headspace of the Mason Jars on day 2, 3, 4, 8, 10, 15, 23, 31, 44, 65, 80, and 95. For each measurement of CO$_2$ respiration, two samplings of container atmosphere were carried out, and the time in-between the two samplings was adapted to the current respiration rates. During the incubation period
of 95 days, the CO₂ concentration, as well as the ¹³C abundance in the respired CO₂, was measured via
gas chromatography isotope ratio mass spectrometry (GC/IRMS) (Delta Plus, Thermo Fisher, Dreieich,
Germany). The CO₂ levels were calibrated against three calibration gases (890, 1500 and 3000 ppm
CO₂; Linde AG, Pullach, Germany). Then, source carbonic acid with known isotopic composition
diluted in helium was used as a lab standard. This standard was in turn calibrated against three
international standards (RM 8562, RM 8563, and RM 8564; International Atomic Energy Agency,
Vienna, Austria) with a dual inlet system. The temperature and water holding capacity were kept
constant at 21 °C and 60%, respectively, along with the incubation period.

**Sampling**

After 95 days of incubation, each microcosm was cut into three horizontal sections with a razor blade,
separating the top, center, and bottom layer (each 1.67-cm high). Subsamples for subsequent microbial
analyses were freeze-dried and stored at 4 °C, and dried aliquots for fractionation were stored in sealed
plastic containers at 20 °C. Furthermore, a few POM particles were selected manually for NanoSIMS
measurements.

**Physical fractionation and subsequent analyses**

The soil was separated into five distinct OM fractions using a combined density and particle size
fractionation scheme³³. Air-dried soil (18–20 g) was gently capillary-saturated with sodium
polytungstate solution (Na₆[H₂W₁₂O₄₀]; 1.8 g cm⁻³) and after 12 h, the free-floating particulate organic
matter (fPOM) was collected using a vacuum pump. oPOM was released from aggregated soil structures
via ultrasonic dispersion (Bandelin, Sonoplus HD 2200; energy input of 440 J ml⁻¹) allowing its
separation from heavier minerals. The excess salt was removed from the oPOM by washing it with
deionized water over a sieve (20-µm mesh size), which yielded an oPOM fraction of < 20 µm
(oPOMsmall). Both fPOM and oPOM fractions were washed for several times using deionized water and
pressure filtration (20-µm mesh) until the solution dropped below an electric conductivity of < 5 µS/cm
via pressure filtration. The oPOMsmall fraction was cleaned via saturation with deionized water for 24 h.
While sand and coarse silt fractions were separated by wet sieving, mineral fractions < 20 µm were
separated via sedimentation, and later combined as one MAOM fraction. The C, N, and ¹³C contents
were determined for freeze-dried and milled OM fractions, as well as milled bulk soil, via dry
combustion with an isotope ratio mass spectrometer (Delta V Advantage, Thermo Fisher, Dreieich, Germany) coupled with an elemental analyzer (Euro EA, Eurovector, Milano, Italy). Acetanilide was used as a lab standard for calibration and to determine the isotope linearity of the system, and was in turn calibrated against several suitable isotope standards (International Atomic Energy Agency, Vienna, Austria). International and lab isotope standards were included in every sequence to create a final $^{13}$C correction. Since the samples did not contain carbonates, the C contents were assumed to be equal to organic C contents.

$^{13}$C Nuclear Magnetic Resonance Spectroscopy

The chemical compositions of the POM fractions were determined via $^{13}$CP-MAS NMR in solid state (Bruker DSX 200, Bruker BioSpin GmbH, Karlsruhe, Germany), where samples were filled into 7-mm zirconium dioxide rotors and spun in a magic angle spinning probe at a rotation speed of 6.8 kHz and 0.01024 s acquisition time. The recorded $^{13}$C spectra were quantified in the following chemical shift regions: alkyl C ($-10$–$45$ ppm), O alkyl C ($45$–$110$ ppm), aromatic C ($110$–$160$ ppm), and carbonyl/carboxyl C ($160$–$220$ ppm). The regions were integrated and an alkyl C/O alkyl C ratio ($-10$–$45$/$45$–$110$ ppm) was computed to describe the degree of aliphaticity of the different fractions. Lastly, the obtained spectra were transformed into OM compound classes via the molecular mixing model with the following chemical shift regions: 0–45, 45–60, 60–95, 95–110, 100–145, 145–165 and 165–215 ppm.

Calculations of litter-derived C in CO$_2$, soil and OM fractions

Along with the incubation period, the amount of C respired per hour was computed as

$$\frac{mg \ CO_2 - C}{h} = \frac{\Delta CO_2 [ppm]}{\Delta t [min]} \cdot \frac{1}{10^6} \cdot \frac{V_{HSP} [ml]}{22.4 \frac{ml}{mmol}} \cdot \frac{T_0 [K]}{T_1 [K]} \cdot 12 \left[ \frac{mg \ CO_2 - C}{mmol} \right] \cdot 60 \ min$$

where $\Delta CO_2/\Delta t$ is CO$_2$ increase over time, $V_{HSP}$ is the volume of the headspace of Mason Jars. The volume of an ideal gas is set at 22.4, and 12 represents the atomic mass of C.

Subsequently, the percentage of respired CO$_2$ originating from the litter was calculated as
where \( \delta^{13}C_{\text{resp}} \) emission gives the \( \delta^{13}C \) for the current \( CO_2 \) emission between the two samplings (‰ V-PDB), \( \delta^{13}C_{\text{control}} \) is the average \( \delta^{13}C \) of the control soils at the time of measurement, and \( \delta^{13}C_{\text{litter}} \) is the \( \delta^{13}C \) signature of the labeled litter. Finally, the respired \( C \) originating from the soil was computed as

\[
CO_2-C_{\text{litter}} \% = \left( \frac{\delta^{13}C_{\text{resp}} - \delta^{13}C_{\text{control}}}{\delta^{13}C_{\text{litter}} - \delta^{13}C_{\text{control}}} \right) \cdot 100
\]

The proportion of litter-derived \( C \) (%) in the OM fractions was calculated as

\[
\text{Litter-derived } C [\%] = \left( \frac{\delta^{13}C_{\text{labeled}} - \delta^{13}C_{\text{control}}}{\delta^{13}C_{\text{litter}} - \delta^{13}C_{\text{control}}} \right) \cdot 100
\]

where \( \delta^{13}C_{\text{labeled}} \) is the \( ^{13}C \) enrichment in labeled samples, \( \delta^{13}C_{\text{control}} \) is the \( ^{13}C \) enrichment in controls (natural abundance level, \( i.e., \) 28 ‰ V-PDB), and \( \delta^{13}C_{\text{litter}} \) is the \( ^{13}C \) enrichment in the added litter \( (i.e., \) 2129 ‰ V-PDB) from which the amount of litter-derived \( C \) within each OM fraction could then be determined as

\[
C_{\text{litter}} [mg] = \frac{\text{litter-derived } C}{100} \times C_{\text{fraction}} \times m
\]

where \( C_{\text{fraction}} \) is the amount of \( C \) in mg g\(^{-1}\), and \( m \) is the recovered mass (g) of each fraction after the fractionation.

**PLFA analyses**

The PLFA patterns were analyzed and adjusted according to the ISO/TS 29843-2:2011F standard. In summary, the soil lipids from 3 g of soil (freeze-dried aliquots) were extracted with a Bligh & Dyer solution [methanol, chloroform, and citrate buffer \( (pH = 4 \pm 0.1) \), 2:1:0.8, v/v/v]. A biphasic system was achieved by adding chloroform and citrate buffer from which the lipid phase was evaporated at 30 °C under a nitrogen stream. The phospholipids were separated from neutral lipids and glycolipids by solid-phase extraction on silica tubes (SPE DSC-Si, 500 mg, Discovery\textsuperscript{®}) and evaporated. The PLFA were turned into fatty acid methyl esters (FAMEs) via alkaline methanolysis and later quantified via gas...
chromatic retention time comparison with a gas chromatograph (GC Agilent HP6890, G1530A, Chemestation, Santa Clara, USA) connected to a flame ionization detector equipped with a capillary column (SGE, BPX5, 60 m × 0.25 mm × 0.25 mm). The FAME concentrations were quantified relative to methyl nonadecanoate (19:0), enabling methylated lipids to be identified. A standard soil was used and extracted in parallel to detect potential deviations between the extraction rounds, expressed in nmol C-FA per g of soil. Mono-unsaturated and cyclopropylated PLFA (C16:1w7c, C18:1w9c, and C18:1w9t) were assigned to gram-negative bacteria, iso- and anteiso-branched PLFA (iC15:0, aC15:0, iC16:0, i-C17:0, C:17, and C18:0) were assigned to gram-positive bacteria and C18:2w6c, C18:3w3 respectively C20:5w3c were assigned to fungi. The total content of bacteria was expressed by adding gram-positive, gram-negative together with the markers C14:0, C16:0, C20:0, and C15:1. Lastly, the $^{13}$C-labeling of FAME was concluded by correcting for the added methyl moieties during methanolysis and relating it to the chain length of fatty acids

$$
\delta^{13}C_{FA} \ [\% V-PDB] = \frac{(C_n + 1) \times \delta^{13}C_{FAME} - \delta^{13}C_{MeOH}}{C_n}
$$

where $\delta^{13}C_{FA}$ represents the $\delta^{13}$C of the fatty acid, $C_n$ the number of C atoms in the fatty acid, $\delta^{13}C_{FAME}$ is the $\delta^{13}$C of the fatty acid methyl ester, and $\delta^{13}C_{MeOH}$ is the $\delta^{13}$C of the methanol used for the methylation (−63 %o) to calculate the isotope ratios of the fatty acids. The relative incorporation of $^{13}$C into four microbial groups was calculated by relating the proportions of each fatty acid to the total $^{13}$C incorporation, and the absolute incorporation of $^{13}$C in each microbial group was calculated by dividing the amount of $^{13}$C enriched fatty acid with the total amount of extracted fatty acid for that particular group.

**SEM and NanoSIMS microspectroscopy**

In order to gain insights on the microscale distribution of the assemblages of litter with microbes and minerals, we used SEM and NanoSIMS. Free POM from non-fractionated soil was hand-picked and fixed onto graphene sample substrates on metal stubs (10 mm in diameter). To avoid the charging phenomena, samples were gold-coated prior to SEM analyses by physical vapor deposition under argon atmosphere (Emitech Sputtercoater SC7620, Gala Instrumente, Bad Schwalbach, Germany). To analyze
the microscale structures of the assemblages of POM, microorganisms and soil minerals of the samples were first analyzed using SEM (Jeol JSM 5900LV, Freising, Germany), and subsequently the spots that best exemplified the microbial transformation on the decaying litter (POM) surface were analyzed using a Cameca NanoSIMS 50 L (Cameca, Gennevilliers, France). For the NanoSIMS measurements, a 270-pA high primary beam was used to locally sputter away impurities and gold coating, and to implant primary ions (Cs+) into the samples surface (impact energy of 16 keV) to enhance the yields of secondary ions. Subsequently, secondary ions were measured using electron multipliers; $^{12}\text{C}^-$, $^{13}\text{C}^-$, $^{12}\text{C}^{14}\text{N}^-$ to display OM fragments and $^{16}\text{O}^-$, $^{28}\text{Si}^-$, $^{27}\text{Al}^{16}\text{O}^-$ and $^{56}\text{Fe}^{16}\text{O}^-$ secondary ions to record the mineral phase. The instrument was tuned to a high mass resolution in order to accurately separate mass isobars at mass 13 ($^{13}\text{C}^-$, $^{12}\text{C}^1\text{H}^-$). The ion images were acquired with a 25 $\times$ 25 $\mu$m field of view, 40 planes and 1 ms pixel$^{-1}$ dwell time for all measurements. Charging effects were compensated for with an electron flood gun if necessary. The acquired measurements were dead time (44 ns) and drift corrected using the OpenMIMS plugin of the ImageJ software. The $^{13}\text{C}^-:(^{12}\text{C}^-+^{13}\text{C}^-)$ and $^{12}\text{C}^{14}\text{N}^-:^{12}\text{C}^-$ ratios were computed for distinct regions of interests which were chosen manually with respect to the major compartments: continuous fragments of fungal hyphae, individual bacteria, EPS patches as well as exposed POM surfaces. To account for instrumental mass fractionation, the electron multipliers were carefully checked, and the control measurements of non-labeled POM samples were conducted regularly along the sessions. Here, the mean $^{13}\text{C}^-:(^{12}\text{C}^-+^{13}\text{C}^-)$ ratios were in line with the level of natural abundance, which meant that a correction of ratios for labeled POM samples was not necessary.

**Statistical analyses**

All parameters were separately tested for normality with Shapiro–Wilk test and for homoscedasticity with Bartlett’s test. In addition, the distribution of the datasets was checked with Q-Q plots. In cases where the assumptions of normality or homoscedasticity were not met, a log-transformation was applied on the raw data and analyses were carried out on the log-transformed data. The differences caused by texture and litter addition were tested using unpaired t tests, and depth differences were tested using one-way analysis of variance with Tukey’s honestly significant difference as the post-hoc test. In cases where the log-transformed data did not meet the requirements for parametrical testing, the unpaired two-samples Wilcoxon test or Kruskal-Wallis test was applied. The statistical findings were considered
significant if the confidence limits were in excess of 95% (p < 0.05). All statistical testing was carried out in the R statistical environment\textsuperscript{41} using agricolae\textsuperscript{42} and ggpubr\textsuperscript{43} packages.
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Author contributions

KW carried out the measurements following the incubation, collected and analyzed data, and wrote the manuscript; AV designed and supervised the experiment and wrote the manuscript; DIS designed and conducted the incubation and respiration measurements, prepared samples for subsequent analyses and collected and analyzed data; CH conducted the NanoSIMS measurements and supported the data evaluation; SS designed and supported the incubation experiment and contributed to the data evaluation of the NanoSIMS measurements; FB conducted and evaluated the GC-IRMS measurements of CO\(_2\) and the EA-IRMS measurements of soil fractions; VP supervised the PLFA extraction, conducted the GC-C-IRMS measurements and evaluated the data, JH provided the labeled plant litter; CC supervised the PLFA and PLFA-SIP extraction and evaluated the data; CWM designed and supervised the experiment and wrote the manuscript. All authors discussed the data and contributed to the final draft.

Data availability

The data supporting the findings of this study are available on request from the corresponding author (KW).

Competing financial interests

The authors declare no competing financial interests.
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