



Assessing soil C stock and C to N ratio of soil organic matter under mixed pine-beech forests at different scales

Marina Getino-Álvarez¹ · Roberto San-Martin² · Hans Pretzsch^{3,5} · Maciej Pach⁴ · Felipe Bravo⁵ · María-Belén Turrión¹

Received: 15 January 2023 / Revised: 17 April 2023 / Accepted: 5 May 2023 / Published online: 24 May 2023
© The Author(s) 2023

Abstract

Healthy soils are the second C sink on Earth, and this sink could last for hundreds or even thousands of years as stable soil organic matter (SOM). Forest soils, in particular, have the potential to store significant amounts of C, however, the amount of C sequestered and the carbon-to-nitrogen (C/N) ratio of soil organic matter (SOM) depend on the vegetation influencing the soil. In the last decades, mixed stands have aroused great interest among the scientific community, but it is still necessary to intensify research on its effect on soils and their C storage capacity. In this study, we assess soil C sequestration potential in soil and litter of mixed and pure stands of Scots pine and beech (*Pinus sylvestris*–*Fagus sylvatica*). Three triplets (9 forest plots), two located in Southern Poland and one in Southern Germany were studied. A total of 40 circular subplots of 5 m radius were selected within the triplets, covering a wide range of species mixture, and soil and litter were sampled. Data were analyzed at two scales (plot-level and microsite-level) to determine which option is more appropriate when studying the mixing effect on SOM. C stock in forest floor ranged between 2.5 and 11.1 Mg C ha⁻¹ and in mineral soil between 39.6 and 337.8 Mg ha⁻¹. According to our findings, the percentage of species mixture primarily impacted the forest floor rather than the mineral soil. On the forest floor, stands with 25–50% pine in the mixture were found to have a C/N ratio between 20 and 30, which indicates an equilibrium state between mineralization and immobilization. In the mineral soil, total organic C was the only variable affected by mixture percentage ($p < 0.1$). Finally, microsite-level scale proved to be the most appropriate when studying tree stand composition effect on SOM, as the plot-level scale diluted or masked some effects.

Keywords C sequestration · Soil organic matter · Mixed forests · *Pinus sylvestris* · *Fagus sylvatica*

Communicated by Agustin Merino.

✉ Marina Getino-Álvarez
marina.getino@uva.es

Roberto San-Martin
roberto.smartin@uva.es

Hans Pretzsch
hans.pretzsch@tum.de

Maciej Pach
rlpach@cyf-kr.edu.pl

Felipe Bravo
felipe.bravo@uva.es

María-Belén Turrión
mariabelen.turrión@uva.es

² Departamento de Estadística e Investigación Operativa, Instituto de Investigación en Gestión Forestal Sostenible (iuFOR), ETS de Ingenierías Agrarias, Universidad de Valladolid, Avda. de Madrid 44, 34004 Palencia, Spain

³ Chair of Forest Growth and Yield Science, School of Life Sciences Weihenstephan, Technical University of Munich, Freising, Germany

⁴ Department of Ecology and Silviculture, Faculty of Forestry, University of Agriculture in Krakow, Krakow, Poland

⁵ Departamento de Producción Vegetal. Instituto de Investigación en Gestión Forestal Sostenible (iuFOR), ETS de Ingenierías Agrarias, Universidad de Valladolid, Avda. de Madrid 44, 34004 Palencia, Spain

¹ Área de Edafología y Química Agrícola, Departamento de Ciencias Agroforestales, Instituto de Investigación en Gestión Forestal Sostenible (iuFOR), ETS de Ingenierías Agrarias, Universidad de Valladolid, Avda. de Madrid 44, 34004 Palencia, Spain

Introduction

Considering their high potential to sequester carbon, soils can play a valuable role in combating climate change. Healthy soils are the second largest C sink on Earth, containing two-thirds of the total C in terrestrial ecosystems (Dixon et al. 1994). This sink may endure for hundreds or even thousands of years as stable soil organic matter (SOM) (Basile-Doelsch et al. 2020). In this context, forest soils play a very important role in mitigating global change, storing around 70% of the world's soil organic carbon (SOC) reserves (Schleuß et al. 2014; Osei et al. 2021). However, depending on the management applied, forest ecosystems may see their capacity to accumulate SOC favored or become sources of CO₂ that returns to the atmosphere (Schlesinger and Andrews 2000).

It's well known that soil and vegetation closely related (Cornwell et al. 2008). The establishment of one type of vegetation or another will depend on soil characteristics, but at the same time, soil characteristics can be modified by the vegetation present. For these reasons, the scientific interest in understanding the factors that influence the amount of C sequestered by forest ecosystems and the processes increasing their stability and, therefore, its residence time in the soil, has aroused great relevance (Wander and Nissen 2004; Turrión et al. 2009; Roman Dobarco 2014). The main source of organic matter (OM) and nutrients in forest ecosystems came from litter and the decomposition of plant roots (Berg and McLaugherty 2020). Depending on tree species composition the leaf litter production and composition will be different and, consequently, the quality and quantity of OM that reaches the soil (Lucas-Borja et al. 2012; Román Dobarco et al. 2021). Thus, it is reasonable to believe that one of the factors that affects the dynamics of C accumulation and decomposition is the tree species composition (Herrero et al. 2016).

In the last decades, the management of mixed stands has become highly relevant. Forestry research has focused on this type of stands due to the solid evidence that mixed forests can provide numerous ecosystem services more efficiently than monocultures (Gamfeldt et al. 2013; Bravo-Oviedo et al. 2014; Pretzsch et al. 2015). Due to the complementary use of space, the basal area of mixed stands is usually higher than the monocultures, which may lead to higher litterfall input (Błońska et al. 2018; Osei et al. 2021). As stated by Pretzsch et al. (2015), the combination of different litter types, especially those with contrasting characteristics (coniferous and broad-leaf species) can improve, in the long term, the storage capacity of carbon and other resources in the soils. Although the studies focused on the effect of mixtures on SOC have increased (Cremer et al. 2016; López-Marcos et al. 2018;

Osei et al. 2021), is still a poorly studied aspect. Studies dealing with mineral soil, are mainly focused on the top-soil layer or up to 20–30 cm depth. However, half of the C stored in soils is located in deeper layers (> 30 cm depth) (Basile-Doelsch et al. 2020).

Leaf litter coming from different tree species will decompose at different rates depending on its chemical quality and recalcitrance. At the same time, litter quality will also influence the soil microbial community, which in turn will influence OM decomposition dynamics (Wang et al. 2015). Recent advances in the knowledge of the dynamics of organic matter indicate that secondary metabolites released by plants, possibly have a greater impact on carbon dynamics than the primary ones, due to their effects on microorganisms and the mobilization of organic matter due to their priming effect (Basile-Doelsch et al. 2020). Priming effects (PE) can be defined as the changes in natural mineralization processes of C and N (acceleration; PE + or reduction; PE-) caused by the addition of different substances to the soil (plant residues, dead microorganisms, organic or mineral fertilizers...) (Kuzyakov et al. 2000; Wang et al. 2015).

Further research should be done to increase the knowledge about tree species mixture effect on SOM decomposition dynamics, and its evolution in deep. Filling this knowledge gap is essential to propose management alternatives that preserve or increase SOC reserves in forest soils, soil fertility, and contribute to climate change mitigation.

In this study, we have focused on admixtures of coniferous and broad-leaf species, particularly in two of the main tree species in Europe: Scots pine (*Pinus sylvestris* L.) and European beech (*Fagus sylvatica* L.). In Europe, Scots pine covers about 12×10^6 ha and European beech 49×10^6 ha. About 1.7×10^6 ha of European forests are mixed stands of Scots pine and European beech. However, some authors have estimated that this type of mixture could cover 32×10^6 ha of European territory (Brus et al. 2012).

To assess the potential advantages of mixed stands versus monospecific stands, a large number of studies have been conducted based on the triplet methodology (Pretzsch et al. 2015; Heym et al. 2017; López-Marcos et al. 2020). The establishment of sets of triplets facilitates the comparison between mixed and monospecific stands (Riofrío et al. 2017). This approach has been used in several studies, mainly focused on the aboveground level, on topics such as stand growth and productivity (Pretzsch et al. 2015; Ruiz-Peinado et al. 2021), tree morphology (Cattaneo et al. 2020; Uzquiano et al. 2021) or understory vegetation (López-Marcos et al. 2020). The triplets' methodology establishes a categorical grouping with three levels: monospecific stand 1, monospecific stand 2 and mixed stand (Heym et al. 2017). In our case, it corresponds to the "stand type" variable which has 3 levels: pure beech, pure pine and mixed. Soils are very heterogeneous, and

many of their properties, such as the quantity and quality of organic matter, can vary within a few meters of distance (Oueslati et al. 2013; Falconer et al. 2015). For this reason, using a microsite scale (local mixture) rather than using a plot scale might be more appropriate for studying the effect of species mixing on SOM. Very little was found in the literature on the microsite approach in mixed forests. For their studies of soil and litter decomposition in mixed stands, Bueis et al. (2017, 2018) used a microsite approach, in this case with circular subplots of 6 m radius in *Pinus halepensis* reforestations Osei et al. (2021) also apply the concept of mixture percentage (quantitative variable instead of the categorical grouping), however, they calculate it at the plot level (triplets' methodology).

In this study, we aim to assess the potential effect of European beech and Scots pine admixture on the C to N ratio of SOM and the amount of C accumulated in forest floor and mineral soil, contributing to increasing the knowledge about the C dynamics in mixed stands. To study the data, two types of scale were used: plot-level (triplets or stand-type approach) and microsite-level (local mixture %). We postulate that the scale used in triplets' approach (plot-level) is too large for SOM studies, and a microsite scale might be more appropriate. Thus, an initial objective of this research is to determine which scale; plot-level or microsite-level, is better to apply when studying the effect of tree stand composition on SOM. Secondly, we want to assess the effect of the percentage of species mixture on (1) the accumulation and decomposition of leaf litter, (2) the distribution of carbon and nitrogen throughout the edaphic profile and (3) the C to N ratio of the organic matter accumulated.

Materials and methods

Study site and experimental design

Nine forest plots (three triplets) of mixed and pure stands of Scots pine (*Pinus sylvestris* L.) and beech (*Fagus sylvatica* L.) were selected for the study. Experimental plots belong to a European network of triplets (COST Action FP 1206 EuMiXFOR). Under this project, sets of three rectangular plots including pure and mixed stands (1 per stand type; pure pine, pure beech and the mixed) of the species under study (*Pinus sylvestris* and *Fagus sylvatica*) were established along a climatic gradient in Europe. Plot size varies between 0.05 and 0.18 ha, including at least 20 trees per species, and mixing proportions varied from 18 to 72% of Scots pine (Pretzsch et al. 2015). Stands within triplet present similar site conditions (soil, climate and topography), age, tree density and management. Stand characteristics such as tree species, diameter at breast height (dbh), spatial location of individual trees or plot size were obtained from the EuMiXFOR data set available in <http://dx.doi.org/10.5061/dryad.8v04m> (Heym et al. 2019).

Out of the EuMiXFOR triplets, we selected three triplets located in different areas, but with similar soil types and climate. Two triplets (Pol_4 and Pol_5) were located in Niepołomice forest (Fig. 1) in southern Poland. Situated in the Vistula River Valley, 25 km east from Cracow, Niepołomice forest (50°01'34.3"N, 20°19'34.6"E) covers an area of 110 km² approximately, mainly dominated by *Pinus sylvestris* stands, alone or mixed with other tree species (Grodzinski et al. 2012). It has an oceanic-continental

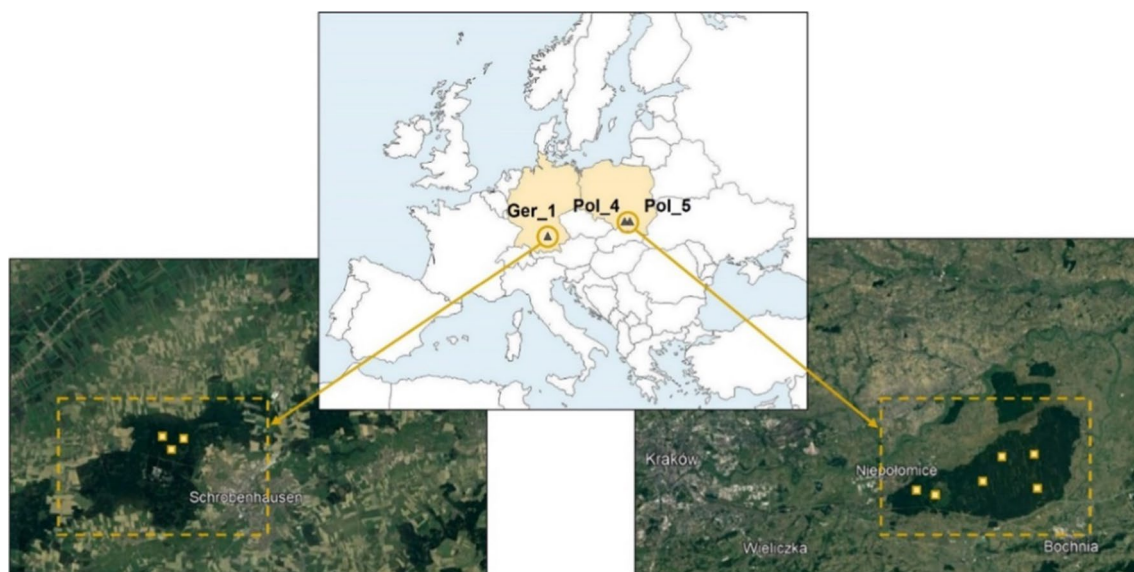


Fig. 1 Study site location: Schrobenuhousen (Germany), Niepołomice (Poland)

climate (Cfb) according to (Köppen 1936) classification, the average annual precipitation ranges between 670 and 715 mm and the average annual temperature between 8.4–8.7°C (Fick and Hijmans 2017; Kapusta et al. 2019; Stanek and Stefanowicz 2019). Plots are located at an altitude of 191–231 m above sea level. Soils are podzols and cambisols (Rozen et al. 2004) developed on Pleistocene sands, with sandy or sandy-loam texture and extremely acid pH (3.2–4.5). The third triplet (Ger_1) was situated on the outskirts of Schrobenhausen, southern Germany (Fig. 1). Schrobenhausen plots are located at an average altitude of 460 m above sea level, with an oceanic-continental climate (Cfb) according to (Köppen 1936) classification, an average annual rainfall between 700 and 900 mm and average annual temperature between 7.5 and 8.5 °C (Heym et al. 2017). Soils are classified as podzols, developed on coarse and fine sand tertiary sediments, with sandy or sandy-loam texture and extremely acid pH (3.8–4.5).

Table 1 illustrates some of the main characteristics of the studied plots and subplots. No silvicultural intervention has

been done in the stands in the last ten years. These triplets have been previously used in other studies as in Błońska et al. (2018) and de Stree et al. (2021).

In order to study the effect of different percentages of species mixture on the C to N ratio of SOM and the amount of C accumulated, circular subplots of 5 m radius were established within the previously described plots following two conditions: (1) no overlapping between subplots and (2) different percentage of species mixture. To locate the subplots, the area of influence of each tree was calculated using Thiessen polygons (a method to analyze proximity and neighborhood). This method establishes the perpendicular bisector between a tree and each one of its nearest neighbors, creating a polygon (area of influence) for each individual (Butler et al. 2014). The percentage of species mixture at each subplot was calculated as a ratio between the sum of Scots pine influence areas and the total area of the subplot, and was expressed as pine percentage (or pine proportion). An example of the circular subplots' establishment is shown in Appendix 1. Under these bases, a total of 40 subplots were established, covering a wide range of species mixture. In

Table 1 Stand characteristics at plot-level or stand type (Source: EuMixFOR database, Heym et al. (2019)) and subplot-level or microsite (authors' own data)

Location	Plot ID	Treat	%Pine	Alt (m)	Slope (°)	Age (years)	BA (m ² ha ⁻¹)	N (trees ha ⁻¹)	Dm (cm)	Ho (m)
Niepolomice (Poland)	Pol_4P	Pine	100	210	0	57	27.4	671.9	22.6	24.6
	Pol_4B	Beech	0	201	0	57	17.6	531.1	19.8	20.0
	Pol_4M	Mixed	15.9	218	4	57	34.2	609.2	24.4	28.7
	Pol_5P	Pine	100	212	0	55	32.6	440.5	30.2	27.2
	Pol_5B	Beech	0	191	0	55	22.8	438.6	24.7	27.8
	Pol_5M	Mixed	43.8	231	0	55	31.0	555.2	25.0	26.6
Schrobenhausen (Germany)	Ger_1P	Pine	100	449	1	57	25.9	264.6	34.7	28.8
	Ger_1B	Beech	0	467	1	57	27.4	952.4	17.6	27.2
	Ger_1M	Mixed	24.2	473	1	57	37.4	1056.8	19.4	26.5

Subplot ID	%Pine	BA (m ² ha ⁻¹)	N (trees ha ⁻¹)
Pol_4P-1	100	32.9	767.8
Pol_4P-2	100	44.8	1407.7
Pol_4P-3	100	41.6	895.8
Pol_4P-4	100	37.7	895.8
Pol_4B-1	0	45.7	511.9
Pol_4B-2	0	53.9	895.8
Pol_4B-3	0	72.8	895.8
Pol_4B-4	0	51.4	767.8
Pol_4M-1	13.7	28.0	639.9
Pol_4M-2	14.3	39.8	1023.8
Pol_4M-3	57.6	30.4	1023.8
Pol_4M-4	17.0	23.7	895.8
Pol_4M-5	19.0	30.3	383.9
Pol_5P-1	100	41.5	767.8
Pol_5P-2	100	40.0	767.8
Pol_5P-3	100	26.5	767.8
Pol_5P-4	100	54.6	1279.7
Pol_5B-1	0	38.4	1023.8
Pol_5B-2	0	53.5	511.9
Pol_5B-3	0	46.2	639.9
Pol_5B-4	0	43.2	767.8
Pol_5M-1	78.2	41.7	639.9
Pol_5M-2	64.2	39.8	895.8
Pol_5M-3	31.8	46.3	639.9
Pol_5M-4	86.5	29.2	767.8
Pol_5M-5	8.4	43.2	895.8
Pol_5M-6	23.1	19.8	511.9
Ger_1P-1	100	33.9	255.9
Ger_1P-2	100	63.7	639.9
Ger_1P-3	99.1	39.9	383.9
Ger_1P-4	100	36.7	511.9
Ger_1B-1	0	24.7	895.8
Ger_1B-2	0	26.5	895.8
Ger_1B-3	0	29.9	1407.7
Ger_1B-4	0	33.1	1151.7
Ger_1M-1	40.9	40.8	767.8
Ger_1M-2	34.6	29.0	1023.8
Ger_1M-3	13.7	38.4	1279.7
Ger_1M-4	63.5	54.5	1151.7
Ger_1M-5	8.1	22.0	767.8

Plot ID: plot identifier; Treat: stand type; %Pine: pine proportion; Alt: elevation above sea level; Slope: Age: mean age; BA: basal area; N: tree density; Dm: mean diameter; Ho: dominant height; Subplot ID: subplot identifier.

general, 4 subplots were established in pure stands and 5 or 6 subplots in mixed stands (according to the plot's dimensions). As a result, the following subplot distribution was obtained: 12 subplots in pine stands, 12 subplots in beech stands and 16 subplots in mixed stands.

Soil and litter sampling

Organic and mineral soil horizons were collected in the 40 subplots established. To provide a representative sample of the area, samples were taken at 3 points 1 m apart from the center of each subplot, and then mixed creating one composite sample per horizon in each subplot. At each point, mineral soil horizons were manually sampled up to 40 cm depth. Organic horizons of the forest floor (FF) were collected using a 25 × 25 cm wooden frame as a reference area.

In addition, to describe and characterize the edaphic profile of the studied plots, a soil pit of at least 60 cm depth was made in all the plots conforming a triplet (1 for each stand type and triplet). The nine soil profiles were described according to the FAO guidelines for soil description (FAO 2006) and classified following the World Reference Base for Soil Resources (IUSS Working Group WRB 2015). Disturbed and undisturbed, soil samples were collected from each mineral horizon, as well as the litter (organic horizon) accumulated on it. Sampling was carried out between July and October 2019.

Laboratory analysis

Forest floor samples were separated into three fractions according to decomposition state as in (López-Marcos et al. 2018): almost undecomposed litter or fresh fraction (FsL), partially decomposed litter or fragmented fraction (FgL) and mostly decomposed organic matter or humified fraction (HmL). Litterfall fractions were dried at 60 °C and weighed to calculate the total dry biomass (B) of each fraction per hectare (Mg ha⁻¹).

Composite soil samples were air dried at room temperature and sieved (Ø 2 mm) for further analysis. Coarse fraction percentage (> 2 mm, CF), earth fraction percentage (< 2 mm, EF), texture according to USDA criteria and pH in water were determined. Undisturbed soil samples were dried, for 24 h at 105 °C, and weighed to calculate the bulk density (bD). In addition, litterfall fractions and soil samples were analyzed for total organic C and N content (TOC, TN) by dry combustion using a LECO CNS928 elemental analyzer. Carbon-to-nitrogen ratios were calculated for litter samples; fresh + fragmented fraction (C/N)_{Fs+FgL} and humified fraction (C/N)_{HmL}, and soil samples.

After analyzing all the plots, one of the pine plots from Niepołomice (Pol_5P) was excluded from the research. Although we are not aware of it, we suspect that soil was

stirred and mixed, modifying the natural sequence of the edaphic profile.

Data analysis

Fresh litter fraction represented a rather small part of the total forest floor and was not present in many samples. Thus, we decided to combine the data of both fractions in a new category of "fresh-fragmented fraction" (Fs + FgL). Although soil samples were collected and analyzed by horizons, data were subsequently converted into depths every 10 cm (0–10 cm; 10–20 cm; 20–30 cm and 30–40 cm) by calculating the weighted means between soil horizons (Appendix 2). In this way, the analysis is facilitated, being able to establish comparisons between depths in different soils.

The stock of C in the mineral soil was calculated for each depth (Cstock₀₋₁₀, Cstock₁₀₋₂₀, Cstock₂₀₋₃₀ and Cstock₃₀₋₄₀) following the next formula: $Cstock_i = TOC_i * bD_i * EF_i * T_i$ being *i*: soil depth (0–10 cm, 10–20 cm, 20–30 cm or 30–40 cm), *TOC*: total organic carbon content (%), *bD*: bulk density (g cm⁻³), *EF*: earth fraction (%), *T*: soil layer thickness (cm).

C stock in forest floor fractions (Cstock_{Fs+FgL}, Cstock_{HmL}) were calculated by multiplying TOC content by fraction biomass. C stock in forest floor (Cstock_{FF}) would be the sum of both fraction Cstocks:

$$Cstock_{FF} = (TOC_{Fs+FgL} * B_{Fs+FgL}) + (TOC_{HmL} * B_{HmL})$$

Finally, C stock in the whole soil profile (Cstock_T) was calculated as the sum of C stock in the whole soil profile and litter fractions C stocks.

Statistical analysis

When analyzing the data, two types of scale were used: plot-level (triplets or stand-type approach) and microsite-level (local mixture %). For the stand-type approach (plot-level) we considered each plot conforming a triplet as an experimental unit. Using the stand-type as a categorical variable with 3 levels: pure beech, pure pine and mixed. In the microsite-level approach rather than considering whole stand (plot) characteristics to explain forest floor and soil properties, we focused on the area surrounding each sampling point (5 m radius), where specific mixture percentage (quantitative variable) per subplot was calculated.

Plot-level or stand-type approach

To analyze the possible effects of stand type (pure pine, pure beech or mixed) on the variables studied (B, TOC, TN, C/N y Cstock) repeated measured linear mixed models (LMM)

were fitted separately for soil and forest floor, both with the following equation:

$$Y_{ijkl} = \mu + T_i + S_j + P_{k(i,j)} + D_l + S_j \times D_l + \xi_{ijkl}$$

where Y was the response variable, μ the general mean, T_i the triplet effect, S_j the stand type effect, $P_{k(i,j)}$ the random effect of the microsite (subplot), were $P_{k(i,j)} \rightarrow N(0, \sigma_{ij}^2)$, D_l the depth effect and $S_j \times D_l$ the interaction between stand type and depth. When the LMM was applied to the forest floor, the depth effect (D_l) was replaced by the litter fraction effect (F_l). The depth (or litter fraction) factor was considered as a repeated measured factor with a variance–covariance structure of heterogeneous autoregressive order one type (arh(1)). Finally, ξ_{ijkl} were the random error of the model, such that $\xi_{ijkl} \rightarrow N(0, \sigma^2)$ and were independent. To attain model convergence, REML (restricted maximum likelihood) variances were calculated. Pairwise t tests were used to compare the estimated means for the different levels of the significant fixed effects.

Microsite-level or local mixture % approach

In this approach, we consider the mixture as a quantitative factor rather than as a fixed factor with three levels, so, for each subplot (microsite) we will use the percentage of pine as a covariate in the mixed linear model. Thus, for each response variable we will have the LMM with the equation:

$$Y_{ijkl} = \mu + T_i + P_{k(i,j)} + D_l + (b_1 + b_{1l}) \cdot x + (b_2 + b_{2l}) \cdot x^2 + \xi_{ijkl}$$

where b_1 and b_2 were the common regression coefficient of x and its square, x^2 , and b_{1l} and b_{2l} the slope deviation of the l -th depth from de common slopes b_1 and b_2 , respectively, x the percentage of pine observed in the microsite (subplot). The rest of the terms of the model are those already explained in the previous case, and as before, when the LMM was applied to the forest floor, the depth effect (D_l) was replaced by the litter fraction effect (F_l).

Finally, in order to check the influence of the stand density on the litter biomass and $C_{stock_{FF}}$, linear correlations between some variables of interest were investigated using the Pearson's coefficient ($p < 0.05$).

All statistical analyses were performed using SAS 9.4 software (SAS Institute 2019).

Results

General soil parameters

Some physical and chemical characteristics of the nine edaphic profiles described are shown in Table 2. The maximum depth reached in the edaphic profiles was 115 cm

(Ger_1P), and the minimum 80 cm (Pol_5B). The texture in the different soil profiles, and horizons, was mainly sandy, varying between loamy sand to sand. All the soils studied showed an extremely acid pH (3.2–3.9) in the topsoil, slightly increasing with depth (4.2–5.9). Compared to the rest of the plots, those corresponding to Pol_4 showed higher TOC content in the first horizon. Soil N contents showed, in all cases, very low values.

Differences at plot level (stand-type approach)

Forest floor

As can be seen in Table 3, a significant effect in the interaction between stand type and litter fractions was found in all the variables studied: litter biomass ($p < 0.001$), TOC content ($p < 0.05$), TN content ($p < 0.001$), C/N ratio ($p < 0.001$) and the C_{stock} ($p < 0.001$).

Average litter biomass values in the pure beech, pine and mixed plots were 11.7, 17.3 and 12.9 Mg/ha, respectively (Fig. 2a). Litter biomass under pine stands was significantly higher than in beech and mixed stands, which did not differ between them. Strong differences ($p < 0.001$) were observed in litter fractions (Fs + FgL and HmL) biomass depending on the stand type (Table 3). Pure beech and mixed stands behave similarly, having significantly higher values of partially or initially decomposed litter (Fs + FgL) and about half the amount of highly decomposed litter (HmL). The opposite was found for pine stands, being HmL higher than Fs + FgL. HmL fraction represented 30% of the total biomass in pure beech and mixed plots but up to 93% in pure pine stands. In addition, no correlation between the total litter biomass and the stand characteristics (BA, N, Dm...) was detected.

Litterfall under pine stands showed the highest TOC content in both fractions (TOC_{Fs+FgL} : 455.9; TOC_{HmL} : 454.8 mg C g⁻¹). The data showed that TOC in the litter fractions in beech and mixed stands decreased with the decomposition state, while in pine stands it does not change (Fig. 3a).

As can be seen in Fig. 3b, TN content was higher in the HmL fraction, showing that TN increased with the humification state. TN_{HmL} was significantly higher in mixed stands (18.7 mg N g⁻¹) compared to beech stands (17.5 mg N g⁻¹), while pine stands did not differ from them. TN in Fs + FgL fraction did differ among the three stand types, being higher in mixed (15.8 mg N g⁻¹), lower in pine (10.9 mg N g⁻¹), and intermediate in beech stands (13.8 mg N g⁻¹). In all cases, total N content increased as the humification state did; TN_{HmL} (18.5 mg N g⁻¹) > TN_{FgL} (13.5 mg N g⁻¹), being more pronounced in pine stands, where the percent change reached a 78%.

C/N ratio (Table 3) in the forest floor ranged between 17.3 and 72.7 with an average of 33.7 across all litter fractions and stand types. As can be seen in Fig. 3c, C/N ratio

Table 2 General soil profile properties

	Depth (cm)	CF (%)	Texture			bD (g cm ⁻³)	pH (H ₂ O)	TOC (mg C g ⁻¹)	TN (mg N g ⁻¹)	C/N
			Sand (%)	Silt (%)	Clay (%)					
Pol_4P										
Ah	0–6	0.0	84.6	5.4	10.0	0.42	3.4	153.69	6.63	23.2
E	6–15	0.0	95.5	1.0	3.5	1.53	3.9	16.37	0.99	16.5
Bhs	15–42	0.0	90.0	4.5	5.5	1.47	4.7	9.91	0.75	13.3
C	>42	0.0	93.5	3.0	3.5	1.76	4.8	1.54	0.29	5.3
Pol_4B										
Ah	0–9	0.0	80.9	7.9	11.3	0.35	3.2	188.62	9.50	23.3
E	9–30	0.0	94.0	2.5	3.5	1.25	3.6	11.76	0.87	13.5
Bhs	30–58	2.7	94.5	1.5	4.0	1.77	4.2	6.29	0.67	9.4
Bs	>58	8.5	93.5	1.5	5.0	1.97	4.3	2.18	0.46	4.7
Pol_4M										
Ah	0–4	0.0	93.7	0.3	6.1	1.22	3.5	74.54	3.35	22.2
E	4–18	0.0	96.0	0.0	4.0	1.64	3.7	8.09	0.57	14.3
Bs	18–42	0.0	93.5	2.5	4.0	1.66	4.3	4.88	0.44	11.0
C	>42	0.0	95.0	1.0	4.0	1.78	4.3	3.38	0.43	7.9
Pol_5B										
Ah	0–9	2.9	88.9	3.5	7.6	1.37	3.5	50.28	2.74	18.3
Bhs	9–28	8.1	90.5	2.0	7.5	1.67	4.0	10.75	0.78	13.7
Bs	28–50	20.5	91.0	5.5	3.5	1.69	4.4	4.80	0.55	8.7
C	>50	0.6	92.0	4.5	3.5	1.78	4.4	1.23	0.38	3.2
Pol_5M										
Ah	0–20	0.0	95.5	1.3	3.3	1.47	3.5	36.36	1.65	22.1
Bhs	20–32	0.0	91.4	3.5	5.0	1.51	4.0	17.62	0.84	21.0
Bs	32–61	0.0	94.0	2.5	3.5	1.72	4.2	4.38	0.38	11.6
C	>61	0.1	95.0	2.5	2.5	1.80	4.2	2.28	0.29	7.9
Ger_1P										
Ah	0–3	2.0	90.4	5.6	4.1	1.11	3.6	70.74	3.35	21.10
A	3–14	5.3	87.7	5.3	7.0	1.25	4.0	15.32	0.65	23.6
Bs	14–66	4.7	90.5	3.7	5.7	1.56	4.5	2.06	0.27	7.7
C	>66	2.1	92.8	3.2	4.0	1.50	4.5	1.21	0.26	4.6
Ger_1B										
Ah	0–6	1.0	82.4	10.0	7.5	1.06	3.9	31.75	1.67	19.1
Bs	6–9	0.7	79.2	11.0	9.8	1.14	4.5	10.99	0.59	18.7
B1	9–55	1.5	82.5	9.5	8.0	1.38	4.5	3.55	0.35	10.1
B2	55–90	0.1	89.7	3.3	7.0	1.46	5.6	1.13	0.17	6.6
C	>90	0.0	92.2	4.0	3.8	1.52	5.9	0.70	0.17	4.1
Ger_1M										
Ah	0–6	17.0	86.4	6.8	6.8	0.85	3.9	55.05	2.89	19.0
Bhs	6–8	28.1	86.9	5.0	8.0	1.36	4.1	18.31	0.77	23.9
B	>8	36.3	90.0	3.7	6.2	1.62	4.5	3.32	0.23	14.3

Depth soil horizon depth; *CF* coarse fraction (>2 mm); sand/silt/clay content following the USDA criteria; *bD* bulk density; *pH* pH value (measured in water 1/2.5); *TOC* total organic carbon content; *TN* total nitrogen content; *C/N* C to N ratio

decreased as the humification state increased. The highest C/N values were found in the Fs + FgL in pine stands, following the sequence: beech = mixed <<< pine. C/N values in HmL showed the same sequence (beech = mixed < pine),

but in this case, C/N in pine stands was quite similar to that on beech and mixed stands.

Total Cstock_{FF} was significantly higher in pine stands, where 92% of the carbon was provided by HmL fraction

Table 3 Statistical results of the mixed model for forest floor and mineral soil at plot level (stand type, triplet methodology)

	<i>p</i> -values	Triplet	Fraction	Stand type	Stand type*Fraction
Forest floor	B	ns	0.0002***	0.0008***	<0.0001***
	TOC	0.0541*	0.0008***	0.006**	0.029**
	TN	0.0052**	<0.0001***	0.0063**	<0.0001***
	C/N	0.0376**	<0.0001***	0.0002***	0.0007***
	Cstock	0.0818*	0.0032**	<0.0001***	<0.0001***
		Triplet	Depth	Stand type	Stand type*Depth
Soil	TOC	<0.0001***	<.0001***	ns	ns
	TN	0.0018**	0.0009***	ns	ns
	C/N	<0.0001***	<.0001***	ns	ns
	Cstock	<0.0001***	<.0001***	ns	ns

B litter biomass; *TOC* organic carbon content; *TN* nitrogen content; *C/N* carbon-to-nitrogen ratio; *Cstock* carbon stock

Significant levels: ***: ($p < 0.001$); **: ($p < 0.05$); *: ($p < 0.1$); ns: no significant

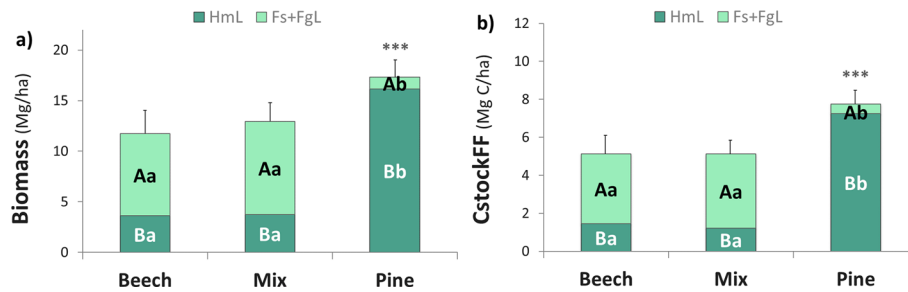


Fig. 2 Forest floor stocks for: **a** Biomass (Mg ha^{-1}) and **b** Total C stock (Mg C ha^{-1}). Different CAPITAL LETTERS show significant differences between litter fractions within each treatment. Different

ent lower-case letters show significant differences among treatments within each litter fraction. *** significant differences among treatments in total values ($p < 0.001$)

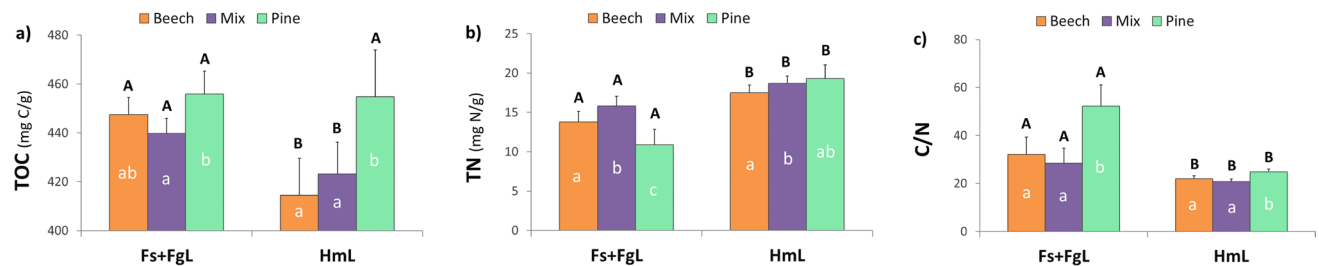


Fig. 3 Forest floor mean values for: **a** Total carbon content (mg C g^{-1}), **b** Total nitrogen content (mg N g^{-1}) and **c** C/N ratio. Different CAPITAL LETTERS show significant differences between litter frac-

tions within each treatment. Different lower-case letters show significant differences among treatments within each litter fraction

(Fig. 2b). The opposite trend was found in mixed and beech stands, which did not significantly differ between them, and, in both cases, 74% of the carbon was provided by the Fs + FgL fraction. As total biomass, no correlation between the $Cstock_{FF}$ and the stand characteristics (BA, N, Dm...) was detected.

Mineral soil

All the variables studied for the mineral soil (TOC, TN, C/N, Cstock) showed the same trend, having higher values on the first 10 cm and decreasing sharply as depth increases (Fig. 4). Except for C/N ratio, which presented a

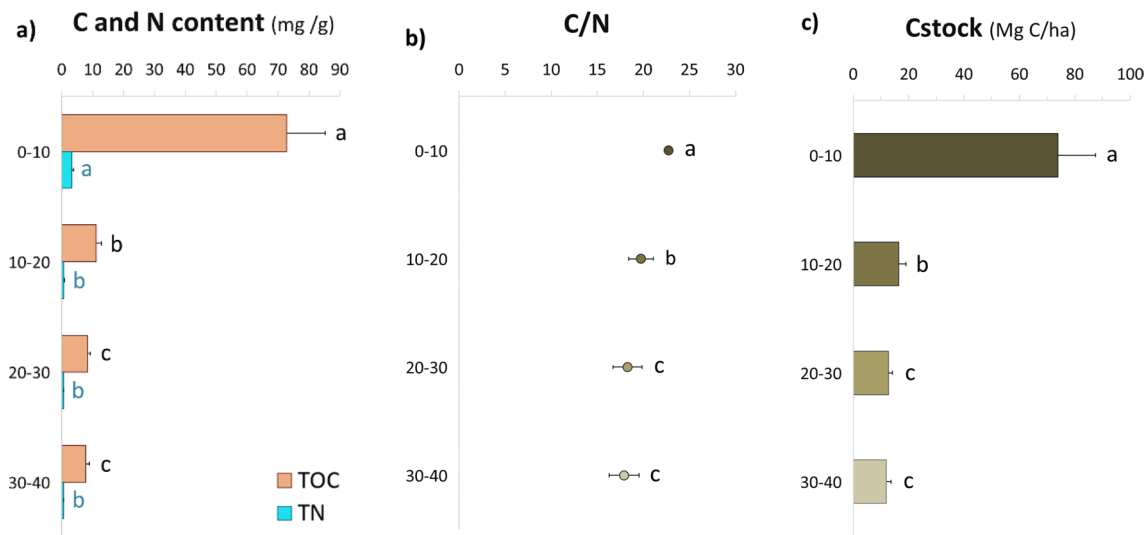


Fig. 4 Mineral soil depths mean values for: **a** C and N content (mg g^{-1}), **b** C/N ratio and **c** Total C stock (Mg C ha^{-1}). Different letters show significant differences between soil depths

smoother decrease (Fig. 4b), the other variables showed a similar decreasing trend, with an average decrease percentage being the following: $_{10 \text{ to } 20} -82\%$, $_{20 \text{ to } 30} -20\%$ and $_{30 \text{ to } 40} -5\%$. Cstock in mineral soil ranged between 39.6 and 337.8 Mg ha^{-1} . The highest values for TOC and Cstock were found in the mineral topsoil layer (0–10) and amounted to 72.8 mg C g^{-1} and 73.9 Mg ha^{-1} , respectively. Total N content followed the same trend, with the highest values in the topsoil layer (3.2 mg N g^{-1}) and very low values in deeper layers ($0.2\text{--}1.2 \text{ mg N g}^{-1}$). C/N ratio was also higher in topsoil layer and slightly decreased with depth (C/N_{0-10} : 22.7; C/N_{10-20} : 19.7; C/N_{20-30} : 18.3; C/N_{30-40} : 17.9). All the variables (TOC, TN, C/N, Cstock) studied for the mineral soil showed significant differences by depth ($p < 0.001$ in all cases) (Table 3). Contrary to expectations, CstockT did not significantly differ among stand types (Table 3). A significant negative correlation was found between CstockT and tree density of the plot ($r = -0.4467$; $p\text{-value} = 0.0063$).

Differences at microsite level (local mixture % approach)

A second approach was analyzing each subplot according to its specific mixture % (microsite-level), rather than considering the stand-type approach (plot-level).

Forest floor

No effect of pine proportion was detected in total biomass (pine and Pine²: ns) when we analyzed it at microsite-level (local mixture %). However, significant differences were found ($p < 0.001$) when differentiating between fractions.

As shown in Fig. 5a, fragmented and humified litter biomass exhibited an opposite trend as pine proportion varied. Fs + FgL biomass increased with pine proportion peaking around 35% and decreasing considerably thereafter. On the contrary, HmL biomass decreased until 25–30% of pine and then increased, representing more than 76% of the total biomass in subplots with the highest presence of pine (> 75% pine).

Regarding TOC, pine stands showed significantly higher C content when analyzed at the plot level ($p < 0.05$; Table 3). However, when studying it at microsite level, this difference does not appear, being only different between litter fractions ($p < 0.001$, Table 4). $\text{TOC}_{\text{Fs+FgL}}$ was higher ($446.2 \text{ mg C g}^{-1}$) than TOC_{HmL} ($404.6 \text{ mg C g}^{-1}$), however, both remained stable regardless of pine proportion (Fig. 5c).

As can be seen in Table 4, a significant effect in the interaction between litter fractions and square pine proportion was found on TN content, C/N ratio and the Cstock ($p < 0.05$; $p < 0.05$; $p < 0.001$, respectively).

TN in Fs + FgL and HmL showed different trends depending on the percentage of pine in the mixture (Fig. 5d). TN in Fs + FgL increased with pine proportion peaking around 35–40% (16.8 mg N g^{-1}) and decreasing thereafter. In general, the TN content was higher in the humified fraction (18.6 mg N g^{-1}) and did not vary with pine proportion.

C/N ratio ranged between 18.9 and 67.3 along all litter fractions and sampling points. C/N decreased with the humification state, being the average values 34.7 for Fs + FgL and 21.7 for HmL. As can be seen in Fig. 5e, C/N ratio in Fs + FgL litter decreased at first, getting a minimum around 40% of pine in the mixture, and then increased reaching values almost 2 times higher than the ones in stands with

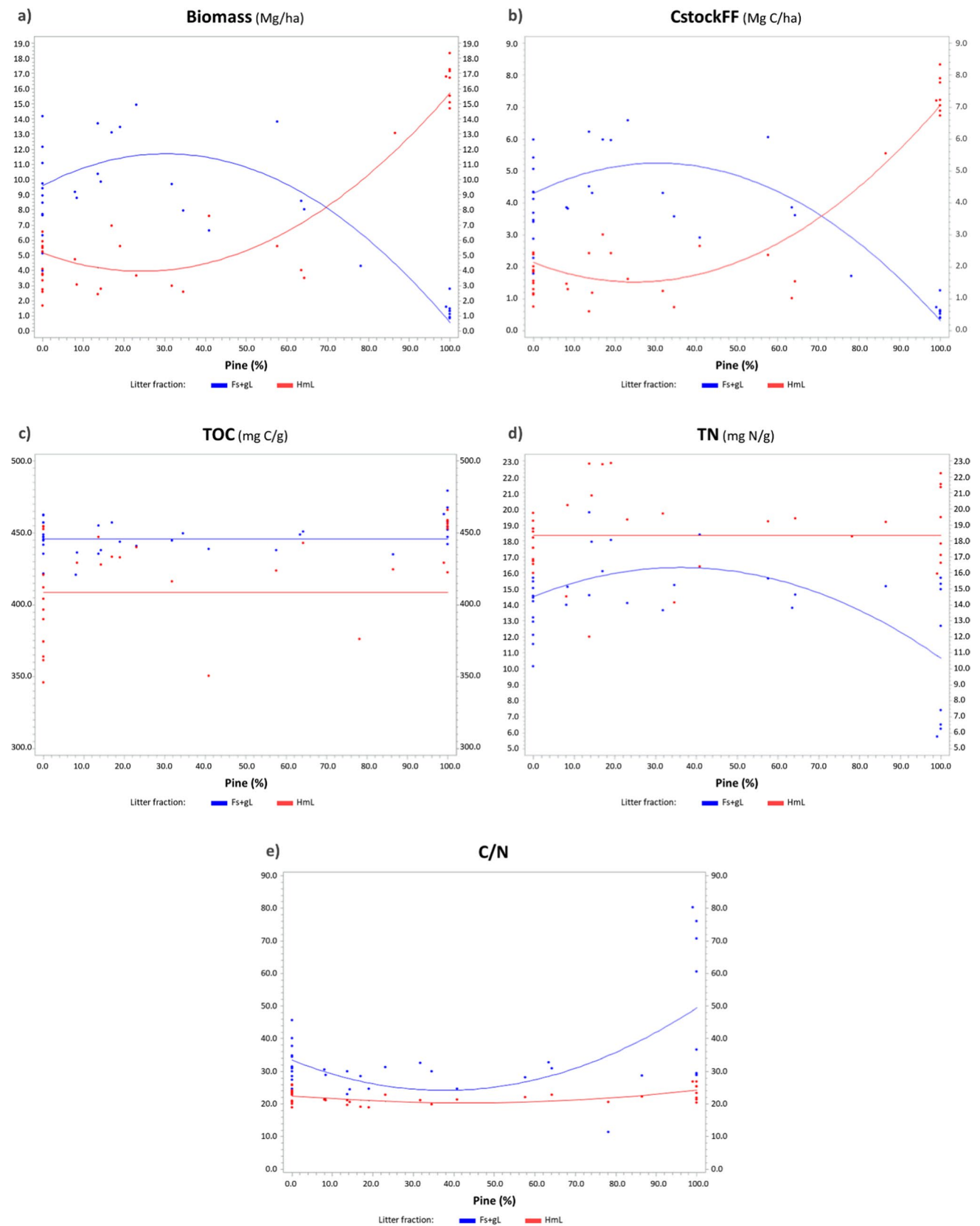


Fig. 5 Forest floor evolution of (a) Biomass (Mg ha^{-1}), b Total C stock (Mg C ha^{-1}), c Total C content (mg C g^{-1}), d Total N content (mg N g^{-1}) and e C/N ratio with pine proportion. Note: Blue line—fresh + fragmented fraction (F+gL); Red line—humified fraction (HmL)

Table 4 Statistical results of the mixed model for forest floor and mineral soil at microsite-level (local mixture %)

	<i>p</i> -values	Triplet	Fraction	Pine	Pine*Fraction	Pine ²	Pine ² *Fraction
Forest floor	B	ns	<.0001***	ns	<.0001***	ns	<0.0001***
	TOC	0.0003***	<0.0001***	ns	ns	ns	ns
	TN	0.0153**	<0.0001***	ns	0.0397**	0.0791*	0.0035**
	C/N	0.0498**	0.0006***	0.0338 **	0.0646*	0.0063**	0.0123**
	Cstock	0.0908*	<0.0001***	ns	<0.0001***	ns	<0.0001***
		Triplet	Depth	Pine	Pine*Depth	Pine ²	Pine ² *Depth
Soil	TOC	<.0001***	<.0001***	0.071*	ns	0.0822*	ns
	TN	<.0001***	<.0001***	ns	ns	ns	ns
	C/N	<.0001***	0.0112***	ns	ns	ns	ns
	Cstock	<.0001***	<.0001***	ns	ns	ns	ns

B litter biomass; *TOC* organic carbon content; *TN* nitrogen content; *C/N* carbon-to-nitrogen ratio; *Cstock* carbon stock

Significant levels: ***($p < 0.001$); **($p < 0.05$); *($p < 0.1$); ns: no significant

a low presence of pine (0–30%). So does C/N ratio in the HmL increasing much lighter, but significantly. The lowest values in the C/N ratio were reached in intermediate mixtures (25–50%) where, in addition, there were no differences between the fractions; $(C/N)_{Fs+FgL} = (C/N)_{HmL}$.

Cstock in forest floor ($Cstock_{FF}$) ranged between 2.5 and 11.1 Mg C ha⁻¹ with an average of 6.9 Mg C ha⁻¹ across all subplots studied. As $Cstock_{FF}$ is calculated from the litter biomass and its C content, in this case, $Cstock_{FF}$ was highly dependent on the amount of litter biomass accumulated. Although no differences were found in totals, the behavior of $Cstock_{FF}$ with pine proportion was the opposite in each fraction ($p < 0.001$). In mixtures with less than 70% of pine, Fs + FgL fraction accumulated more C than the HmL. $Cstock_{Fs+FgL}$ increased until 30% of mixture and then decreased considerably as pine proportion increased. $Cstock_{HmL}$ showed the opposite trend (first decreased and then increased). The lines intersect around 70% of pine, becoming $Cstock_{HmL} > Cstock_{Fs+FgL}$ (Fig. 5b).

As in the plot-level approach, no significant correlation between the total litter biomass, neither $Cstock_{FF}$, and the stand characteristics (BA, N, Dm...) was detected.

Mineral soil

As seen before, the first 10 cm of the mineral soil showed the highest values for all variables studied. As with TOC, Cstock was higher in the first 10 cm of soil, representing on average 65% of the total carbon accumulated in the profiles.

Total organic carbon was the only variable affected by pine proportion. Although the interaction was not significant (Table 4), the quadratic and linear trends were ($p < 0.1$). Thus, the effect of pine proportion on TOC is the same for all depths.

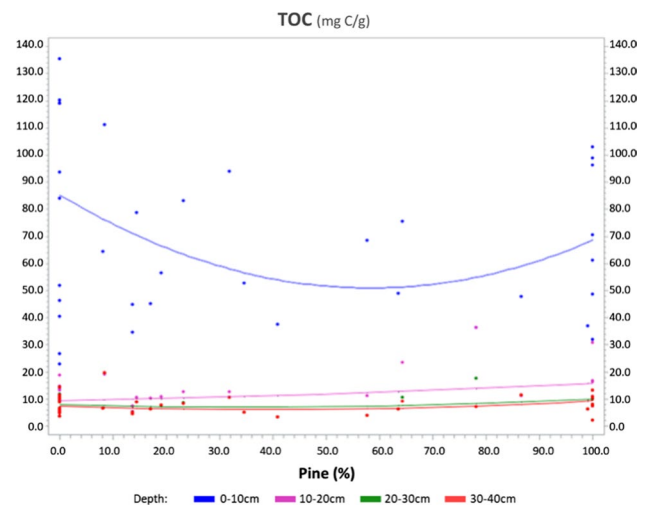


Fig. 6 Total C content (mg C g⁻¹) in mineral soil. Note: Blue line—0–10 cm; Pink line—10–20 cm; Green line—20–30 cm; Red line—30–40 cm

In general, TOC decreased as the presence of pine in the mixture increased, reaching a minimum value in stands with 60% pine, and slightly increased thereafter (Fig. 6). Despite this increase, the carbon content in the pine-dominated stands did not exceed the carbon content of the stands with low or no presence of pine (<20% pine).

No effect of pine proportion was found in mineral soil for TN content, C/N ratio neither Cstock. Significant differences were found only between soil depths ($p < 0.001$; $p < 0.001$; $p < 0.001$). No significant correlation between the $Cstock_T$ and the subplot characteristics (BA, N...) was detected.

Discussion

Plot-level vs microsite-level approach

The microsite-level approach was consistent with most of the differences detected with the plot-level approach, however, it provided us with greater insight into how different mixing percentages affect SOM. According to Michalet et al. (2015), in a mixed forest, the individual trees of different species interact locally with each other. In this sense, by using the %mixture approach, this interaction can be detected. Similarly to us, Yeste et al. (2021) used a 4 m circle around the sampling point as the influence area in their studies on the effect of pine-beech mixtures on soil and root properties.

Moreover, by replacing "Stand type" by "Pine proportion" in the model, we were able to appreciate different trends, including a quadratic evolution (Table 4: Pine²), in several variables studied (Fig. 5). Our results show that, when analyzing at plot-level, litter fractions in mixed and beech stands did not show significant differences in biomass, TOC, C/N and Cstock, except for TN. Meanwhile, pine stands differ significantly from the rest. However, with the microsite-level analysis we were able to get a more detailed perspective of the effect of those intermediate mixtures on SOM (Fig. 5). For example, we were able to identify the optimal mixture percentage for each variable studied by observing the mixture percentage at which a maximum or minimum value is reached.

Regarding mineral soil, in both approaches, differences between depths were detected. However, when analyzing TOC at microsite-level, in addition to detect differences by depth, Pine² was significant at 90%. Thus, there was a quadratic effect of pine proportion on TOC, and that effect was the same for all depths. The results found by López-Marcos et al. (2021) highlight the fundamental role of scale in the study of species richness and forest ecosystem functioning in mixed stands of *Pinus sylvestris* and *Pinus pinaster*. More specifically these authors evidenced an overyield on a small spatial scale related to the higher density and basal area in mixtures compared to monospecific plots, due to the more intimate mixture of both species of *Pinus* at a neighborhood scale. This more efficient use of space in the mixed plots was related to the complementarity of niches in terms of using soil resources (water and soil fertility). In our case, mixed plots also had a higher density and basal area than pure plots (Table 1), which may reflect a more efficient use of soil resources.

Due to the consistency of the results, the additional information provided, and the fact that soil properties can vary even within a few meters distance (Falconer et al. 2015). We decided to bet on the microsite-level approach

to study the effect of species mixing on SOM. From our point of view, by not taking into account the mixture percentage of individual sampling points, we lose valuable information concerning how the local tree composition affects the soil, since the effect is diluted by the mean value of the plot.

It should also be noted that, significant differences observed between sites were not expected. Including different experimental sites in the same study could have some advantages and disadvantages. As a benefit, since the study is not restricted to a single site, we might be able to obtain more representative results. The downside is that differences between sites can increase the variability of data. Despite this, we decided to include all three sites in the study since they presented similar characteristics, such as texture, pH, sequence of horizons, soil type and climate. The causes of these differences are unclear to us. It is therefore necessary to study these plots more closely in order to explain these differences.

Differences at microsite level (local mixture %)

Does the different species mixture affect litter quantity and its C to N ratio?

Our results confirmed a strong effect of pine proportion on litter fractions biomass; nevertheless, total biomass was not affected. Numerous authors have observed a positive and significant effect of BA on litter production and its accumulation in the soil (Lado-Monserrat et al. 2015; Bueis et al. 2017). However, no correlation between the total litter biomass and the basal area, neither tree density, of the subplots was detected in our study. Out of the litter biomass, in stands with a high presence of pine, 80% was provided by the humified litter. This result might be explained by the fact that pine litter has more chemically recalcitrant compounds (Wang et al. 2016; Berg and McLaugherty 2020), and higher C/N ratio. Higher recalcitrance hinders further decomposition of litter, accumulating large amounts of the humified fraction which turn into lower C input into the soil (López-Marcos et al. 2018). In the same way, C/N ratios in litter are related to nitrogen immobilization and mineralization during decomposition (Swift et al. 1979). As said by Wang et al. (2015) plant residues with a higher C/N ratio would produce an acceleration in the mineralization of SOM (positive priming effect), rapidly leading to an N limitation (negative priming effect). Therefore, the microbes will need additional N to balance out the excess of C. If they have N-rich litter, like beech litter, then they can continue working, otherwise, if the N-rich litter is not available on the soil, the litter will accumulate.

In mixtures with less than 50% of pine, more than 70% of the litter biomass was provided by Fs + FgL fraction. In

general, the Fs + FgL litter is associated with lower stability than HmL litter, being, the C accumulated in that fraction, more likely to return into the atmosphere as a consequence of the mineralization process (Córdova et al. 2018). However, our results showed that the lowest C/N ratio values ($C/N = 20\text{--}25$) were reached in intermediate mixtures (pine percentages between 25 and 50%). We postulate that the presence of beech leaves may be causing a positive priming effect (Wang et al. 2015), accelerating the decomposition of the residues that are more difficult to degrade (higher and more recalcitrant C/N ratios). It would also explain why, although the basal area in those mixtures was higher, the total litter biomass accumulated did not exceed the amounts accumulated in subplots with less tree density. Moreover, in those mixture percentages, no differences between the fractions were detected; $(C/N)_{Fs+FgL} = (C/N)_{HmL}$. These results, therefore, suggest that, in intermediate mixtures (25–50% pine), although litter was physically differentiated as fragmented or humified, chemically it had a similar humification state. Numerous works have emphasized the importance of C/N ratio in terms of litter quality and humification (Kooch et al. 2017). A C to N ratio of 20–25 results in an equilibrium state between mineralization and immobilization (Brust 2019). Lower C/N ratios are related to a high humification degree and, therefore, a more stable and better quality of SOM (Błońska et al. 2018; Berg and McLaugherty 2020; Martins et al. 2011). The highest C to N ratio was found in the Fs + FgL fraction in pine-dominated stands. However, in those stands, 92% of the $C_{stock_{FF}}$ was found in humified fractions (HmL), which exhibited a C/N ratio of approximately 20–25 (Fig. 5e). Similar results were shown by Laganière et al. (2013) when studying the effect of the composition of forest stands on the C stock and its stability in Canadian forests. However, we must not forget that the C storage in forest floor could be vulnerable to future changes in environmental conditions. The increase in temperature may favor C mineralization and, therefore, the loss of the accumulated C. Similarly, in the event of natural disasters such as forest fires, this C would be lost by burning. Therefore, the optimum C to N ratio detected in the forest floor of intermediate mixtures (25–50%) does not necessarily imply a great advantage in terms of C accumulation, since it would be far more interesting if this C is accumulated in mineral soil, where it could be protected for longer periods.

One unexpected finding was that, in mixtures with low to medium presence of pine (10–40% pine), TN content in Fs + FgL litter increased as pine proportion did, reaching a maximum around 40% of pine in the mixture. Thereafter, TN content decreased as pine proportion increased, reaching the lowest TN values ($TN_{100\%} \ll TN_{0\%}$). It is somewhat surprising that the presence of pine needles, which have lower N content than beech leaves (Berg and McLaugherty 2020), increases TN in Fs + FgL fraction. The expected pattern

would have been a decrease in TN content, and what we found is a synergistic effect (Fig. 5d). The reason for this is not clear but it may have something to do with the fact that when more labile OM is added, it facilitates microbial community growth. As reported by Wang et al. (2015) the quality of litter inputs may influence soil microbial community structure, which will preferentially consume these labile OM (substrate preference). In general, under N-limited but C-rich conditions, the soil microbial community can rapidly absorb N (Kuzyakov et al. 2000), immobilizing large amounts of this nutrient in their tissues. This sequestered N will form part of the biomass of microorganisms present in the litter and may be the reason for the increase in N detected. Due to differences in rooting patterns, beech and pine mixtures create a complementary use of root space (Pretzsch et al. 2015), and therefore greater soil exploration (Rothe and Binkley 2001) and access to nutrients (Augusto et al. 2002; Błońska et al. 2016). In addition, some authors (Prescott 2002; Cremer et al. 2016) have reported that a more diverse canopy in mixed stands can improve nutritional and biological diversity (Rothe and Binkley 2001).

Does the different species mixture affect the mineral soil characteristics?

Regarding the mineral soil, our results showed higher values in the first 10 cm for all the studied parameters and a sharply decrease as depth increases. Demonstrating that soil organic carbon is vertically distributed with a strong concentration gradient that decreases from the surface to deeper layers (Basile-Doelsch et al. 2020). Similar results were found by Jandl et al. (2021) in their studies about soil organic carbon stocks in mixed-deciduous and coniferous forests in Austria. TOC was the only parameter that varied with the mixture percentage. Pine proportion had a slight, but almost significantly ($p < 0.1$) effect on TOC for all soil depths, decreasing up to 60% of pine and slightly increasing thereafter.

Around 69% of the TOC and 64% of the C stock in the whole profile (C_{stockT}) were located in the first 10 cm. These results are logical and consistent with previous studies (Angst et al. 2018, 2019; Wiesmeier et al. 2019; Lavalley et al. 2020; Mayer et al. 2020) as topsoil receives a higher contribution of organic matter from the decomposition of the litterfall.

Contrary to expectations, this study did not find a significant difference in C stocks across the different mixture percentages (Table 4). Similar results were found by Cremer et al. (2016) in their studies on mixed stands of beech and coniferous (Douglas fir and Norway spruce). Species mixture had a very small and inconsistent effect on C and N content in the mineral soil, and they did not detect an increase in C stocks in the upper layer of the mineral soil. Rehschuh et al. (2021) suggested that synergistic or antagonistic

interactions in species mixtures may be responsible for the fact that the mixing ratio does not have a great impact on organic C reserves in forest soils. Another possible explanation may be that, due to the intermediate age of the stands (55 years), the changes detected in the litter quantity and its C to N ratio have not reverted to the mineral soil. In their study, López-Marcos et al. (2018) did find an effect of the admixture on soil C stock and TOC, however, the age of their stands ranged between 44 and 151 years.

Our results show that the percentage of species mixture mainly influenced the quantity and the C to N ratio of the organic matter in the forest litter rather than the mineral soil. There was only limited evidence of any tree species' effect on mineral TOC. This finding supports previous research, the results obtained by Vesterdal et al. (2013), in which these authors showed that C storage in the mineral soil seemed to be more influenced by the type of soil or the climate rather than by the tree species, while the effect of tree species on C pools in litter was more consistent.

Conclusions

Our results show that the percentage of species mixture mainly influenced the amount of C accumulated and the C to N ratio of organic matter in the forest floor rather than in the mineral soil. A C to N ratio of 20–25 was found in the forest floor of stands with 25–50% of pine in the mixture, indicating an equilibrium state between mineralization and immobilization. Mineral soil properties were barely affected by species mixture proportion, and the distribution of carbon and nitrogen along the soil profile was the same in all cases.

Returning to the hypothesis that the scale used in plot-level approach is too large for SOM studies, and a smaller scale might be more appropriate, our results showed that the plot-level approach dilutes or masks some effects that can be observed at the microsite-level. Thus, it is now possible to state that microsite-level scale (local mixture %) is a better option when studying the effect of tree stand composition on SOM.

Appendix 1

See Figs. 7 and 8.

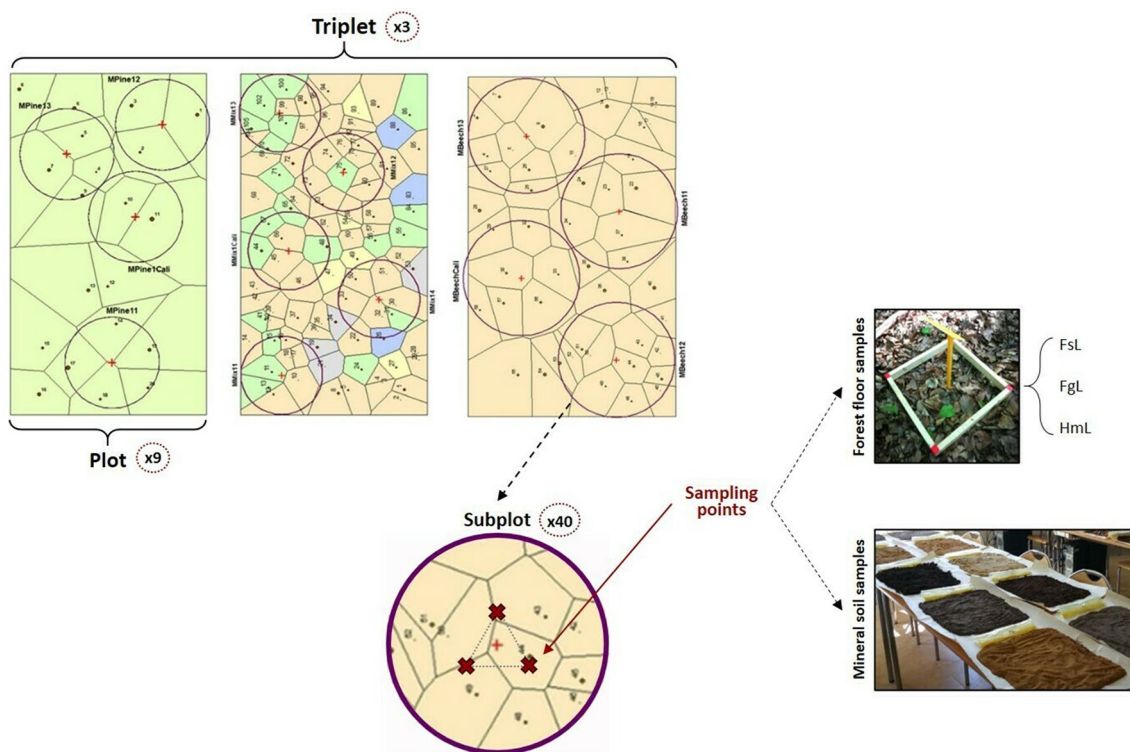


Fig. 7 Experimental design example (Triplet: Ger_1). Rectangular plots from left to right: pure pine plot (100% pine); mixed plot (63%, 40%, 34%, 13%, 8% pine); pure beech plot (0% pine). Tree positions and their influence area are shown by black points and the Thiessen

polygons, respectively. Tree species: *Pinus sylvestris* (green), *Fagus sylvatica* (orange), *Picea abies* (blue), *Larix decidua* (gray) and *Quercus* (yellow)

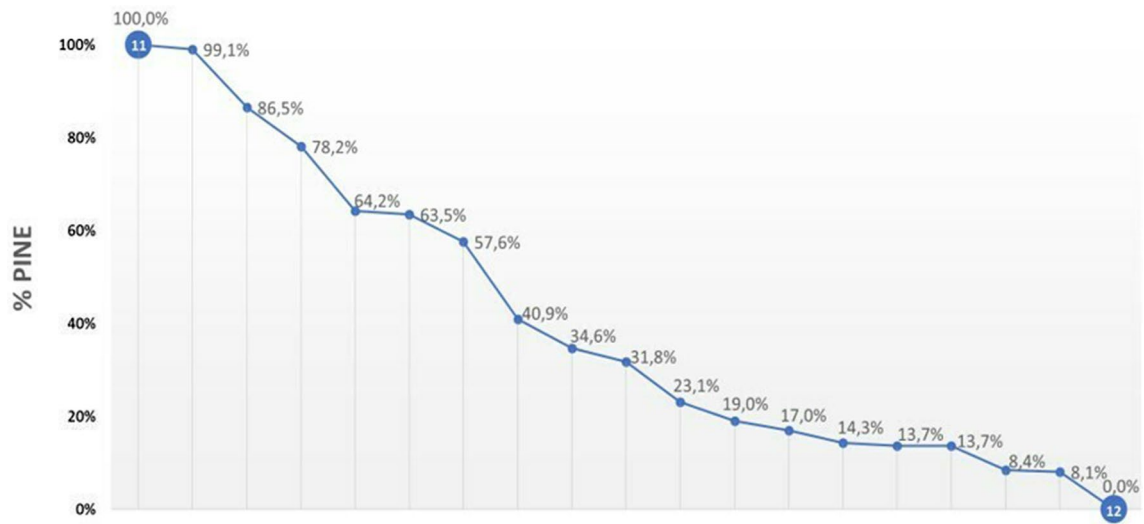


Fig. 8 Species mixture percentage covered. Total number of subplots: 40

Appendix 2

See Fig. 9.

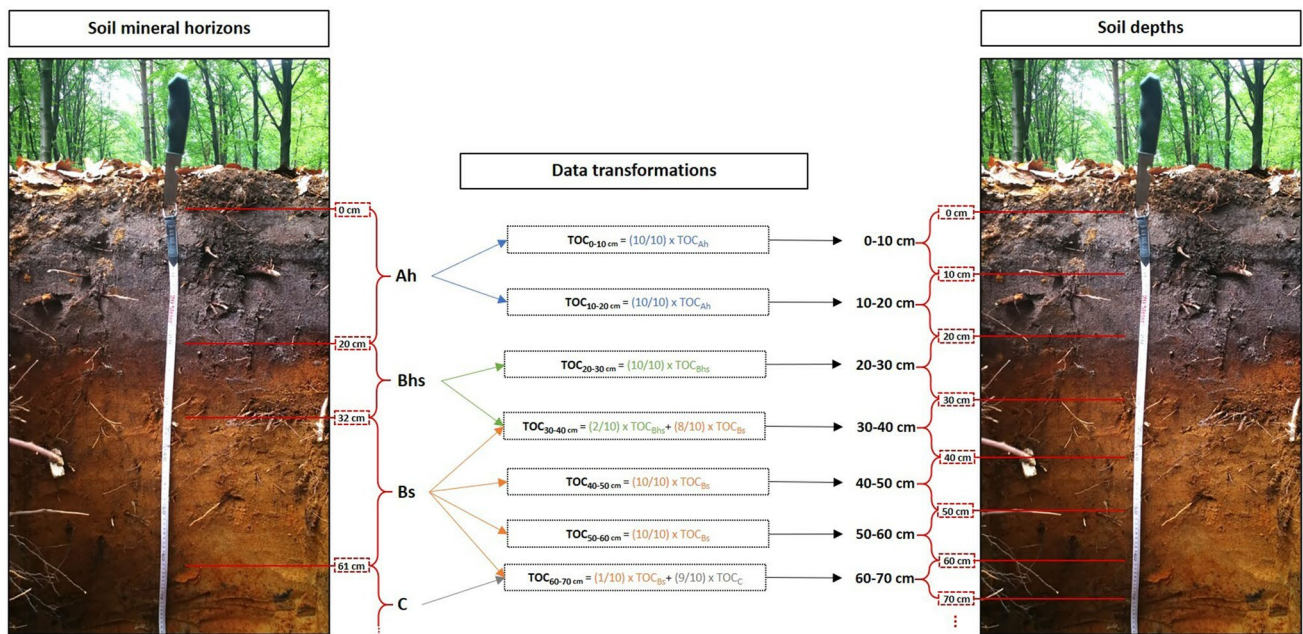


Fig. 9 Example of transformation of variables from horizons to depths. Soil profile Pol_5M

Acknowledgements This research has been possible thanks to the people who participated in the EuMIXFOR project, establishing the experimental triplets that were sampled in this study. Finally, we would like to thank the invaluable help of all the people who have participated in field work, laboratory analysis and with statistical and forestry assistance.

Author contributions MBT and FB contributed to research idea. MGA contributed to field and laboratory work. MGA, HP and MP contributed to data acquisition. MBT and FB contributed to funding acquisition. RSM contributed to statistical analyses. MGA, RSM and MBT contributed to data analysis and comment on the results and discussion. MGA contributed to writing the original draft. MGA, RSM, MBT and FB contributed to reviewing, checking and improving the manuscript; final version checked by all the authors.

Funding Open Access funding provided thanks to the CRUE-CSIC agreement with Springer Nature. This research was supported by the Spanish Ministry of Science and Innovation, financing a predoctoral contract for the first author (FPU 2016-BOE 01-17-2017) and the current project "IMFLEX: integrated forest management along complexity gradients" (PID2021-126275OB-C22), by the Junta de Castilla y León (Spain) through the projects "Servicios ecosistémicos de los suelos bajo masas forestales mixtas versus masas puras. Efecto del tipo de masa en la fertilidad edáfica, retención de agua y secuestro de carbono" (REF. V096G19), "CLU-2019-01 and CL-EI-2021-05-iuFOR Institute Unit of Excellence of the University of Valladolid" and cofinanced by the European Regional Development Fund. Finally, the field work in Poland and Germany was possible thanks to two research stays financed by the CARE4C project (funded by the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie Grant Agreement No 778322).

Availability of data and material Data are available from the corresponding author on reasonable request.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

References

- Angst G, Greiner M, Häusler W, Hertel D, Kirfel K, Kögel-Knabner I, Leuschner C, Messinger J, Mueller CW, Rethemeyer J (2018) Soil organic carbon stocks in topsoil and subsoil controlled by parent material, carbon input in the rhizosphere, and microbial-derived compounds. *Soil Biol Biochem* 122:19–30. <https://doi.org/10.1016/j.soilbio.2018.03.026>
- Angst G, Chorover J, Eissenstat DM, Freeman KH, Hobbie SE, Mueller CW, Mueller KE, Oleksyn J, Reich PB, Trumbore S (2019) Soil organic carbon stability in forests: Distinct effects of tree species identity and traits. *Glob Chang Biol* 25:1529–1546. <https://doi.org/10.1111/gcb.14548>
- Augusto L, Ranger J, Binkley D, Rothe A (2002) Impact of several common tree species of European temperate forests on soil fertility. *Ann for Sci* 59:233–253. <https://doi.org/10.1051/forest:2002020>
- Basile-Doelsch I, Balesdent J, Pellerin S (2020) Reviews and syntheses: the mechanisms underlying carbon storage in soil. *Biogeosciences* 17:5223–5242. <https://doi.org/10.5194/bg-17-5223-2020>
- Berg B, McLaugherty C (2020) Plant litter: decomposition, humus formation, carbon sequestration. Springer, Cham
- Błońska E, Lasota J, Zwyczaj M, Piaszczyk W (2016) Stand mixing effect on enzyme activity and other soil properties. *Soil Sci Annu* 67:173–178. <https://doi.org/10.1515/ssa-2016-0021>
- Błońska E, Gruba P, Klamerus-Iwan A, Lasota J, Pach M, Pretzsch H (2018) What characteristics of soil fertility can improve in mixed stands of scots pine and European beech compared with monospecific stands? *Commun Soil Sci Plant Anal* 49:237–247. <https://doi.org/10.1080/00103624.2017.1421658>
- Bravo-Oviedo A, Pretzsch H, Ammer C et al (2014) European mixed forests: definition and research perspectives. *For Syst* 23:518. <https://doi.org/10.5424/fs/2014233-06256>
- Brus DJ, Goedhart PW, Gunia K, Heidema AH, Hengeveld GM, Nabuurs GJ, Walvoort DJJ (2012) Statistical mapping of tree species over Europe. *Eur J for Res* 131:145–157. <https://doi.org/10.1007/s10342-011-0513-5>
- Brust GE (2019) Management strategies for organic vegetable fertility. In: Safety and practice for organic food. Elsevier, pp 193–212
- Bueis T, Bravo F, Pando V, Turrión MB (2017) Influencia de la densidad del arbolado sobre el desfronde y su reciclado en pinares de repoblación del norte de España. *Bosque (valdivia)* 38:401–407. <https://doi.org/10.4067/S0717-92002017000200017>
- Bueis T, Bravo F, Pando V, Turrión MB (2018) Local basal area affects needle litterfall, nutrient concentration, and nutrient release during decomposition in *Pinus halepensis* Mill. plantations in Spain. *Ann for Sci* 75:21. <https://doi.org/10.1007/s13595-018-0699-5>
- Butler BJ, Hewes JH, Liknes GC, Nelson MD, Snyder SA (2014) A comparison of techniques for generating forest ownership spatial products. *Appl Geogr* 46:21–34. <https://doi.org/10.1016/j.apgeog.2013.09.020>
- Cattaneo N, Schneider R, Bravo F, Bravo-Oviedo A (2020) Interspecific competition of tree congeners induces changes in crown architecture in Mediterranean pine mixtures. *For Ecol Manage* 476:118471. <https://doi.org/10.1016/j.foreco.2020.118471>
- Córdova SC, Archontoulis SV, Castellano MJ, Dietzel RN, Mueller KE, Olk DC (2018) Plant litter quality affects the accumulation rate, composition, and stability of mineral-associated soil organic matter. *Soil Biol Biochem* 125:115–124. <https://doi.org/10.1016/j.soilbio.2018.07.010>
- Cornwell WK, Cornelissen JHC, Amatangelo K et al (2008) Plant species traits are the predominant control on litter decomposition rates within biomes worldwide. *Ecol Lett* 11:1065–1071. <https://doi.org/10.1111/j.1461-0248.2008.01219.x>
- Cremer M, Kern NV, Prietzel J (2016) Soil organic carbon and nitrogen stocks under pure and mixed stands of European beech, Douglas fir and Norway spruce. *For Ecol Manage* 367:30–40. <https://doi.org/10.1016/j.foreco.2016.02.020>
- de Streef G, Ammer C, Annighöfer P et al (2021) Mixing has limited impacts on the foliar nutrition of European beech and Scots pine trees across Europe. *For Ecol Manage* 479:118551. <https://doi.org/10.1016/j.foreco.2020.118551>

- Dixon RK, Brown S, Houghton RA, Solomon AM, Trexler MC, Wisniewski J (1994) Carbon pools and flux of global forest ecosystems. *Science* 263:185–190. <https://doi.org/10.1126/science.263.5144.185>
- Falconer RE, Battaia G, Baveye P, Chenu C, Otten W, Schmidt S (2015) Microscale heterogeneity explains experimental variability and non-linearity in soil organic matter mineralisation. *PLoS ONE* 10:e0123774. <https://doi.org/10.1371/journal.pone.0123774>
- FAO (2006) Guidelines for soil description. Fourth edition. Rome
- Fick SE, Hijmans RJ (2017) WorldClim 2: new 1-km spatial resolution climate surfaces for global land areas. *Int J Climatol* 37:4302–4315. <https://doi.org/10.1002/joc.5086>
- Gamfeldt L, Snäll T, Bagchi R et al (2013) Higher levels of multiple ecosystem services are found in forests with more tree species. *Nat Commun* 4:1340. <https://doi.org/10.1038/ncomms2328>
- Grodzinski W, Weiner J, Maycock P (eds) (2012) Forest ecosystems in industrial regions: studies on the cycling of energy nutrients and pollutants in the Niepolomice forest southern Poland
- Herrero C, Turrión MB, Pando V, Bravo F (2016) Carbon content of forest floor and mineral soil in Mediterranean *Pinus* spp. and Oak stands in acid soils in Northern Spain. *For Syst* 25:e065. <https://doi.org/10.5424/fs/2016252-09149>
- Heym M, Ruíz-Peinado R, del Río M et al (2017) EuMIXFOR empirical forest mensuration and ring width data from pure and mixed stands of Scots pine (*Pinus sylvestris* L.) and European beech (*Fagus sylvatica* L.) through Europe. *Ann for Sci* 74:63. <https://doi.org/10.1007/s13595-017-0660-z>
- Heym M, Ruíz-Peinado R, Del Río M, et al (2019) Data from: EuMIXFOR empirical forest mensuration and ring width data from pure and mixed stands of Scots pine (*Pinus sylvestris* L.) and European beech (*Fagus sylvatica* L.) through Europe. Dryad, Dataset
- IUSS Working Group WRB (2015) World reference base for soil resources 2014, update 2015 International soil classification system for naming soils and creating legends for soil maps. World Soil Resources Reports No. 106. FAO, Rome
- Jandl R, Ledermann T, Kindermann G, Weiss P (2021) Soil organic carbon stocks in mixed-deciduous and coniferous forests in Austria. *Front for Global Change* 4:1–14. <https://doi.org/10.3389/ffgc.2021.688851>
- Kapusta P, Stanek M, Szarek-Lukaszewska G, Godzik B (2019) Long-term moss monitoring of atmospheric deposition near a large steelworks reveals the growing importance of local non-industrial sources of pollution. *Chemosphere* 230:29–39. <https://doi.org/10.1016/j.chemosphere.2019.05.058>
- Kooch Y, Samadzadeh B, Hosseini SM (2017) The effects of broad-leaved tree species on litter quality and soil properties in a plain forest stand. *Catena (amst)* 150:223–229. <https://doi.org/10.1016/j.catena.2016.11.023>
- Köppen W (1936) Das geographische System der Klimate. Hand-buch der Klimatologie 7–30
- Kuzyakov Y, Friedel JK, Stahr K (2000) Review of mechanisms and quantification of priming effects. *Soil Biol Biochem* 32:1485–1498. [https://doi.org/10.1016/S0038-0717\(00\)00084-5](https://doi.org/10.1016/S0038-0717(00)00084-5)
- Lado-Monserrat L, Lidón A, Bautista I (2015) Litterfall, litter decomposition and associated nutrient fluxes in *Pinus halepensis*: influence of tree removal intensity in a Mediterranean forest. *Eur J for Res* 134:833–844. <https://doi.org/10.1007/s10342-015-0893-z>
- Laganière J, Bergeron Y, Brassard BW, Cavard X, Chen HYH, Paré D (2013) Stability of soil carbon stocks varies with forest composition in the Canadian Boreal Biome. *Ecosystems* 16:852–865. <https://doi.org/10.1007/s10021-013-9658-z>
- Lavallee JM, Soong JL, Cotrufo MF (2020) Conceptualizing soil organic matter into particulate and mineral-associated forms to address global change in the 21st century. *Glob Chang Biol* 26:261–273. <https://doi.org/10.1111/gcb.14859>
- López-Marcos D, Bravo F, Jonard M, Martínez-Ruiz C, Ponette Q, Titeux H, Turrión MB (2018) Soil carbon stocks and exchangeable cations in monospecific and mixed pine forests. *Eur J for Res* 137:831–847. <https://doi.org/10.1007/s10342-018-1143-y>
- López-Marcos D, Turrión MB, Bravo F, Martínez-Ruiz C (2020) Can mixed pine forests conserve understory richness by improving the establishment of understory species typical of native oak forests? *Ann for Sci* 77:15. <https://doi.org/10.1007/s13595-020-0919-7>
- López-Marcos D, Turrión MB, Bravo F, Martínez-Ruiz C (2021) Overyielding in mixed pine forests with belowground complementarity: impacts on understory. *Eur J for Res* 140:777–791. <https://doi.org/10.1007/s10342-021-01365-0>
- Lucas-Borja ME, Andrés M, Bastida F, Candel D, Jindo K, Moreno JL (2012) Soil microbial community structure and activity in monospecific and mixed forest stands, under Mediterranean humid conditions. *Plant Soil* 354:359–370. <https://doi.org/10.1007/s11104-011-1072-8>
- Martins T, Saab SC, Milori DMBP, et al (2011) Soil organic matter humification under different tillage managements evaluated by Laser Induced Fluorescence (LIF) and C/N ratio. *Soil Tillage Res* 111:231–235. <https://doi.org/10.1016/j.still.2010.10.009>
- Mayer M, Prescott CE, Abaker WEA et al (2020) Tamm review: influence of forest management activities on soil organic carbon stocks: a knowledge synthesis. *For Ecol Manage* 466:118127. <https://doi.org/10.1016/j.foreco.2020.118127>
- Michalet R, An L, Chen S, Ding C, Guo P, Wang X, Wang Y, Xiao S (2015) Communities: are they groups of hidden interactions? *J Veg Sci* 26:207–218. <https://doi.org/10.1111/jvs.12226>
- Osei R, Titeux H, Bielak K et al (2021) Tree species identity drives soil organic carbon storage more than species mixing in major two-species mixtures (pine, oak, beech) in Europe. *For Ecol Manage* 481:118752. <https://doi.org/10.1016/j.foreco.2020.118752>
- Oueslati I, Allamano P, Bonifacio E, Claps P (2013) Vegetation and topographic control on spatial variability of soil organic carbon. *Pedosphere* 23:48–58. [https://doi.org/10.1016/S1002-0160\(12\)60079-4](https://doi.org/10.1016/S1002-0160(12)60079-4)
- Prescott CE (2002) The influence of the forest canopy on nutrient cycling. *Tree Physiol* 22:1193–1200
- Pretzsch H, del Río M, Ammer C et al (2015) Growth and yield of mixed versus pure stands of Scots pine (*Pinus sylvestris* L.) and European beech (*Fagus sylvatica* L.) analysed along a productivity gradient through Europe. *Eur J for Res* 134:927–947. <https://doi.org/10.1007/s10342-015-0900-4>
- Rehusch S, Dannenmann M, Jonard M, Rennenberg H, Wiesmeier M (2021) Impact of European beech forest diversification on soil organic carbon and total nitrogen stocks—a meta-analysis. *Front for Global Change* 4:606669. <https://doi.org/10.3389/ffgc.2021.606669>
- Riofrío J, del Río M, Pretzsch H, Bravo F (2017) Changes in structural heterogeneity and stand productivity by mixing Scots pine and Maritime pine. *For Ecol Manage* 405:219–228. <https://doi.org/10.1016/j.foreco.2017.09.036>
- Román Dobarco M, Jacobson AR, Van Miegruet H (2021) Chemical composition of soil organic carbon from mixed aspen-conifer forests characterized with Fourier transform infrared spectroscopy. *Eur J Soil Sci* 72:1410–1430. <https://doi.org/10.1111/ejss.13065>
- Roman Dobarco M (2014) Influence of stand composition on soil organic carbon stabilization and biochemistry in Aspen and Conifer Forests of Utah. Utah State University
- Rothe A, Binkley D (2001) Nutritional interactions in mixed species forests: a synthesis. *Can J for Res* 31:1855–1870. <https://doi.org/10.1139/cjfr-31-11-1855>
- Rozen A, Sobczyk KP, Niklińska M (2004) Heavy metal concentrations in Enchytraeidae (Oligochaeta) in the Niepolomice Forest. *Ecotoxicol Environ Saf* 57:81–88. <https://doi.org/10.1016/j.ecoenv.2003.08.006>

- Ruiz-Peinado R, Pretzsch H, Löf M et al (2021) Mixing effects on scots pine (*Pinus sylvestris* L.) and Norway spruce (*Picea abies* (L.) Karst) productivity along a climatic gradient across Europe. *For Ecol Manage* 482:1188. <https://doi.org/10.1016/J.FORECO.2020.118834>
- Schlesinger WH, Andrews JA (2000) Soil respiration and the global carbon cycle. *Biogeochemistry* 48:7–20
- Schleuß P-M, Fender AC, Heitkamp F, Jungkunst HF, Leuschner C (2014) Higher subsoil carbon storage in species-rich than species-poor temperate forests. *Environ Res Lett* 9:014007. <https://doi.org/10.1088/1748-9326/9/1/014007>
- Stanek M, Stefanowicz AM (2019) Invasive *Quercus rubra* negatively affected soil microbial communities relative to native *Quercus robur* in a semi-natural forest. *Sci Total Environ* 696:133977. <https://doi.org/10.1016/j.scitotenv.2019.133977>
- Swift MJ, Heal OW, Anderson JM, Anderson JM (1979) *Decomposition in terrestrial ecosystems*. Blackwell. University of California Press, Berkeley and Los Angeles
- Turrión M-B, Schneider K, Gallardo JF (2009) Carbon accumulation in Umbrisols under *Quercus pyrenaica* forests: effects of bedrock and annual precipitation. *Catena (amst)* 79:1–8. <https://doi.org/10.1016/j.catena.2009.04.004>
- Uzquiano S, Barbeito I, Bravo F, Ehbrecht M, San Martín R, Seidel D (2021) Quantifying crown morphology of mixed pine-oak forests using terrestrial laser scanning. *Remote Sens (basel)* 13:4955. <https://doi.org/10.3390/rs13234955>
- Vesterdal L, Clarke N, Sigurdsson BD, Gundersen P (2013) Do tree species influence soil carbon stocks in temperate and boreal forests? *For Ecol Manage* 309:4–18. <https://doi.org/10.1016/j.foreco.2013.01.017>
- Wander M, Nissen T (2004) Value of soil organic carbon in agricultural lands. *Mitig Adapt Strateg Glob Chang* 9:417–431
- Wang H, Bai E, Boutton TW, Hu G, Jiang P, Xu W (2015) Quality of fresh organic matter affects priming of soil organic matter and substrate utilization patterns of microbes. *Sci Rep* 5:10102. <https://doi.org/10.1038/srep10102>
- Wang J, Sun OJ, Sun X, Tang Z, You Y (2016) A comparison of decomposition dynamics among green tree leaves, partially decomposed tree leaf litter and their mixture in a warm temperate forest ecosystem. *J for Res (harbin)* 27:1037–1045. <https://doi.org/10.1007/s11676-016-0248-8>
- Wiesmeier M, Urbanski L, Hobbey E et al (2019) Soil organic carbon storage as a key function of soils: a review of drivers and indicators at various scales. *Geoderma* 333:149–162. <https://doi.org/10.1016/j.geoderma.2018.07.026>
- Yeste A, Blanco JA, Elizalde-Arbilla M, Imbert JB, Zozaya-Vela H (2021) *Pinus sylvestris* L. and *Fagus sylvatica* L. effects on soil and root properties and their interactions in a mixed forest on the Southwestern Pyrenees. *For Ecol Manage* 481:1187. <https://doi.org/10.1016/j.foreco.2020.118726>

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.