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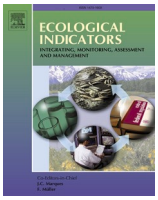

Loss of soil carbon and nitrogen indicates climate change-induced alterations in a temperate forest ecosystem

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ABSTRACT

Climate warming is expected to influence terrestrial biogeochemical cycles by modifying the quality and quantity of plant litter input to soils. Although a growing number of studies recognize the importance of plant litter input in influencing the loss of soil organic matter (SOM) through a phenomenon called the priming effect (PE), the exact mechanisms behind PE are not well known. Importantly, most PE research is based on short term pot experiments in which fresh organic matter (FOM) input is represented by a single addition of compounds of unnaturally simple chemical composition. Furthermore, only a few studies exist in which the PE was explored in terms of organic C (SOC) and total N content in the soil. Here, we report results of a 3-year long litter manipulation study conducted under natural conditions in a broadleaved Korean pine forest in N-E China. We show that the extra supply (twice the normal input) of aboveground tree litter composing of conifer needles, leaves and small twigs was associated not only with slightly decreased SOC (by 5%) but especially that of soil total N (STN) (by 15%) content in the top soil (0–5 cm depth). In contrast, removal of litter resulted in an increased (ca. 15%) amount of both SOC and STN during the study when compared to control soils receiving natural litter input. Despite the enhanced leaf litter decomposition rate in the treatment receiving extra litter, the changes in SOC and STN were related neither to soil microbial biomass nor to community composition. The amount of N lost (40.0 g m^{-2}) in the soil due to litter addition was ca. three times the amount of N added (12.3 g m^{-2}) via the litter, while the amount of C lost (238 g m^{-2}) was about one third of that added (940 g m^{-2}), suggesting that soil N in our research site is more prone to the PE than soil C. As we did not manipulate belowground FOM input, our results suggest that input of aboveground litter rather than that by roots explained the PE in our study. Results of our long-term study conducted under natural conditions in undisturbed forest soils highlight the large potential of recalcitrant, aboveground litter to affect the PE, which should not go unnoticed when predicting the role of forest soils under conditions (such as climate warming) when these soils act as C sinks.

1. Introduction

Soils – whether natural or managed – and their functions are critical in ensuring the provision of various ecosystem services in terrestrial ecosystems (Adhikari and Hartemink, 2016). One of the most intriguing findings in ecology is the central role of fresh organic carbon (C), such as aboveground litter and root exudates, in the decomposition of old soil organic matter (SOM). Virtually all soils contain large storages of organic C as SOM, more than twice the amount of C bound in terrestrial vegetation (Schaphoff et al., 2006) and the atmosphere (Schimel et al., 1995). However, this C is a difficult resource for soil microbes and other soil biota because it takes more energy to decompose it than what can be obtained from it (Lehmann and Kleber, 2015). Due to its relatively high N concentration (Baisden et al., 2002), old SOM is, however, a valuable resource and becomes usable for the decomposer biota if it can simultaneously use other, easily utilizable C sources (Paterson, 2009; Kuzyakov, 2010). This is why labile, “easy” C, released by fresh plant litter and plant roots, can accelerate the decomposition of old SOM, a

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process called the priming effect (PE) (Kuzyakov et al., 2000).

In the context of increasing atmospheric CO_2 concentrations and rising temperatures, net primary productivity (de Graaff et al., 2006; Friend 2010) and input of C in litter fall and root-derived C to the soil (Norby et al., 2002; Trueman and Gonzalez-Meler, 2005) are predicted to increase, which may increase soil organic carbon (SOC) storage (Wieder et al., 2015; Chen et al., 2019). However, the priming effect can have significant consequences on the ability of terrestrial ecosystems to sequestrate C to their soils; in case increasing input of new C augments the priming effect (e.g. Carney et al., 2007; Kuzyakov, 2010), increases in C stocks could be minor or the stocks could, counter-intuitively, even decrease. So far, generally the priming effect research has focused on the effects of root exudates. For example, according to a *meta*-analysis by Huo et al. (2017), processes in the plant rhizosphere, mostly due to living roots, enhance SOC decomposition by over 50% above the rate of unplanted soil controls. Also, fresh plant litter entering the soils from above the ground can act as a significant priming agent (Prévost-Bouré et al., 2010; Wang et al., 2014a). A study by Sun et al. (2019) reports that the addition of exogenous organic C stimulates native SOC decomposition by over 45% (i.e., inducing a positive PE), with the highest value in cropland soils (ca. 60%) and the lowest in forest soils (ca. 26%).

Despite accumulating evidence suggesting the potential influence of the PE on SOM pools in various soil types, there is no clear understanding if the potential increases in productivity due to climate change drivers and consequential increased litter inputs to soil will lead to higher soil C stocks or instead, due to a positive soil priming, to lower soil C stocks. Most studies have been conducted as short-term laboratory incubation experiments, which fail in providing realistic, long-term positive priming mechanisms under field conditions (Ostle et al., 2009; Zhou et al., 2021). Furthermore, in virtually all studies the PE has been estimated by quantifying the proportion of CO_2-C released from the "old" SOC via microbial respiration, rather than quantifying the actual change in SOC stock after a resource pulse. Also the influence of the fresh organic matter pulse on soil N stocks is poorly studied, although one prominent mechanism leading to an acceleration of SOM mineralization relates to soil N. The "microbial N mining hypothesis" (see Chen et al., 2014) suggests that, when organic matter amendment induces N demand, microorganisms use the energy of that fresh, labile resource to synthesize enzymes. These enzymes target the mineralization of mineral associated soil organic N (SON) stored in the soil. This would induce an increase in SOM mineralization (Guenet et al., 2012; Dijkstra et al., 2013) or fragmentation of SOM to smaller constituents, which, in theory, should also manifest as a reduced SON content. Despite the close association between C and N in the biogeochemical cycle (Vitousek et al., 1997), long-term data on such a PE-induced influence on soil N content is scarce (but see Sayer et al., 2021; Man et al., 2022). Recent findings suggest that not only recalcitrance of the litter but also a complex combination of physical, chemical and biological drivers in the soil can control the rate at which OM accumulates in the soils (Cotrufo and Lavallee, 2022), thus potentially affecting the magnitude PE. For example, soil fauna stimulate decomposition rate and soil nutrient dynamics through fragmenting litter and grazing upon soil microbes (Setälä and Huhta, 1991; Nielsen, 2019), which would be expected to indirectly influence the magnitude of the PE. However, virtually nothing is known about the impacts of soil fauna on the PE.

Here we present a 3-year long field study, conducted in a mature, mixed Korean pine (*Pinus koraiensis* Sieb. et Zucc.) – Mongolian oak *(Quercus mongolica* Fisch. ex Ledeb.) forest in NE-China, aiming at exploring the long-term effects of above-ground litter addition or removal on SOC and STN content. The experimental design had the following treatments with 10 replicates per treatment: (1) Control (Con) receiving natural litter input (see below); (2) Doubled litter (DL) receiving doubled litter input every autumn, and (3) No new litter (NNL) receiving no new litter in any autumn of 2015, 2016 and 2017. As the soil rhizosphere was left untouched in each of the 3 litter manipulation treatments (see Materials and Methods), we were able to (i) concentrate on the effects of the PE induced by the added/removed above-ground litter, and (ii) explore these effects without disturbing the soils and their decomposer biota underneath the litter.

We posed the following hypotheses:

- The addition of fresh tree leaf litter primes the soil by activating the soil decomposer biota (microbes and soil fauna), thus leading to a positive PE (reduction of SOC).
- Besides SOC, the content of STN will also decrease as a consequence of positive priming. This is because stimulated microbial growth increases N-acquiring enzyme production and thus N mineralization (Wang et al.,2015), which, in the presence of roots, leads to a lowered STN content.
- The removal of leaf litter at the end of the three study years reduces or has no effect on SOC and STN content.
- Litter decomposition rate is higher in treatment plots receiving extra litter compared to control plots with normal litter input or plots in which yearly litter input is excluded. This is because the biomass of soil decomposer organisms (microbes and soil fauna) is expected to be higher in soils receiving extra litter compared to soils with normal or no litter input.
- Finally, we expect that litter-derived PE is larger in the uppermost (0–5 cm deep) soil layer than beneath it (5–10 cm deep) due to the closer contact between litter and soil in the tops soil layer.

2. Materials and Methods

2.1. Site description

The experiment was conducted in a mixed broad-leaved *Pinus koraiensis* forest (41°41′49″ − 42°25′18″N, 127°42′55″ − 128°16′48″E), at the Changbai Mountain Forest Research Station, Chinese Academy of Sciences, Antu, Jilin, China. The site situates in a typical temperate continental monsoon region, with an average annual temperature of 3.8 ℃, annual precipitation of 600–900 mm. The study site is located at the No.1 site (128°05′ E, 42°24′ N) of the Research Station of Changbai Mountain Forest Ecosystems, Chinese Academy of Sciences, at an altitude of 766 m a.s.l. The dominant tree species at the Changbai Mountain site are Korean pine (*Pinus koraiensis* Sieb. et Zucc.), Mongolian oak (*Quercus mongolica* Fisch. ex Ledeb.), *Fraxinus mandsurica* and *Tilia amurensis* Rupr. The main shrub species are *Euonymus alatus*, *Philadelphus schrenkii*, *Corylus mandshurica*, *Lonicera japonica*, *Deutzia scabra*, and the main herbaceous species are *Anemone raddeana*, *A. cathayensis*, *Funaria officinalis*, *Cyperus microiria*, *Filipendula palmate*, *Adonis vernalis*, and *Brachybotrys paridiformis*. The average age of the dominant tree species is ca. 300 years. The soil at the study site is sandy loam, which belongs to dark brown soil and is developed from loose volcanic limestone. Soil pH (1:5 air-dried soil/distilled water) ranges from 3.8 to 4.4 in the ca. 5 cm deep O horizon, and 4.1 to 4.9 in the uppermost mineral soil layer (5 to 10 cm depth). For further details of the site, see Zhang et al. (2021).

2.2. Experimental design

The experiment with three litter manipulation treatments was established in June 2015. Ten replicate blocks, each about 400 m^2 in size, were randomly chosen in an area of ca. 2 ha. Four litter traps made of nylon cloth (2 mm mesh), each 1 m^2 in area and standing ca. 1 m above soil surface, were first placed in each block to collect tree litter that was needed in the experiment. At the same time, three litter frames (marking the boundaries of experimental plots), were randomly placed at each block. Each litter-frame, composing of a rectangular frame made of 1 cm (diameter) PVC tubing upon which a coarse plastic mesh (1.0 cm openings) was stretched, was gently anchored on the soil surface using metal rods. The purpose of the litter-frames was to (i) keep the experimentally manipulated litter (see below) underneath the frames, and (ii)

prevent the naturally abscising litter from entering the soil surface. Minimum distance between the experimental plots within a block was 3 m. One plot at each block was subjected to one of the three main treatments: (1) Control (Con) receiving natural litter input (see below), but with a litter-frame placed over the litter layer; (2) Doubled litter (DL) receiving doubled litter input every autumn, and (3) No new litter (NNL) receiving no new litter in any autumn of 2015, 2016 and 2017, and the litter-frame on top of the soil preventing litter from entering the soil. Two auxiliary treatments were established to evaluate e.g., the effect of litter frame as such on the studied variables (see Supplemental Method 1). At each block, prior to litter-frame installment, all vegetation (herbs and saplings) was carefully removed and up-rooted from the plot soils. Our experimental design rendered possible to sample intact soils that were disrupted by e.g. experimental procedures.

2.3. Litter experiment preparation

In late October in 2015–2017, i.e. at the end of each litter-fall period, litter in the four litter traps was separately collected from each block in large bags and transported to the laboratory for litter mass determination. Cones and twigs larger than 1 mm in diameter were removed. The litter was let to dry overnight in the laboratory, after which its mass was stabilized to represent room-dry litter. The room-dried litter within a block was pooled, and a quarter was reserved for Con, two quarters for DL, and the rest for chemical analyses. Litter mass varied slightly between years and blocks, the average (±SD) litter input being 312.7±74 g dry mass m^{-2}. Consequently, Con received ca. 313 g and DL ca. 626 g of litter in the fall of 2015, 2016, and 2017. The mean C and N concentration of the added litter mix were 48.8% and 0.65%, respectively. In the field, the litter that had accumulated on top of the frame mesh was removed before placing experimental litter underneath the frames. Thereafter litter abscising from nearby vegetation was let to accumulate on top of the litter frames.

2.4. Decomposition of litter

To investigate the influence of litter manipulation on litter decomposition rate (i.e. activity of the soil decomposer biota), a litterbag experiment was set up in treatments Con, NNL and DL. In October 2015, senesced *Tilia amurensis* litter (representing ca. 8.5% of the total litter mass; C/N ratio = 36.6) was collected from underneath a mature *T. amurensis* tree close to the experimental site. The litter was oven dried (65 °C for 15 h) and 1 g of oven dry litter was placed into litterbags (10×10 cm in size) with either 100 μm (excluding most soil fauna) or 2 mm (allowing access of virtually all soil fauna, including some soil macrofauna) openings. In the experimental plots the litterbags were placed underneath the litter layer (treatments Con, DL) left uncovered (treatment NNL). Within plots, the litterbags were placed ca. 20 cm apart from the litter-frame edge and each other. Each plot of the four treatments received 3 fine-mesh and 3 coarse-mesh bags, resulting in a total of 180 litterbags (3 bags × 2 mesh-size × 3 treatments × 10 blocks). One litterbag of both mesh-sizes was removed from each plot in each October during the three consecutive study years. Litter in the bags was then carefully cleaned to remove debris in the laboratory, dried (70 °C, 24 h) and weighed for dry mass.

2.5. Soil analyses

Three soil subsamples (10 cm deep) were collected from each plot using an auger (2 cm diameter). As the time during which changes in soil characteristics was expected to be rather long, the soils were not sampled every year but first in October 2015 and the second time in October 2018, respectively. Each soil core, situated at least 10 cm apart from the litterbags, was first divided into 0–5 cm and 5–10 cm soil layers. After that, samples collected from the same block were combined into one composite sample for each layer, resulting in120 soil samples (3 treatments × 10 blocks × 2 soil layers × 2 years).

In the laboratory, roots were removed and the soil was gently homogenized by passing it through a 2 mm mesh sieve and divided into two parts. One part was stored at −80 °C prior to DNA extraction and sequencing (see below), and the other part was air-dried for measuring total organic carbon and total nitrogen. The total soil C (representing SOC due to the negligible amount of inorganic C at our study site) and nitrogen (TN) concentration using a Vario Max CNS elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany). A suite of other variables, including soil pH, C/N ratio, available phosphorus (AP) and total phosphorus (TP) were quantified to explain variation in the PE (for more details, see Supplemental Method 2).

2.6. Soil microbial community

Soil total DNA was extracted from a 0.5 g subsample using the Omega Soil DNA Kit (M5635-02, Omega Bio-tek, Norcross, GA, USA), according to the manufacturer's instructions. PCR amplification of the bacterial 16S rRNA gene V3-V4 region and the fungal ITS2 gene were performed using primer sets of 338F/806R and ITS3F/ITS4R, respectively (Pires et al., 2012; Zhao et al., 2020; Zou et al., 2020). Pair-end 2×300 bp sequencing was conducted using the MiSeq system with the Illumina MiSeq Reagent Kit v3 (Shanghai Personal Biotechnology Co., Ltd, Shanghai, China). Microbiome bioinformatics (data on relative abundances) was performed using QIIME 2 (Bolyen et al., 2018). The paired-end raw reads were demultiplexed with the demux plugin, followed by primer cutting using the cutadapt plugin (Martin, 2011). Then, the sequences were quality filtered and denoised using the DADA2 pipeline with the following parameters: truncLen = 240, 220, minLen = 175, maxEE = 2, 2, and truncQ = 2 (Callahan et al., 2016). We used learnErrors(), derepFastq(), dada()and mergePairs() functions to control sequence quality and remove all sequencing errors with default parameters. Then, the singleton amplicon sequence variants (ASVs) were removed. Afterwards, the bacteria taxonomic identity was determined with the SILVA v132 database (https://www.arb-silva.de) (Quast et al., 2013), the fungal taxonomic identity was determined with the UNITE database (Koljalg et al., 2013). Data sets of bacterial and fungal sequences are archived at the National Center for Biotechnology Information Sequence Read Archive (https://www.ncbi.nlm.nih.gov/) under BioProject ID PRJNA818458.

The effects of litter addition and removal on the biomass of soil bacteria (16S gene copies) and fungi (internal transcribed spacer (ITS) gene copies) were assessed using quantitative real-time PCR (qPCR) on a StepOnePlus™ System (ABI, CA, USA) with AceQ® qPCR SYBR® Green Master Mix (Q112-02, Vazyme) (Daniell et al., 2012). We used the same primers as were used in Miseq DNA sequencing. Each reaction contained 10 μL of 2 × SYBR real-time PCR premixture and 0.4 μL of each PCR primer (10 μM). Thermocycler conditions included an initial denaturation step at 95 °C for 5 min, and then 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. Standard curves were generated with plasmid vectors containing PCR products, and the products were linearized by PCR amplification with the primers followed by purification as previously described. For each qPCR assay, no-template control, standard, and sample reactions were performed in three replications, and a dissociation curve was generated at the end of each run to check product specificity. The fluorescence of SYBR Green was measured at the end of each extension step, and this fluorescence was normalized to that of the ROX reference dye. The efficiency of the 16S qPCR assay was 99% and that of the ITS qPCR assay was 88%. The biomarker-based estimate was converted to copy number (interpreted as biomass) of each gene in each reaction and expressed as copy number per soil dry mass.

During the field samplings, also soil fauna (Nematoda and Enchytraeidae) were extracted from the soils. For more details, see Supplemental Method 3.

2.7. Statistical analyses

All statistical analyses were carried out in R (version 3.6.1, R Core Team, 2019). Normality of the data, including soil characteristics, litter mass loss, number of nematode individuals, enchytraeid worm biomass, and soil microbial biomass (gene copies), was evaluated using histograms and the Shapiro-Wilks test. Data were either Ln or square-root transformed, where necessary. The *lmerTest()* function (lmerTest package, Kuznetsova et al., 2017) was used to create the linear mixed models (LMM). LMM were used to test the effect of litter manipulation (a factor with three levels: Con, DL, NNL), sampling year (a factor with two levels: 2015, 2018), and their interactions on soil characteristics (including SOC, TN, C/N ratio, pH, TP and AP) at two depths (0–5 and 5–10 cm), separately. A Post-hoc Tukey test of the LMM was performed using *emmeans()* function (emmeans package, Lenth, 2018) to test the effects of litter manipulation on soil characteristics in the top soil layer (0–5 cm) between the two sampling years to remove the potential between block variation in terms of litter quality and quantity and thus explore the occurrence of the PE. Similarly, LMM were performed to test the effect of litter manipulation (same as above), litterbag mesh-size (a factor with two levels: coarse and fine), sampling year (a factor with three levels: 2016, 2017 and 2018), and their interactions on tree leaf litter decomposition. As litterbag mesh-size had a significant ($p < 0.001$) effect on litter mass loss, LMM were then performed for coarse and fine mesh, separately, followed by a Post-hoc Tukey test. Similar LMM were used to test the effect of litter manipulation and sampling year, and their interactions on the number of nematode individuals and enchytraeid worm biomass. As soil microbial samples were taken at the end of the experiment (year 2018) only, LMM excluding the factor of sampling year were used to test the effect of litter manipulation on soil microbial biomass. In all models, block identity was included as a random factor.

For the analysis of community structure of soil microbes and soil nematodes, non-metric multidimensional scaling (NMDS) analysis was performed, based on the Bray-Curtis distance matrix. Significances was tested by performing permutational multivariate analysis of variance (PERMANOVA). Metastats was used to detect differentially abundant taxa using bacterial taxonomic data from Silva Classifier and fungal data from UNITE Classifier controlling the false discovery rate at 10% for each level of the taxonomy.

3. Results

3.1. Soil characteristics as influenced by litter manipulations

The concentration of SOC in the uppermost soil layer (0–5 cm depth) was slightly but not statistically significantly decreased during the three study years in DL, while it increased slightly but insignificantly in NNL (Fig. 1). In Con, no change in SOC was observed. Soil TN, its concentration decreased significantly ($P = 0.011$) in DL, and increased slightly ($P = 0.163$) in NNL during the course of the study (Fig. 1, Tables 1 and 2). In general, the addition of litter (DL) decreased SOC and soil TN content by 5 and 15%, respectively, while litter removal (NNL) was associated with increased (ca. 15%) amounts of both SOC and soil TN during the study (Fig. 1, see the panels describing changes in C and N stocks embedded in the bar graphs).

In terms of soil in the deeper layer (5–10 cm), litter addition/removal had no significant effects on any of the measured soil properties.

Table 1
LMM results, testing the effects of litter treatment (DL = Double Litter; Con = Control; NNL = No New Litter) and sampling year (2015, 2018) on SOC, total N, C/N ratio, pH, total P and available P in the top soil layer (0–5 cm depth).

Variable	Intercept	Con	NNL	2018	Con × 2018	NNL × 2018
SOC	2.421	−0.177	**−0.197**	−0.083	0.104	0.194
	(0.077)	(0.097)	**(0.094)**	(0.094)	(0.136)	(0.134)
	<0.001	0.076	**0.042**	0.387	0.449	0.153
TN	0.988	**−0.083**	**−0.157**	**−0.090**	0.053	**0.137**
	(0.029)	**(0.035)**	**(0.034)**	**(0.034)**	(0.048)	**(0.048)**
	<0.001	**0.022**	**<0.001**	**0.011**	0.277	**0.006**
C/N ratio	11.702	−0.003	**1.794**	**1.271**	−0.021	−1.229
	(0.345)	(0.493)	**(0.480)**	**(0.480)**	(0.688)	(0.678)
	<0.001	0.995	**0.001**	**0.011**	0.976	0.077
pH	1.668	0.044	0.043	−0.035	0.001	−0.051
	(0.031)	(0.046)	(0.044)	(0.043)	(0.064)	(0.062)
	<0.001	0.345	0.338	0.423	0.982	0.411
TP	0.147	−0.017	0.015	0.006	−0.011	−0.014
	(0.020)	(0.028)	(0.028)	(0.028)	(0.040)	(0.040)
	<0.001	0.543	0.584	0.826	0.789	0.723
AP	0.003	−0.000	0.000	0.001	−0.001	**−0.002**
	(0.001)	(0.001)	(0.001)	(0.001)	(0.001)	**(0.001)**
	<0.001	0.870	0.854	0.273	0.251	**0.035**

The double litter input and 2015 are in the intercept. Coefficients, standard errors (in parentheses) and p-values are presented. Significant effects ($p < 0.05$) are highlighted in bold. Soil total N was square-root transformed. Soil organic carbon, pH were Ln transformed.

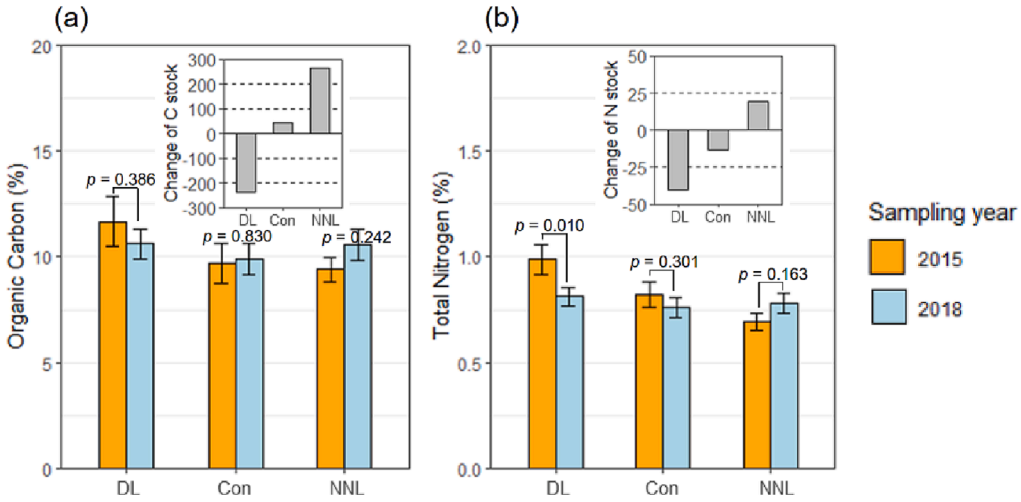

Fig. 1. Influence of litter manipulation (DL = Double Litter addition; Con = Control; NNL = No New Litter) on soil organic carbon (a) and soil total nitrogen (b) (mean ± SE; n = 10). Yellow bars denote soil characteristics in the beginning (year 2015) and blue bars at the end (year 2018) of the study in the uppermost (0–5 cm depth) soil layer. The small panels within the plots describe the change of carbon and nitrogen stock during the course of the study. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Post-hoc Tukey test results, testing the effects of treatment (DL = Double Litter; Con = Control; NNL = No New Litter) on SOC, total N, C/N ratio, pH total P and available P in the top soil layer (0–5 cm) between the two sampling years (2015, 2018).

Treatment	Sampling year	SOC (%)		TN (%)		C/N ratio		pH		TP (%)		AP (%)	
		Estimate	*p*-Value	Estimate	*p*-Value	Estimate	*p*-Value	Estimate	*p*-Value	Estimate	*p*-Value	Estimate	*p*-Value
DL	2015–2018	0.083	0.387	**0.090**	**0.011**	**−1.271**	**0.011**	0.035	0.424	−0.006	0.826	−0.001	0.273
Con	2015–2018	−0.021	0.830	0.036	0.302	**−1.250**	**0.015**	0.034	0.482	0.004	0.874	0.000	0.596
NNL	2015–2018	−0.112	0.243	−0.048	0.163	−0.042	0.930	0.086	0.059	0.008	0.779	0.001	0.056

Significant effects ($p < 0.05$) are in bold. Soil total N was square-root transformed. Soil organic carbon, pH were Ln transformed.

Concentrations of both SOC and TN were more than twice lower in the deeper soil layers than in the top soil, with no statistically significant differences between treatments (Supplemental Fig. 1 & Table 1).

3.2. Litter mass loss

As litterbag mesh-size had a significant ($p < 0.001$) effect on litter mass loss (Supplemental Table 2), LMM were conducted for coarse (allowing the entrance of most soil fauna) and fine (excluding most soil fauna) mesh, separately. In terms of litterbags with fine mesh, both litter manipulation ($p < 0.001$) and year ($p < 0.001$) had a significant effect on *Tilia* litter decomposition rate. In terms of litter manipulation (data of the three samplings combined), litterbags in DL had marginally significantly ($p = 0.066$) less litter remaining than those in Con, and significantly ($p < 0.001$) less litter remaining compared to those in NNL. Litter decomposition did not differ significantly ($p = 0.163$) between Con and NNL (Supplemental Table 3). For coarse-mesh litterbags, litter manipulation did not influence litter decomposition significantly ($p = 0.313$), while sampling time (year) had a significant ($p < 0.001$) effect on litter mass loss (Fig. 2, Table 3).

Irrespective of litter treatment, the presence of soil fauna (comparison between litterbags with coarse mesh and fine mesh) increased litter mass loss significantly ($P < 0.001$) throughout the study, especially so from the second study year onwards (Fig. 2, Supplemental Table 2).

3.3. Soil microbes and fauna

3.3.1. Microbial community

After quality filtering, a total of 2,665,857 and 1,967,040 high-quality sequences were obtained from the soil bacterial and fungal community, respectively, which could be classified into 171,260 and 15,685 OTUs (For the alpha diversity of bacteria and fungi in the soil, see Supplemental Fig. 2). Biomass of soil bacteria and fungi (estimated by the gene copy numbers of bacteria (16S) or fungi (ITS)) at the end of the study (year 2018) were not significantly affected by the litter treatment (Fig. 3, Supplemental Table 4). Neither was the community composition of soil bacteria (Fig. 4a; $P = 0.995$) and fungi (Fig. 4b; $P = 0.865$) responsive to litter treatments at the end of the study. Similarly, the abundance of bacteria and fungi at the genus level were unresponsive to the treatments (violin graphs, Supplemental Fig. 3 and Supplemental Fig. 4).

The relative abundance of soil bacterial groups did not differ between the litter manipulation treatments, the three most abundant groups in each treatment being Proteobacteria > Acidobacteria > Verrucomicrobia (Supplemental Fig. 5a). Neither were the most abundant fungal groups affected by the litter treatments: irrespective of treatment, Basidiomycota and Ascmycota were the two dominant groups, with Zycomycota the third most common group (Supplemental Fig. 5b).

3.3.2. Nematodes and enchytraide

Litter manipulation showed no significant influence neither on total number of nematodes nor enchytraeid worm biomass during the course of the study (See Supplemental Fig. 6).

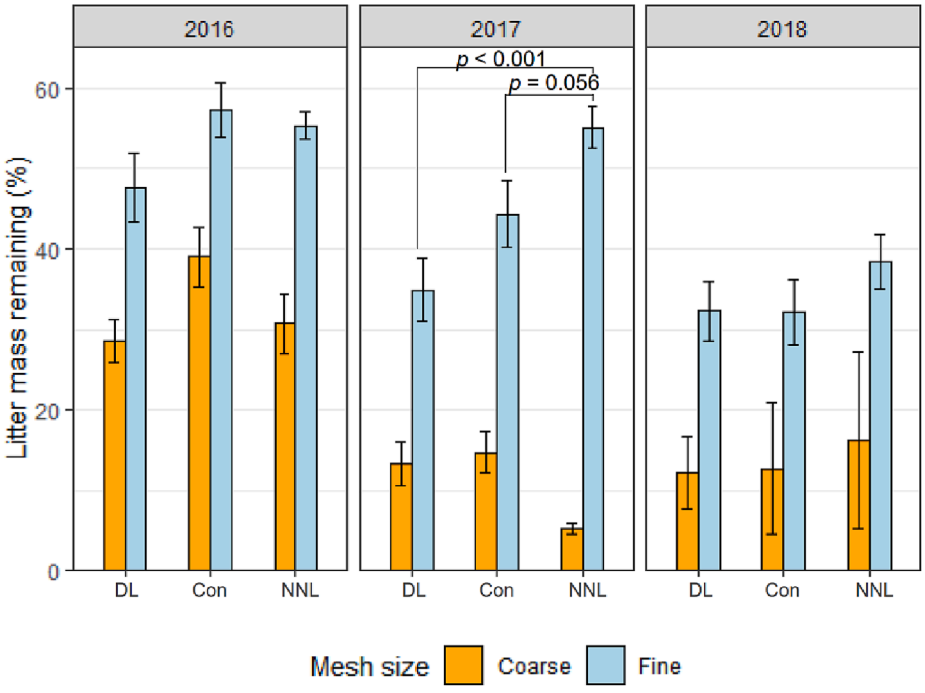

Fig. 2. Effect of litterbag mesh-size (coarse mesh, 2 mm, orange bars; fine-mesh, 100 μm, light blue bars) and litter manipulation (DL = Double Litter; Con = Control; NNL = No New Litter) on *Tilia* litter decomposition (% mass remaining, mean ± SE) across the three study years. The main effects of litter mesh-size, litter manipulation and sampling year, and their interactions are presented in Supplemental Table 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
LMM results, testing the effects of litter treatment (DL = Double Litter; Con = Control; NNL = No New Litter) and sampling year (2016, 2017, 2018) on *Tilia* litter decomposition (mass remaining, %).

Variable	Intercept	Con	NNL	2017	2018	Con × 2017	NNL × 2017	Con × 2018	NNL × 2018
Litter mass remaining (%)-**fine mesh**	47.597	**9.647**	7.739	**−12.721**	**−14.781**	−0.212	12.509	−10.026	−2.180
	(3.451)	**(4.605)**	(4.605)	**(4.605)**	**(4.902)**	(6.512)	(6.512)	(6.807)	(6.725)
	<0.001	**0.040**	0.097	**0.007**	**0.004**	0.974	0.059	0.145	0.747
Litter mass remaining (%)-**coarse mesh**	28.516	**10.495**	2.226	**−15.159**	**−16.523**	−9.117	−10.389	−10.529	0.801
	(3.315)	**(4.436)**	(4.436)	**(4.436)**	**(4.917)**	(6.274)	(6.274)	(7.358)	(8.228)
	<0.001	**0.021**	0.618	**0.001**	**0.001**	0.152	0.103	0.158	0.923

The double litter input and 2016 are in the intercept. Coefficients, standard errors (in parentheses) and p-values are presented. Significant effects ($p < 0.05$) are highlighted in bold.

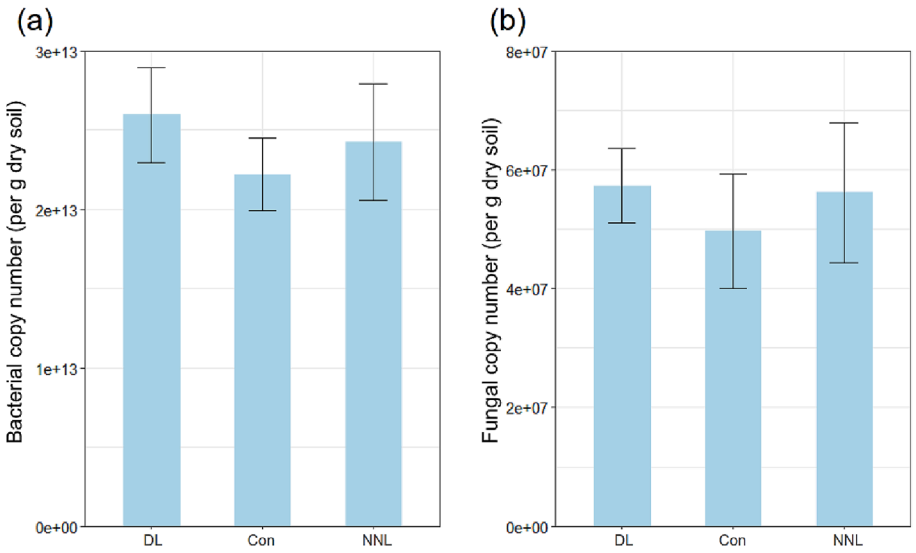

Fig. 3. Soil bacterial (a) and fungal biomass (b) (gene copy numbers per g dry soil, mean ± SE) in the three treatments (DL = Double Litter; Con = Control; NNL = No New Litter) at the end of the study (October 2018). LMM results are presented in Supplemental Table 4.

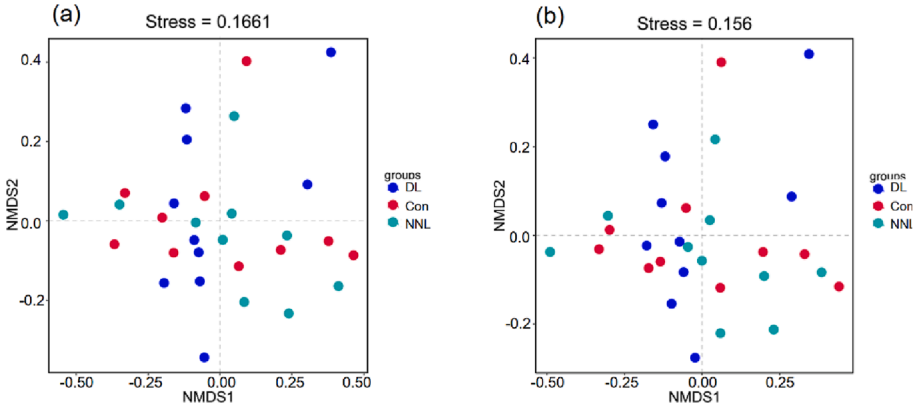

Fig. 4. Non-metric multidimensional scaling (NMDS) plot for bacterial (a) and fungal (b) communities in relation to the three litter manipulation treatments (DL = Double Litter; Con = Control; NNL = No New Litter).

4. Discussion

4.1. Litter addition primes C and N mineralisation

Climate warming is predicted to increase plant production in various biomes, including temperate forests (Friend, 2010). We hypothesised that the addition of fresh leaf litter on top of soil in a temperate mixed old-growth forest primes the soil leading to a reduction in soil organic matter, specifically SOC but also soil total N. In terms of SOC, our hypothesis is supported by our data and the results of recent reviews/*meta*-analyses (Huo et al., 2017; Sun et al., 2019): the addition of litter tended to decrease soil SOC by ca. 5 % during the 3-year study. Even though the positive PE in systems receiving extra litter was not statistically significant, the 15% increase in SOC in NNL soils subjected to litter removal adds credibility to the occurrence of the PE. Our study design made it possible to explore the absolute, long-term losses/gains of C in the soil, i.e. the "real priming effect", sensu Kuzyakov et al. (2000), in which increased C loss is due to the decomposition of SOM

rather than turnover of microbial compounds. While the PE is commonly determined via quantifying short-term mineralisation (i.e. CO_2 production) of SOC, only a few studies exist in which the PE has been quantified by following actual changes in SOC content over a 3-year period (Sayer et al., 2021; Man et al., 2022). Results of our 3-year field study suggest that SOC content – considered to be rather stable in undisturbed temperate and boreal forest soils (Augusto et al., 2002; Vesterdal et al., 2013) – can be subject to both a positive and negative PE via alterations in above-ground litter input. In case ongoing climate warming will associate with increased NPP (Carney et al., 2007), the results of our study infer a reduction of SOC stocks in temperate mixed forest soils, similarly to those of other ecosystems.

Similar to soil C, mineralisation of soil organic N (SON) should also be expected to increase due to litter addition given the close link between C and N in the biogeochemical cycle (Vitousek et al., 1997). Consequently, enhanced mineralisation of SOM associated with a positive PE has been suggested to derive from an increased demand for microbes to mine N in SOM to meet their energy and nutrient needs (the so-called nutrient mining theory; see Dijkstra et al., 2013; Chen et al., 2014). Another mechanism tackling soil N dynamics and leading to a positive PE, the co-metabolism theory, suggests that FOM input stimulates microbial growth and therefore an increase of enzymes production leading to an accelerated mineralization of SON (and SOC) (see Guenet et al., 2012; Wang et al., 2015).

Despite the close link between C and N, research on the priming effect has almost exclusively focused on soil organic carbon (SOC) mineralization in the rhizosphere soil, while far less attention has been given to quantifying relationships between the PE and soil N dynamics (Jiang et al., 2021). Indeed, in light of the two theories described above and supporting our hypothesis, concentration of STN was significantly reduced (by ca. 15%) by the addition of extra litter, but statistically marginally increased (by ca.15%) in soils with no litter input. That input of fresh organic resource into soils can induce the PE of SON has previously been documented in controlled laboratory/greenhouse studies (e.g. Zhu et al., 2014; Kieloaho et al., 2016; Yin et al., 2018; Jiang et al., 2021). However, these studies investigated FOM induced effects on gross N mineralisation rates rather than the effect of a FOM-induced PE on soil N stocks per se. Furthermore, previous studies exploring the relationships between FOM addition, the PE and soil N dynamics almost entirely deal with the effects of belowground, root derived input of FOM. However, as it is unlikely that the leaf litter manipulations produced significant, unintentional impact on root activities in our study, our results strongly suggest that the reduced amounts of C and particularly that of N in the forest soil were due to input of above-ground rather than below-ground (e.g. root exudate) resources. Besides, the addition of easily degradable plant material such as root exudates to soil is believed to result in a short-term change in turnover of soil SOM (Wu et al., 1993; Hamer and Marschner, 2002; Blagodatskaya et al., 2007; Kuzyakov et al., 2007). Given the 3-year long duration of our study, and the yearly input of rather recalcitrant plant material, our study adds to the current knowledge that the input of structurally complex aboveground litter also induces a positive PE, which manifests as significantly reduced STN content in temperate forest soils. This is partly in line with a *meta*-analysis by Huo et al. (2017) according to which the PE is positively correlated with aboveground plant biomass, but not with root biomass.

Based on the measured soil C and N concentrations, %C (48.8) and % N (0.65) in the litter and the top soil bulk density value (0.45; Yang et al., 2007) in our study site, the double litter input was associated with a loss of 238 g C m^{-2} and 40 g of N m^{-2} during the 3-year period, while soils of the NNL treatment gained 265 g C m^{-2} and 19 g of N m^{-2} during the same period. In Con (Control) the amount of C stayed practically the same during the study period. The amount of N lost in DL was ca. three times the amount of N added via the litter mixture, while the amount of C lost is about one third of that added (940 g). This novel finding indicates that, when compared to C, soil N stock in our research site is much more prone to the PE. This is expected given that N is commonly tightly cycled within an ecosystem, while C flows through the system.

4.2. Mechanistic understanding of the priming effect

Even though all the patterns and drivers of the PE are not well understood (Chen et al., 2019), there is a general consensus that the PE is ultimately controlled by soil microbes and their activity (Kuzyakov et al., 2000; Paterson, 2009; Sulman et al., 2014; Di Lonardo et al., 2018). Consequently, we hypothesized that the potential PE due to FOM addition in systems with double litter addition manifests itself as enhanced biomass and/or altered community composition of soil decomposer microbes. Somewhat surprisingly, and in contrast with previous studies (Kuzyakov, 2010; Wang et al., 2014b; Xiao et al., 2015), we found little evidence that the positive PE (i.e. reduced SOC and STN during the course of the study) in the DL system associated with microbial biomass or community composition, even though resource input for primary decomposers in DL during the study was substantial (ca. 1878 g (dry mass) m^{-2}). Neither were the biomass nor community composition of soil nematodes – known to respond rapidly to their microbial prey (Nielsen, 2019) – responsive to the litter treatment. Based on visual observations at our study site, it is clear that much of the litter added during the previous year(s) decomposed, even though visibly more litter remained on DL compared to Con soils. Even much of the recalcitrant litter deriving from Korean pines had turned into unidentifiable detritus during the three study years, which refers to the presence of an active decomposer community at our study site (see Zhang et al., 2021).

Other studies have also found that mineralisation of organic matter does not necessarily relate to microbial biomass or community composition. In their study using agricultural soils, the addition of labile C primed the mineralization of 2–13 month aged SOM, while the mechanism for this priming was unrelated to microbial growth dynamics (Rousk et al., 2015). As a matter of fact, Kemmitt et al. (2008) suggested that organic matter mineralization is independent of microbial biomass size, community structure or specific activity. Instead, these authors suggest that the PE is governed by abiological processes – called the Regulatory Gate Hypothesis – which convert non-bioavailable SOM into bioavailable SOM, and thus cannot be affected by the characteristics of microbial populations (Kemmitt et al., 2008). However, as litter manipulation had no clear effects on soil abiotic properties such as soil pH, concentrations of tot-P and available P, and soil moisture content (the mean varying between 25.3 and 33.7% during the 3-year study period, results not shown), the observed PE in our study unlikely relates to differences in soil abiotic conditions.

Supporting our findings, there is no unequivocal evidence implying that increased microbial biomass is necessary to induce the PE, but that a change in microbial process rates and/or community assemblages prove to be more important. It is well established that the activity of microbes rather than their biomass per se often determines soil process rates (Swift et al., 1979). However, although the NMDS analysis covering all copies of fungi and bacteria failed in distinguishing differences in microbial communities between the litter treatments, some genera of soil bacteria and fungi were responsive to litter treatments (see the violin graphs, Supplemental Figs. 3 and 4). Due to the considerable functional redundancy among soil microbiota (Nielsen et al., 2011; Louca et al., 2018), it remains open whether these data predict true functional differences among soil microbes between the litter treatments. For example, changes in microbial composition may not be fully reflected by DNA sequencing because a large pool of relic DNA persisting in soil for weeks to years after cell death may buffer these changes (Carini et al., 2016). In addition, as functional predictions based on microbial composition does not necessarily provide a reliable understanding on the relationships between soil functions and microbial community composition (Guenet et al., 2012; Wang et al., 2015; Wagg et al. 2019), the factors causing the observed positive PE remain open.

There is, however, indirect evidence suggesting that the activity of

the soil decomposer microbiota was influenced by the litter treatment: compared to Con and especially NNL, the addition of new litter in DL significantly boosted the decomposition rate of linden litter enclosed in the fine-mesh litterbags. This supports our hypothesis and is in accordance with (i) the PE theory predicting that resource addition stimulates SOM mineralisation rates and (ii) our observation on reduced SOC and STN content in the DL treatment. However, litter manipulation did not influence litter mass loss in the coarse mesh litterbags that allows the entrance of soil macrofauna. After two years (October 2017), ca. 90% of the linden litter had been lost with only faecal pellets and some leaf petioles remaining in the litterbags. This alludes to the importance of the soil macrofauna in influencing C and N dynamics through regulating the quality and quantity of resources available for soil microbes, and possibly controlling the degree by which the PE is influenced by FOM input at our site.

It is surprising that the potential contribution of the microbe-detritivorous soil fauna in influencing the rate of the PE has been ignored even though their role in stimulating microbial activity, decomposition rate and NPP is well established (Wardle et al., 2004; Nielsen, 2019). In our study, the biomass of enchytraeid worms that feed on detritus and soil microbes (Didden, 1993) were higher in DL than in NNL soils, indicating greater resource availability in the former. As these worms are key fauna in stimulating C and N mineralisation in forest soils (Huhta et al., 1998; Laakso and Setälä, 1999), the high numbers of enchytraeids may, at least partly, explain the positive PE, i.e. the decreased SOC and STN in DL during the study. The role of soil fauna in inducing the PE may thus be analogous to the microbial oriented "co-metabolism" and/or "nutrient mining" hypotheses", according to which nutrient and C mineralisation by microbes is triggered by the addition of FOM in the soil (see e.g. Fontaine and Barot, 2005; Guenet et al., 2012; Dijkstra et al., 2013). Whether the observed PE (loss of soil C and N) derives purely from changes in microbial communities, or whether the altered soil fauna also had an impact on PE remains open. Nevertheless, as the soils in our study site are N-limited (Zhao et al., 2014), it is likely that the phenomena related to the PE support the N-mining hypothesis, according to which N in the soil is controlled by the availability of fresh C (see also Chen et al., 2014; Zhou et al., 2021). This is why the microbial mining of SOM can be intense and eventually exceed the formation of new SOM, thus leading to the net destruction of SOM and release of mineral nutrients. In contrast, when soluble nutrients are not limiting, the microbial mining of SOM should decrease, leading to a greater sequestration of nutrients in SOM (Fontaine and Barot, 2005). This might explain the significant increase in STN with time in the upper layer of the NNL soils where mineralisation rates of organic N and thus loss of N via leaching and/or denitrification was minimal.

5. Conclusions

To the best of our knowledge, our long-term study is the first that explored the priming effect on SOM under repeated input of fresh, structurally complex set of organic matter in non-disturbed soils. We found evidence of a real PE, i.e. that content of both C and particularly that of N decreased in the top soil due to the decomposition of SOM (see Kuzyakov et al., 2000) compared to the situation before FOM addition. The importance of FOM input on SOM dynamics was further strengthened by the observation that the scarcity of "fresh C" in NNL soils was associated with the increased contents of SOC and STN, apparently due to impaired microbial decomposition of the stable organic carbon pool (see Fontaine et al., 2007). However, the PE was not explained by microbial biomass and community composition.

Given that the non-destructive experimental procedure unlikely influenced root production, our results also suggest that, at least in a mixed temperate forest, the importance of root-derived SOM in affecting the PE in the uppermost (0–5 cm deep) soils is negligible compared to FOM input from above. This may appear puzzling as (i) fine root input to soil carbon in the same forest site has been reported to be 1.2 times larger than leaf litterfall (Wang et al., 2016) and (ii) plant roots are reported to provide a large amount of available C and energy for microorganisms (the 'microbial activation' theory by Blagodatskaya and Kuzyakov, 2013). Our results thus infer that the enhancement in aboveground litter production due, e.g. to fertilization and climate warming (Norby et al., 2002; Trueman and Gonzalez-Meler, 2005) can lead to reduced stocks of SOC and STN in the top soil. In case labile C inputs via root exudation will also increase in deeper soil layers as suggested by a study in a boreal forest (Karhu et al., 2016), the PE can significantly reduce the value of forest soils as a C sink.

That the content of both C and N in the NNL soils without litter addition increased during the 3-year study is difficult to explain. It is possible that in the absence of leaf litter induced PE, the input (production) of fine roots exceeded its output (decomposition of fine roots). It is also possible that a reduction in the biomass of some soil fauna – known to be of pivotal importance in controlling litter decomposition via feeding upon litter and stimulating the activity of decomposer microbes (Coleman et al., 2017; Nielsen, 2019) – in the NNL relates to the observed lowered SOM mineralisation rate in NNL soils. Irrespective of the mechanism, our results emphasise the important role of aboveground litter in controlling C and N dynamics in the upper layer of temperate forest soils. We conclude that – in terms of forests management – planting trees (such as conifers) that produce recalcitrant litter may halt the negative consequences due to PE. Future studies are needed to show whether excess litter input primes SOM dynamics at deeper depths of temperate forests.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2023.110055.

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