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# Nitrogen fertilisation of boreal forest soil increases soil carbon pool through elevated microbial necromass formation but also modifies tree secondary metabolism

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ABSTRACT

Forests contain significant amounts of the global carbon (C) pool with the major fraction stored belowground. Nitrogen (N) fertilisation of forest soils may increase biomass production and soil organic C pools, providing a strategy for climate change mitigation.

Here we aimed to elucidate the mechanisms behind the increase in soil C due to N addition using a long–term fertilisation experiment on a Scots pine stand with a combination of chemistry, microbiology and greenhouse gas fluxes.

Our results showed that N fertilisation increased C stocks, microbial biomass, necromass and the activity of extracellular enzymes, with no significant increase in greenhouse gas production. Moreover, N fertilisation decreased the production of a group of plant secondary metabolites, tannins. These profound changes were observed in the organic layer of the soil, and differences in mineral soil were less detectable.

Mechanistically, N fertilisation increased the C stock via elevated litter input and higher transfer of root C to soil microorganisms increasing fungal biomass and further necromass, which was stabilised in the soil. Our study supports the view that management strategies to increase microbial necromass in persistent C pools could lead to elevated C stabilization, though caution should be taken regarding potential changes in plant metabolism.

## 1. Introduction

Boreal forests contain a significant proportion of global carbon (C) pools, with the major fraction stored belowground as soil organic C (SOC) (Crowther et al., 2016; Baldrian et al., 2023). Boreal forests act as important net C sinks (Pan et al., 2011), as they sequester more C in plant biomass and further in SOC, than what is released due to decomposition (Crowther et al., 2016). The size of SOC flux is determined by the balance between the rate of SOC accumulation and decomposition that is affected by environmental conditions and forest management, including nitrogen (N) deposition and N fertilisation (Mäkipää et al., 2023). Since the C cycle is interlinked with the N cycle (Davidson, 2009), N enrichment experiments have been found to affect SOC pools, mainly in the direction of SOC increases (Tang et al., 2023). Thus, enhanced N supply may be considered a potential climate change mitigation measure that increases SOC stock (Mayer et al., 2020). Although numerous N fertilisation studies have been conducted, it is still necessary to clarify the mechanisms that are the main drivers of C accumulation. Such mechanistic understanding is required to provide recommendations for management aimed at mitigating climate change.

SOC transformations are driven by microorganisms (fungi and bacteria), their interactions, and their interactions with plants (Baldrian et al., 2012; Adamczyk, 2021; Fanin et al., 2022). SOC transformations are affected by climatic conditions, quality and quantity of litter input, and soil characteristics (Mathieu et al., 2015). Free–living fungi and certain fungi living in symbiosis with trees (ectomycorrhizal fungi, ECM) and ericoid shrubs (ericoid mycorrhizal fungi, ERM) are powerful SOC decomposers (Kohler et al., 2015; Brabcová et al., 2018; Lindahl et al., 2021). Saprotrophic fungi are effective degraders of lignin and other polyphenolic compounds, whereas most ECM do not degrade polyphenolics (Lindahl and Tunlid, 2015). This is a consequence of evolutionary changes in ECM genomes leading to the loss of most (but

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not all) enzymes involved in the degradation of polyphenolics such as laccase and peroxidase (Kohler et al., 2015; Lindahl and Tunlid, 2015). However, certain ECM species (e.g. *Cortinarius*) can also use oxidoreductases, such as Mn-peroxidases, to degrade polyphenolics (Kohler et al., 2015; Lindahl and Tunlid, 2015). Recently, revealed negative correlation between Mn and soil C has underlined the importance of Mn-peroxidases in the decomposition of persistent SOC (Stendahl et al., 2017; Neupane et al., 2023). Because a large fraction of forest soil N is bound to polyphenolics (i.e., tannins and lignin), the ability to decompose polyphenolics is crucial for unlocking the N bound within these compounds (Northup et al., 1995; Adamczyk et al., 2011, 2019a). In N-limited environments low N availability increases microbial decomposition of more persistent SOC (including polyphenolics with bound N) using labile substrates. This "microbial nitrogen mining" may be suppressed by a high soil N supply. Thus, microbial N limitation may increase the decomposition of SOC (Craine et al., 2007). In addition to their involvement in SOC decomposition, microorganisms are involved in SOC accumulation (Jörgensen et al., 2022). More precisely, microorganisms decompose litter generating microbial biomass, whose residues, ornecromass, are stabilised in the soil *via* multiple mechanisms. These mechanisms include reactions with minerals (Liang et al., 2017; Xiao et al., 2023), leading to formation of mineral–associated organic matter (MAOM) (Cotrufo et al., 2015; Neurath et al., 2021). Recently, it has been proposed that fungal necromass may also be stabilised through interactions with root-derived tannins, leading to the formation of persistent complexes (Adamczyk et al., 2019b; Adamczyk, 2021). Although microbial living biomass C is 2 % of the total SOC, the significance of microbial necromass is underlined by recent findings in which a large part of SOC (50–80 %) is directly derived from microbial necromass (Amelung et al., 1999; Miltner et al., 2012; Liang et al., 2019). The concept of amino sugars as proxies for microbial necromass has been suggested previously (Amelung et al., 1999; Liang et al., 2017) with muramic acid as an indicator of bacterial necromass and glucosamine (monomer of chitin) as an indicator of fungal necromass (Amelung et al., 1999). Muramic acid is highly specific for bacteria and glucosamine for fungi if corrected for the bacterial glucosamine (Joergensen, 2018). Though chitin (and thus glucosamine) can be found also in exoskeleton og micro-arthropods, their contribution to soil glucosamine content is negligible (Joergensen, 2018). Other amino sugars, i.e., mannosamine and galactosamine are not specific for any group of organisms, thus these are unspecific microbial markers (Joergensen, 2018).

To date, the following mechanisms of SOC accumulation due to N fertilisation have been proposed: 1) soil acidification suppressing microbial activities (Matson et al., 1999); 2) changes in fungal communities and their enzyme activities leading to 2a) to suppression of decomposition (Frey et al., 2004; Bonner et al., 2019); or alternatively, 2b) elevated microbial growth and C use efficiency via reduced mineral C protection (Feng et al., 2022) and 3) increased tree biomass production and a resulting increase in C input to the soil (Mäkipää et al., 1998), leading further to the increase of microbial necromass and its stabilization (Hasegawa et al., 2021), 4) alternatively N fertilisation change plant species abundance and diversity, leading to modifications of C stabilization mechanisms. In line with the first mechanism, N fertilisation may decrease pH via elevated nitrification, which is a well–known acidification process (Matson et al., 1999). This decrease in soil pH leads to a decrease in microbial metabolism thereby decelerating SOC decomposition (Ye et al., 2018; Tang et al., 2023). The second mechanism involves the suppression of polyphenolic decomposition owing to the decreased activity of oxidative enzymes following a relative decrease in the abundance of polyphenol decomposers (Jian et al., 2016; Bonner et al., 2019; Jörgensen et al., 2022). An alternative to the second mechanism, thus increased microbial growth and activity due to lower mineral C protection arises from the exudation of simple organic acids by microbes, leading to the release of mineral-bound C (Feng et al., 2022). The third mechanism of SOC stabilization under N addition relies on the increase in microbial biomass due to the elevated input of litter (Forsmark et al., 2020) and higher transfer of root C to the ECM, thus leading to enhanced formation of microbial necromass, which is stabilised in the soil through numerous processes, including the formation of MAOM (Cotrufo et al., 2015) and potentially also *via* complexing with root–derived tannins (Adamczyk et al., 2019a). The fourth mechanism involves changes in ground vegetation abundance and diversity. Such vegetation change could lead to differences in litter quantity and quality as well as in symbiotic ericoid mycorrhizal fungi abundance and their activities (Sullivan and Sullivan, 2018; Jetsonen et al., 2024).

The discrepancy between studies attempting to identify the drivers of C accumulation due to enhanced N supply may be due to experimental limitations that do not consider all the potential mechanisms and processes. Here we used a long–term experiment originally established in 1959 to understand the impact of fertilisation on tree growth and to study the effect of N fertilisation on changes in SOC pools. We used a wide set of measurements including soil chemistry, greenhouse gas ($CO_2$, $CH_4$, and $N_2O$) fluxes, microbial biomass, necromass, and metabarcoding. We aimed to identify the main phenomenon behind the increased SOC pool due to N load. As a null hypothesis (H0), we propose that N fertilisation does not cause any changes in SOC and that all measures are similar between the control and N fertilisation treatments. We propose the following hypotheses to explain the potentially elevated SOC after N addition: H1) N fertilisation decreases pH decelerating microbial growth and activities leading to increased SOC. Thus, lower pH, microbial growth, lower enzymatic activities, and reduced heterotrophic $CO_2$ respiration and $N_2O$ emissions would confirm H1. H2a,b) The increase in SOC due to N fertilisation is caused by changes in the fungal community composition in favour of fungi unable to degrade polyphenolics over effective degraders of polyphenolics with potent peroxidases. This community change leads to elevated concentration of polyphenolics such as lignin and tannins building up SOC and locking up N. Thus, the dominance of fungi unable to effectively degrade polyphenolics, decrease of oxidoreductase activities and increase of polyphenolic concentration (tannins and lignin) would confirm H2a. An alternate hypothesis (H2b), which also stems from increased microbial growth and activities based on decelerated mineral C protection, would be confirmed via a decrease in mineral-associated organic C pool after N addition, H3) the increase in SOC derived from enhanced C input in the form of either litter or photosynthates trafficked by tree roots and thus increased microbial biomass and consequent formation of microbial necromass which is stabilised in the soil. If present, this mechanism could be confirmed *via* elevated plant litter input, increased microbial biomass and necromass, and elevated concentrations of MAOM–bound C. H4) N fertilisation changes ground vegetation, and thus, its litter quantity and quality elevating SOC.

## 2. Materials and methods

### 2.1. Study site, experimental design and sample collection

The forest site Karstula is located in central Finland (62°54′43.343″N; 24°34′16.021″E), dominated by *Pinus sylvestris* and ground vegetation by *Vaccinium vitis–idaea*, *Calluna vulgaris*, and lichens. The soil type was fine sand with an average organic layer of 4.3 cm and a soil pH of approximately 4. The site is classified as a nutrient-poor xeric heath forest. A fertilisation experiment was established in 1959 to study the impact of nutrients fertilisation on tree growth. The experiment has a factorial design with eight plots of 25 × 25 m size (7 plots for different combinations of fertilizers treatments and 1 control plot) replicated in 3 blocks (n = 24). The distance between the N groups is 100 m and for controls 300 m. The distance (buffer zone without treatment) between plots was at least 10 m. Information of all fertilisation treatments was described in Richy et al. (2024). To examine the effect of N addition in this study, only N fertilisation and control (no fertilizer) treatments were included. N treatment was performed to understory seven times from 1960 to

**Table 1**
Soil pH, C and N concentrations in different soil layers on control and N fertilized plots. Mean value with standard deviation in brackets. Statistically significant differences between control and N fertilisation treatment are marked with different letters. MAOM-GlcN – glucosamine, marker of fungal necromass, in MAOM fraction. MurA – muramic acid.

| | Organic layer | | Mineral layer | |
|---|---|---|---|---|
| | Control | N fertilisation | Control | N fertilisation |
| pH | 4.5 (0.2) | 4.4 (0.1) | 3.7 (0.1) | 3.7 (0.2) |
| C stock, tC $ha^{-1}$ | 10.6 (0.6)$^{b}$ | 16.3 (1.9)$^{a}$ | 27.8 (5.4) | 24.9 (5.3) |
| C mg $g^{-1}$ | 268 (18)$^{a}$ | 307 (32)$^{b}$ | 26 (3.8) | 26.2 (20.1) |
| N mg $g^{-1}$ | 6.1 (0.7)$^{a}$ | 8.0 (0.8)$^{b}$ | 0.85 (0.05)$^{a}$ | 0.95 (0.05)$^{b}$ |
| C/N ratio | 43.1 (4.7)$^{a}$ | 38.1 (0.4)$^{b}$ | 30.0 (4.6) | 27.0 (2.6) |
| $NH_4$–N µg $g^{-1}$ DW | 7.8 (1.0)$^{a}$ | 14.2 (1.5)$^{b}$ | 5.3 (1.6) | 5.3 (1.1) |
| Stable SOC mg $g^{-1}$ | 198 (38)$^{a}$ | 258 (33)$^{b}$ | 6.6 (2.1) | 6.3 (1.9) |
| Labile SOC mg $g^{-1}$ | 51 (11) | 51 (27) | 20.0 (7.0) | 21.1 (7.0) |
| MAOM-C, mg $g^{-1}$ | | | 19.4 (2.4)$^{a}$ | 22.2 (2.4)$^{b}$ |
| POM-C, mg g | | | 13.2 (5.8) | 12.3 (0.5) |
| MAOM-GlcN mg $g^{-1}$ DW | | | 0.18 (0.02)$^{a}$ | 0.23 (0.05)$^{b}$ |
| MAOM-MurA µg $g^{-1}$ DW | | | 4.0 (4.0) | 5.5 (3.9) |

**Table 2**
Tree stand basal area, litterfall (mean ± se) and relative abundance of plant root species according to DNA–based assessment of root samples and vegetation survey i.e., visual estimation of plant cover. Statistically significant differences marked with different letters. See also Table S3 for statistical summary (p values and size effects).

| | Control | N fertilisation |
|---|---|---|
| tree stand basal area, $m^2$ $ha^{-1}$ | 18–25 | 25–35 |
| litterfall g $m^{-2}$ $month^{-1}$ | 15.7 (2.9)$^{a}$ | 23.6 (5.4)$^{b}$ |
| **Relative abundance of plant root species according to DNA-based assessment** | | |
| *Vaccinium vitis-idaea* | 34.6 (28.7) | 61.5 (33.6) |
| *Vaccinium myrtillus* | 29.1 (29.0) | 24.7 (23.1) |
| *Calluna vulgaris* | 7.3 (12.6) | 0.69 (1.6) |
| *Empetrum nigrum* | 5.3 ((13.0) | 0.1 (0.2) |
| *Betula pendula* | 3.4 ((8.2) | 0.1 (0.1) |
| **Vegetation survey, plant cover %** | | |
| *V. vitis-idaea* | 15.3 (11.7) | 30.8 (15.5) |
| *V. myrtillus* | 2.6 (5.2) | 5.8 (2.5) |
| *Empetrum nigrum* | 1.6 (3.1) | 1.8 (2.1) |
| *Calluna vulgaris* | 3.5 (2.2) | 16.8 (12.3) |

2020, once every decade with potassium nitrate/saltpetre (180 kg N $ha^{-1}$). Ca addition (liming) was also used to avoid long-term soil acidification. In September 2021, soils were sampled from N fertilisation and control treatments (n = 6). From each plot, three composite samples were collected from different locations and each composite sample consisted of five soil cores sampled in a row then pooled (N = 12). The distance between locations is at least 20 m (Fig. S1), and previous publication showed that soil from a few meters away in this study site can be treated as independent (Häkkinen et al., 2011).

Samples for chemical analyses were taken from two soil layers, the organic layer and the upper mineral layer (0–20 cm), sieved at the site using a 2 mm sieve and transported in cold boxes to the laboratory where they were immediately freeze–dried and finely milled, with the exception of subsamples for inorganic N analyses. The fine roots removed by sieving were collected into plastic bags, freeze–dried, and finely milled. For ammonium and nitrate analyses, the samples were immediately transported to the laboratory and extracted with 1 M KCl and frozen. For C stocks estimation we also measured the bulk density and stoniness (see below). Moreover, for fractionation into MAOM and POM soil was not milled. Soil samples for DNA analyses were collected up to a depth of 10 cm and were sieved (on a 5 mm sieve) snap-frozen at the site, stored at −24 °C, and then freeze–dried.

Understory vegetation was surveyed on a 1-$m^2$ quadrat from each soil sampling point at the time of the soil sampling in September. All vascular plant species and their cover were recorded (Table 2).

### 2.2. Tree inventory, litterfall, needle collection

A forest inventory of fertilisation experiments was conducted once a decade. During each forest inventory campaign, all trees were measured for diameter at breast height (DBH), whereas tree height and crown base heights were measured for a sub-sample of trees. For stand level estimation, stem volume and basal area tree level measurements were upscaled from each block. Litter (including needles, twigs and cones) was collected monthly during the growing season using collectors and then weighted (Lehtonen et al., 2016; Tupek et al., 2024).

Needles were collected in average every 1–2 months during snow free season. Collection was made from six litter collectors which were located at the center of each treatment square. Diameter of a collector is 80 cm. Litter from all collectors of each square were mixed to the same paper bag.

### 2.3. Soil C stocks, stable and labile SOC

For soil C stocks estimation, soil sampling was conducted in the same way as described above, with separation into organic and inorganic layers. The collected samples were dried, weighed, and sieved, and C and N contents were then analysed using dry combustion (LECO TruMac CN, LECO Corporation, USA). The bulk density was determined from the dried and undisturbed samples. Stoniness was assessed in the field using the rod-penetration technique. Soil C stocks were calculated by multiplying the C content by bulk density and correcting for rock fragment content (Poeplau et al., 2017).

Chemically stable SOC was assessed using acid hydrolysis fractionation (Kallenbach et al., 2016). Freeze-dried and milled soil samples (0.25 g) were treated with 12 mL 6 M HCl and heated at 105 °C for 18 h. After heating, the acid–hydrolysable fraction was decanted and the remaining non–acid–hydrolysable (i.e., stable) residue was rinsed four times with ultrapure water and isolated by centrifugation (2000g, 5min). Chemically stable C was then determined based on the differences in the C remaining in the non–acid–hydrolysable residues and the initial C content of the sample.

The total C content was determined using an elemental CN analyzer (LECO, USA).

### 2.4. Mineral–associated organic matter and particulate organic matter

Soil fractionation into mineral–associated organic matter (MAOM) and particulate organic matter (POM) was conducted as described previously (Just et al., 2021) with an ultrasonication step. Briefly, 5 g of dried and 2 mm sieved soil (not milled) was mixed with water after the addition of glass beads and sonicated for 1 h, followed by 18 h on a shaker at 200 rpm. Then, soil samples were rinsed onto a 53 µm sieve and divided into POM (fraction left on sieve) and MAOM (fraction passed through the sieve). The samples were collected, dried, and milled, and the C content in these fractions was measured using an elemental analyzer (LECO, USA).

### 2.5. Condensed tannin and lignin concentration

The condensed tannin content in the roots, needles and soil was measured using a modified acid-butanol assay, as previously described (Adamczyk et al., 2016). This method involves the HCl–catalysed depolymerisation of condensed tannins in butanol to yield a pink–red anthocyanidin product, the absorbance of which is measured spectrophotometrically. As a standard we used condensed tannins extracted from Norway pine needles, which were well characterised (as in Adamczyk et al., 2011). See Methods S1 for a precise description.

The concentration of lignin in the soil was measured using an acetyl

bromide assay (Danise et al., 2020). Briefly, 25 mg of freeze-dried and milled soil was washed with 70 % acetone, and sonicated. The dry precipitate was used to estimate lignin concentration after reaction with acetyl bromide under acidic conditions provided by glacial acetic acid. The product of this reaction was mixed with hydroxylamine and the absorbance was measured at 280 nm with a ClarioStar plate reader (BMGLabtech, Germany) using UV microplates (Corning Inc., USA). As a standard, we used commercial lignin (product number 370959, Sigma-Aldrich). See Methods S2 for precise description.

### 2.6. Inorganic nitrogen (ammonium, nitrate)

The ammonium and nitrate concentrations were measured in a 1 M KCl soil extract, as described previously (Adamczyk et al., 2019b). The ammonium–N assay was based on a modified indophenol method and for $NO_3$–N on the reduction of nitrate by vanadium (III). The absorbance was measured using a ClarioStar plate reader (BMGLabtech, Germany). See Methods S3 for precise description.

### 2.7. Fungal biomass and microbial necromass

Fungal biomass was measured using its biomarker, ergosterol, with high–performance liquid chromatography (HPLC) (Frostegård and Bååth, 1996) with a reverse–phase column (Phenomenex C18 150 × 4.6 mm, 100 A) as described by (Adamczyk et al., 2023) for bulk soil samples (freeze-dried and milled) and roots (freeze-dried and milled). The ergosterol results were converted to fungal biomass using equations proposed by (Wallander et al., 2013), see Eq (1). See Methods S4 for precise description.

$$FB\ (\mu g\ g-1\ soil) = ergosterol\ (\mu g\ g-1\ soil) x\ f\ x\ Rf \quad (1)$$

where f = 250 (1/4 x 1000, mg biomass µg-1 ergsoterol), and Rf = 1.61 (correction factor for average percent recovery, 1/0.62).

Microbial necromass was studied using its marker, the amino sugar glucosamine for fungal necromass and muramic acid for bacterial necromass (Adamczyk et al., 2024) for the bulk soil samples (no fractionation) and separately for the POM and MAOM fractions. The same method was applied to soil and root samples. Briefly, freeze–dried samples were washed with 0.2 M NaOH to remove unbound amino acids and amino sugars. The samples were then washed with water to remove the NaOH and hydrolysed with 6 M HCl. Released amino sugars were derivatized with 9–fluorenylmethyloxycarbonyl and amino sugar concentrations were measured with HPLC (Arc HPLC, Waters, MA, USA) equipped with fluorescence detector as described by Adamczyk et al. (2024) after separation in a Hewlett Packard ODS Hypersil (5 µm, 250 × 4.6 mm) column. Commercial amino sugars at various concentrations were used as external standards. Glucosamine concentrations were converted into fungal necromass C using equations described earlier (Amelung et al., 1999; Hu et al., 2024), see Eq (2) and Eq (3). See Methods S4 for precise description.

$$Bacterial\ necromass\ C\ (\mu g\ g-1) = murA\ x\ 45 \quad (2)$$

$$Fungal\ necromass\ C\ (\mu g\ g-1) = \left(\frac{GlcN}{179.2} - \frac{2xMurA}{251.2}\right) x179.2x9 \quad (3)$$

where 45 is the conversion factor of muramic acid (MurA) to bacterial necromass. Fungal necromass C was calculated by subtracting bacterial glucosamine (GlcN) from total GlcN and based on the average molar ratio of MurA and GlcN in bacterial cells of 1–2. Additionally, 179.2 and 251.2 were the molecular weights of GlcN and MurA, respectively, and 9 was the conversion factor of fungal GluN to fungal necromass C.

### 2.8. DNA extraction and sequencing of plant and fungal PCR amplicons

Soil samples for DNA analyses were collected up to a depth of 10 cm (including the organic and topmost part of the mineral soil). DNA was extracted in triplicates from 250 mg of freeze-dried soil using a modified Miller method (Sagova-Mareckova et al., 2008). Roots were first homogenised in liquid N, and DNA was extracted in duplicate from 250 mg of freeze-dried material, using the DNeasy Plant Mini Kit (Qiagen, Germany), following the manufacturer's recommendation. The replicates were pooled and cleaned using the Geneclean Turbo Kit (MP Biomedicals, USA).

The fungal and plant ITS2 regions were amplified from soil and root samples, respectively, as described previously (Moravcová et al., 2023). Briefly, the fungal ITS2 region was amplified using the barcoded gITS4/ITS4 primer pair (Ihrmark et al., 2012), whereas the plant ITS2 region was amplified using barcoded ITS-S2F and ITS4 primers (Chen et al., 2010). Amplifications were performed in triplicates. After quality checking, the triplicates were pooled and cleaned using a MinElute Kit (Qiagen, Germany). Sequencing libraries were prepared using the TruSeq PCR-free kit (Illumina, USA) and sequenced on Illumina MiSeq.

Sequence data were processed separately for plant and fungal ITS2 regions following the SEED 2.0 pipeline (Větrovský et al., 2018). Briefly, fastq-join (Aronesty, 2013) was used to join the paired-end reads, followed by quality filtering with a mean quality score of 30. The ITS2 region was extracted from both datasets using ITSx, v1.0.8 (Nilsson et al., 2010), resulting in a dataset used for OTU clustering with the UPARSE algorithm implemented in USEARCH (Edgar, 2013). BLASTn was used to identify OTU representative sequences against the UNITE database (version 8.2 (Nilsson et al., 2019), for fungi and GenBank combined with the PLANiTS database (Banchi et al., 2020) for plants. Sequences identified as non-fungal or non-plant were discarded. Putative ecological guilds for fungi were assigned based on the FungalTraits database (Põlme et al., 2020), primarily using the "primary_lifestyle" category. Since ericoid mycorrhizal (ErM) fungi are not explicitly classified under this category, OTUs were considered potentially ErM if this lifestyle was indicated in either the "secondary_lifestyle" or "Comment_on_lifestyle_template" fields of the database. For fungal genus-level taxonomic assignments, we applied the criteria from (Tedersoo et al., 2014) using a minimum sequence similarity threshold of 90 %. If this threshold was met, the best hit was selected based on the lowest e-value, with a threshold $e^{-40}$ for fungi. Ecological traits were assigned only to genera that met these criteria. The sequence data supporting the findings of this study are available in the NCBI database under the project number PRJNA1225681.

### 2.9. Enzymatic activities

The extracellular enzymatic activities were measured as previously described (Pritsch et al., 2011). Fresh soil (equal to 1 g of dry weight) was homogenised in a slurry with 50 mM sodium acetate buffer. The buffer solution was adjusted to the average soil pH of the organic layer of the soil (4.5) and the average pH of the soil mineral layer (3.7). Soil slurries were homogenised for 2 min in a mortar. The resulting suspensions were continuously stirred using a magnetic stir plate. Oxidative enzymes (phenol oxidase EC 1.14.18.1 and peroxidase EC 1.11.1.x) were measured according to (Marx et al., 2001). One mL of the soil suspension (prepared as above) was mixed with 1 mL of 20 mM DOPA solution (L–3, 4–dihydroxyphenylalanin). The enzymatic activity was expressed as nmol of DOPA per 1 g of dry mass per hour. Activities of acid phosphatase (EC 3.1.3.2), N–acetylglucosaminidase (3.2.1.30), β–glucosidase (EC 3.2.1.21), β–xylosidase (EC 3.2.1.37), cellobiohydrolase (EC 3.2.1.91) and leucine aminopeptidase (EC 3.4.11.1) were measured using fluorometric substrates (Sigma–Aldrich). The enzymatic activity was expressed as nmol of MU or AMC per 1 g of soil dry mass per hour. See Methods S5 for precise description.

### 2.10. Fluxes of $CO_2$, $CH_4$, and $N_2O$, soil temperature and moisture

The soil $CO_2$ emissions, and $CH_4$ and $N_2O$ net exchange were

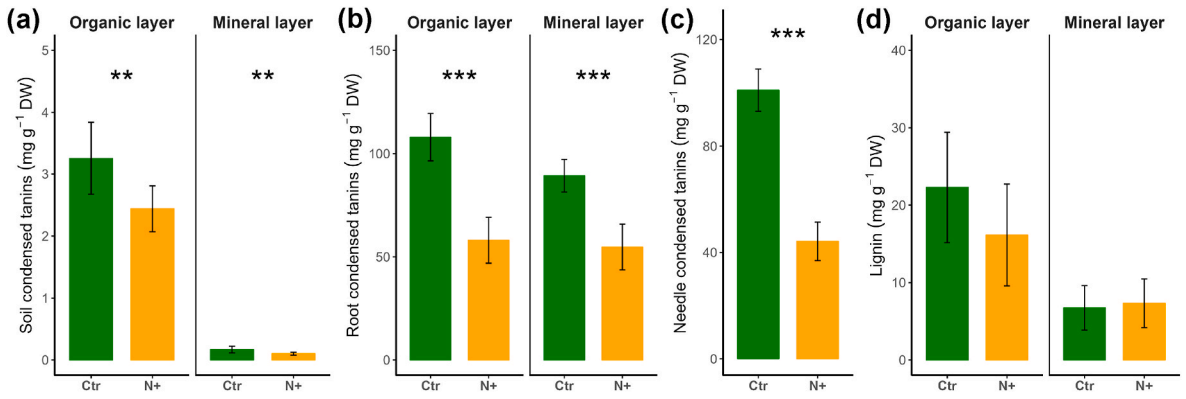


**Fig. 1.** Concentration of condensed tannins in soil (a), roots (b), and needles (c) and lignin concentration in the soil (d) on control (Ctr) and N fertilized (N+) plots (mean ± sd). Replicate = 6 for each treatment. Statistically significant differences between treatments (separately for organic and mineral layers) are marked with asterisks: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

measured biweekly during the vegetative seasons of 2021, 2022, and 2023 in 12 sets of two bare soil plots with a diameter of 30 cm (6 x 2 sets for the control and 6 x 2 plots for the N fertilisation treatment). Each set of plots was trenched (the roots were cut and removed from the trench) in May 2021 into areas of 1 × 2 m to separate the signal of autotrophic respiration (from the tree roots and understory) from the soil heterotrophic respiration. Ingrowth of roots was prevented with a vertical installation of a geotextile water permeable fabric (Ťupek et al., 2019). $CO_2$ fluxes were measured both from trenched area and out of trench, they were regarded as heterotrophic respiration and total respiration, respectively. A non-transparent dynamic chamber (21.7 L) equipped with a small fan for air mixing was connected via Teflon tubing to the LI–COR LI–7810 $CH_4/CO_2/H_2O$ or LI–7820 $N_2O/H_2O$ trace gas analyzer (LI-COR Corporate, USA), and manually placed air tightly on the soil (inside the pre-cut 2 cm deep ridge along the circumference of the chamber). The measurements were conducted for 3 min. The GHG fluxes were during years 2021–2023 measured every two weeks after the snowmelt in spring, throughout summer, and until late autumn accounting for the typical seasonality pattern of soil temperature known as the main driver of microbial respiration. The linearity of the concentration evolution inside the chamber headspace over time was monitored for each measurement. Only stable concentration data were accepted for the flux calculation with the formulas as in (Zhao, 2019) and from the period after 30 s of the recorded measurement start and 15 s before recorded measurement end. Soil Scout Oy sensors for soil temperature and soil moisture monitoring were installed at a depth of 5 cm for each of the six replicates of the two group plots and these were operated continuously at 15 min intervals since June 2020. To account for differences in soil C stock in soil $CO_2$ emissions, the respiration values were for each plot normalized by the nearest measurement of soil organic C stock (in kg C m$^{-2}$ of 1 m soil depth) and expressed as C fraction of SOC per hour (μg C g$^{-1}$ SOC hour$^{-1}$).

### 2.11. Statistical analyses

To determine the impact of N fertilisation on soil chemistry, microbial biomass markers and enzyme activities, linear mixed-effects models were used. We ascribed treatment as fixed effect and Block as random effect. The data was checked for normal distribution before statistical analysis and there was no need for transformation due to lack of normality. Statistical analysis were performed using lme () function within "nlme" package in R 4.3.3 (R Core Team, 2022). For GHG emissions, one one-way ANOVA test was used to determine if there were statistically significant differences in mean GHG emissions among the N fertilisation treatment and control groups. Specifically, for comparing means across groups we used the compare_means function of the ggpubr package with the method = "anova". Normality and homogeneity of variances were checked using Q-Q plots, the Shapiro-Wilk test and Levene's test. The $CO_2$ data were normalized by soil C stocks for spatial comparability, due to dependency of respiration on C stock sizes, and not due to data violating the normality assumption. Correlations were estimated using Spearman test. Statistical significance was set at $p < 0.05$. All DNA analyses were conducted using R 4.2.2 (R Core Team, 2022). The "vegan" (Oksanen et al., 2001) and "phyloseq" (McMurdie and Holmes, 2013) packages were used for fungal community composition analyses. First, singletons were discarded and the OTU table was rarefied to a common sampling depth of 8228 sequences per sample (plant root data) and 5433 (fungal data) using the function rrarefy () in *vegan*. Relative abundances were calculated to assess the taxonomic proportion of plant communities, and fungal communities and their respective ecology categories. Differences in the relative abundance of fungal ecological groups (ERM, ECM, and saprotrophs) and genera (*Cortinarius*, *Lactarius*, and *Russula*), as well as most abundant plant species between the N fertilisation treatment and the control were tested using the Wilcoxon test for unpaired samples, as implemented in R 'stats'. Bray–Curtis dissimilarity was used to construct a fungal community dissimilarity matrix, using Hellinger transformed OTU table. PERMANOVA, as implemented in adonis2 function in *vegan*, was perfomed to test the significance of treatment (control, nitrogen) on fungal community composition and community composition of the most common fungal guilds.

## 3. Results

### 3.1. Soil pH, nitrogen, carbon pools and POM, MAOM fractions

In the soil organic layer, C stocks and C and N concentrations were higher in the N fertilisation treatment than in the control ($p = 1.664E-11$ for C stock; $p = 0.012$ for C concentration; $p = 2.4E-5$ for N concentration), whereas in the mineral layer N was higher in N fertilisation ($p = 0.001$) and C did not differ between treatments ($p = 0.347$ for C stock; $p = 0.870$ for C concentration; Table 1, Fig. S2). In the soil organic layer, also the C to N ratio was lower under N fertilisation ($p = 0.004$ in organic layer; $p = 0.177$ in mineral layer). Differences in pH were not significant ($p = 0.200$ in organic layer; $p = 0.970$ in mineral layer; Table 1).

The concentration of ammonium was higher under N fertilisation than in the control treatment in organic layer ($p = 2.2E-16$) and there was no difference in mineral layer ($p = 0.970$). Concentration of nitrate was below the detection limit in organic and mineral layers.

The stable SOC pool was elevated under the N fertilisation treatment in the soil organic layer ($p = 0.001$), but not in the mineral layer ($p = 0.810$). The labile SOC pool did not differ between the treatments ($p = 0.970$ in organic layer; $p = 0.800$ in mineral layer; Table 1).

The POM–C fractions did not differ significantly ($p = 0.620$). Higher

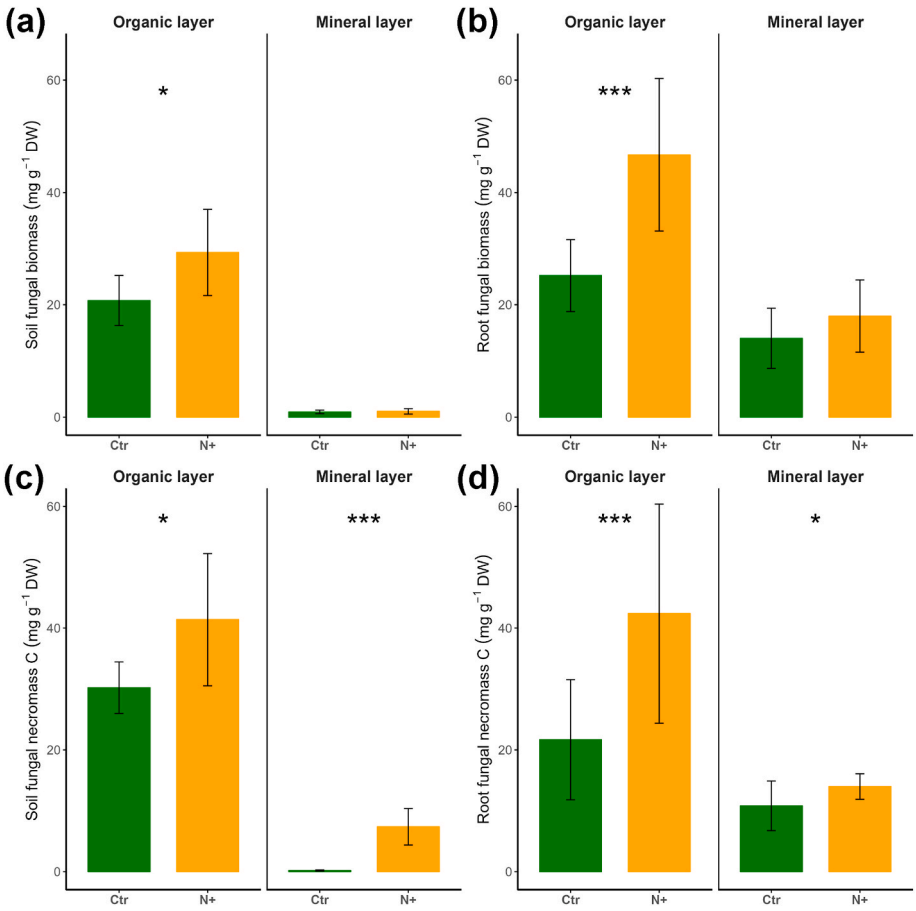


**Fig. 2.** Fungal biomass in soil (a) and roots (b), fungal necromass in soil (c) and roots (d) under control (Ctr) and N fertilisation (N+) treatment (mean ± sd). Replicate = 6 for each treatment. Statistically significant differences between treatments (separately for organic and mineral layers) are marked with asterisks: *$p <$ 0.05, **$p < 0.01$, ***$p < 0.001$.

MAOM–C under N fertilisation was observed ($p$ = 4.3E-7; Table 1). Glucosamine (a marker of fungal necromass) was significantly higher in the MAOM–C fraction under N fertilisation ($p$ = 2.2E-16). MAOM-C in mineral layer was significantly correlated with fungal necromass (r = 0.695, $p$ = 0.001). We detected also low amounts of muramic acid (bacterial necromass marker) in MAOM fraction, but with no difference between treatments (Table 1).

### 3.2. Condensed tannins and lignin concentration

The concentration of condensed tannins in the soil was lower under N fertilisation than in the control in the organic and mineral layers ($p$ = 0.003 in organic layer; $p$ = 0.005 in mineral layer, (Fig. 1a). The concentrations of condensed tannins in the roots followed the same pattern in the soil organic and mineral layers ($p$ = 1.5E-14 in organic layer; $p$ = 2.2E-16 in mineral layer, (Fig. 1b). Condensed tannin concentrations in the tree needles were also significantly lower for N fertilisation treatment ($p$ = 2.2E-16, Fig. 1c). The concentration of lignin did not differ significantly between the treatments ($p$ = 0.110 in organic layer; $p$ = 0.730 in mineral layer, Fig. 1d).

### 3.3. Tree growth, relative abundance of plant species and vegetation survey

The stand basal area and litterfall all were higher in the N fertilized plots (p = 0.04 for litterfall), indicating that tree growth was significantly enhanced compared to the control site (Table 2). Moreover, as estimated in (Richy et al., 2024) from the same study site, N treatment in our experimental site resulted in greater root biomass (11.4 ± 1.4 for control and 15.1 ± 0.7 tn/ha for N addition), and root growth (0.32 ± 0.03 for control and 0.50 ± 0.01 tn/ha/yr for N addition).

Neither the vegetation survey cover nor the molecular barcoding of plant roots differed significantly between the control and N fertilized treatments (Table 2). Under N fertilisation, we found slightly more roots of *Vaccinium vitis-idaea* ($p$ = 0.230), but less *Empetrum nigrum* ($p$ = 0.900). The vegetation survey cover also suggested slight differences in *Calluna vulgaris* ($p$ = 0.462), however, this difference was not seen in the metabarcoding for roots ($p$ = 0.410).

### 3.4. Fungal biomass and necromass in bulk soil and in roots

The concentration of ergosterol (a marker of fungal biomass) was higher under the N fertilisation treatment in the soil organic layer ($p$ = 0.017, Fig. 2a) and in the roots ($p$ = 0.001, Fig. 2b), however, there was no significant difference in the mineral layer of the soil ($p$ = 0.660, Fig. 2a) nor the roots ($p$ = 0.250, Fig. 2b). Glucosamine concentration (a marker of fungal necromass) was significantly higher in the N fertilisation treatment in the soil organic layer ($p$ = 0.019, Fig. 2c) and mineral soil ($p$ = 1.9E-12) as well as in the roots ($p$ = 0.001 in organic layer; $p$ = 0.038 in mineral layer. Fig. 2d). The concentration of muramic acid (a marker of bacterial necromass) was below the detection limit (which is 4 μg g$^{-1}$ DW) The bacterial community structure can be found in Richy et al. (2024).

**Table 3**
Average relative abundance (%) of the fungal functional groups in control and N fertilized treatments in first 10 cm of soil (including organic layer and topmost part of the mineral layer). The OTU table was trimmed depending on the ecology for ericoid, ectomycorrhizal fungi and saprotrophs, and at the genus level for *Cortinarius*, *Russula*, *Lactarius*, white rot saprotrophs, non-white saprotrophs. No statistically significant differences between control and N fertilisation treatment. See Table S2 for size effects and exact p values.

| | Control | N fertilisation |
|---|---|---|
| Ericoid mycorrhiza | 5.57 (2.63) | 5.79 (2.85) |
| Ectomycorrhiza | 22.04 (13.70) | 19.99 (10.88) |
| White rot saprothrophs | 0.06 (0.04) | 0.13 (0.14) |
| Non-white saprothrophs | 46.65 (14.31) | 49.21 (9.06) |
| *Cortinarius* | 0.66 (0.88) | 0.15 (0.19) |
| *Lactarius* | 0.27 (0.29) | 1.08 (2.08) |
| *Russula* | 7.47 (11.6) | 4.31 (6.74) |

### 3.5. Relative abundance of fungal functional groups

PERMANOVA analysis revealed no significant differences in the overall fungal community structure ($p = 0.135$; see Table S1 and Supplementary Materials for representative sequences). The average relative abundance of fungi with distinct primary lifestyle did not differ significantly, showing slightly higher relative abundances of DNA sequences of ECM fungi ($p = 0.164$), ERM fungi ($p = 0.572$) and saprotrophs ($p = 0.129$) in the N fertilisation treatment (Table 3 and Table S2). *Cortinarius* and *Russula* relative abundances were slightly higher ($p = 0.378$, $p = 0.686$, respectively) under control treatment, and *Lactarius* slightly higher under N addition ($p = 0.900$) For composition of fungal communities plotted at the phylum level and for composition of fungal communities categorized by their ecological groups see Fig. S3 and Fig. S4.

### 3.6. Enzyme activities

In the soil organic layer, all enzyme activities were higher in the N fertilisation treatment than in the control treatment ($p = 5.7E-12$ for N–acetylglucosaminidase; $p = 2.2E-16$ for beta–glucosidase; $p = 3.4E-9$ for cellobiosidase; $p = 3.0E-16$ for phosphatase, $p = 8.1E-8$ for beta–xylosidase; $p = 0.0007$ for peroxidase) except phenol oxidase and leucine aminopeptidase ($p = 0.282$, $p = 0.055$, respectively) (Fig. 3a). In the mineral layer, enzymatic activities were higher under N fertilisation for N-acetylglucominidase ($p = 0.03$), phosphatase ($p = 6.1E-5$), and peroxidase ($p = 0.02$) (Fig. 3b).

### 3.7. Soil $CO_2$, $CH_4$, and $N_2O$ fluxes, temperature and moisture

Soil heterotrophic respiration (Rh) during the vegetative season was significantly larger ($p = 8.92e-05$) in N fertilized plots (0.58 ± 0.01 SE, g $CO_2$ $m^2$ $hour^{-1}$) than in control plots (0.46 ± 0.01 SE, g $CO_2$ $m^2$ $hour^{-1}$) (Fig. 4a). However, the Rh normalized with SOC stocks showed no significant difference between N fertilisation (mean 28.3 ± 1.1 SE, µg C $g^{-1}$ SOC $hour^{-1}$) and the control (mean 28.6 ± 1.1 SE, µg C $g^{-1}$ SOC $hour^{-1}$) ($p = 0.831$) (Fig. 4b).

The soil mean net $CH_4$ oxidation was significantly reduced ($p = 4.24e-24$) between control ($-0.21$ $\pm 5^{e-3}$ SE mg $CH_4$ $m^2$ $hour^{-1}$) and N fertilisation ($-0.14$ $\pm 2^{e-3}$ SE mg $CH_4$ $m^2$ $hour^{-1}$) (Fig. 4c). The mean soil net $N_2O$ emission of control were on average zero ($-0.03$ ± 0.1 SE ug $N_2O$ $m^2$ $hour^{-1}$) and under N fertilisation showed a marginally significant increase to 0.5 ± 0.17 SE ug $N_2O$ $m^2$ $hour^{-1}$ ($p = 0.029$) (Fig. 4d).

The mean soil temperatures at 5 cm depth (T) corresponding to gas flux measurements were not significantly different between the control (12.6 ± 0.17 SE °C) and the N fertilized plots (12.0 ± 0.16 SE °C) (Fig. 4e). The volumetric soil moisture at a 5 cm depth (SWC) was, on average, the same for control and N fertilized plots (0.31 $\pm 3^{e-3}$ SE $m^3$

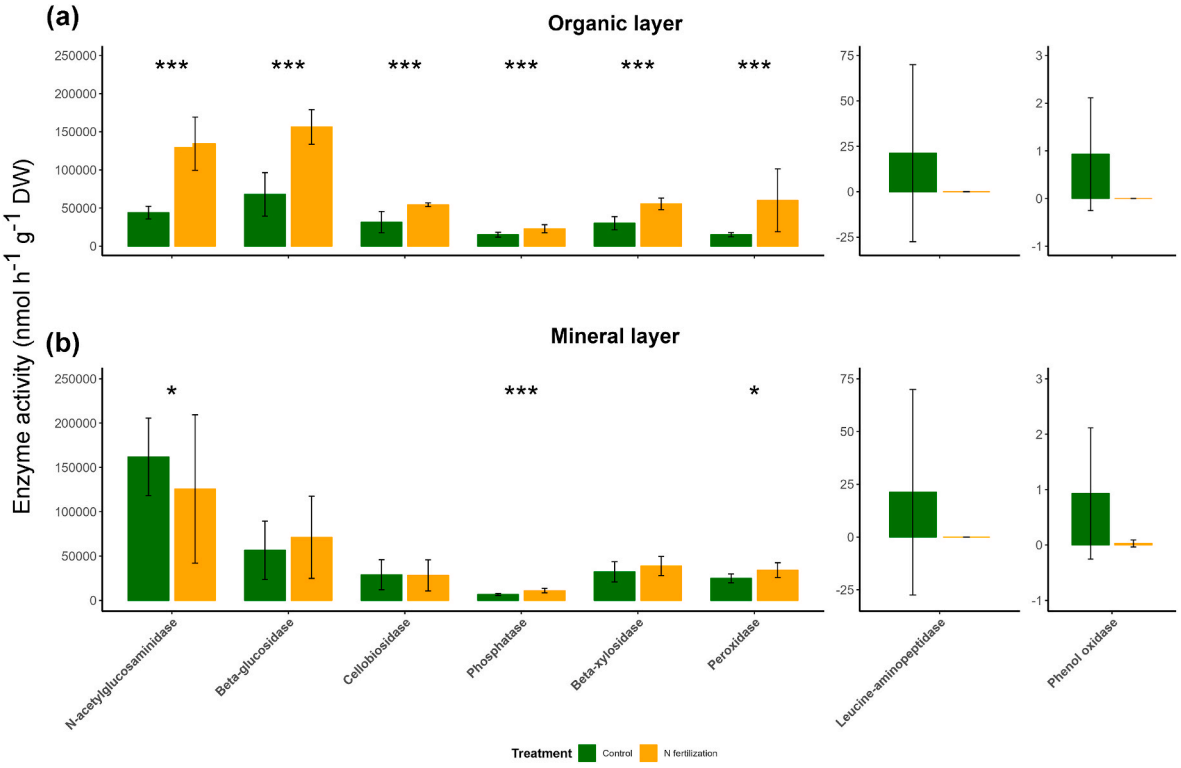


**Fig. 3.** Enzymatic activities under control and N fertilisation treatment: (a) in the organic and (b) mineral layers (mean ± sd). Results presented as the mean and error bars represent standard deviation. Replicate = 6 for each treatment. Statistically significant differences between treatments (separately for organic and mineral layers) are marked with asterisks: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

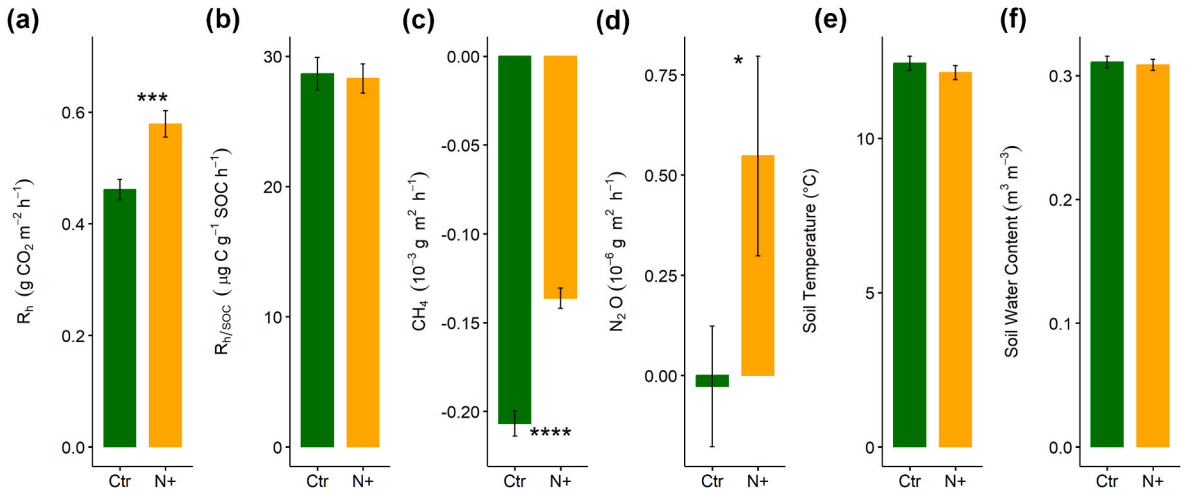


**Fig. 4.** The comparison of the means (±SE) of measured gas flux and microclimate data between the plots with N fertilisation (N+) and controls (Ctr) measured biweekly during the snow-free periods of 2021–2023. The panels show (a) soil heterotrophic respiration (Rh, µg C $g^{-1}$ SOC $hour^{-1}$), (b) soil heterotrophic respiration normalized by SOC stocks (Rh C $SOC^{-1}$ ppm $m^2$ $hour^{-1}$), (c) $CH_4$ oxidation ($10^{-3}$ g $m^2$ $hour^{-1}$), (d) $N_2O$ emissions ($10^{-6}$ g $m^2$ $h^{-1}$), and (e) soil temperature (°C) and (f) volumetric soil water content (m3 $m^{-3}$) at a 5 cm depth.

$m^{-3}$) (Fig. 4f).

### 3.8. Correlations between variables

C stock of the soil was significantly correlated with plant litter ($r = 0.91$, $p < 0.001$), and fungal necromass ($r = 0.58$, $p < 0.05$). Moreover, C stock was also positively correlated with white-rot saprothrops ($r = 0.748$, $p < 0.005$) and oxidoreductases ($r = 0.713$, $p < 0.009$). Fungal biomass was significantly correlated with fungal necromass ($r = 0.818$, $p < 0.001$).

## 4. Discussion

### 4.1. Mechanisms of N fertilisation effect on soil C

We found that N fertilisation caused multilevel changes in the properties and processes at our experimental site. Soil under the control and N fertilized treatments differed in terms of C and N concentrations, tannin concentration, GHG production, microbial biomass and necromass, activity of extracellular enzymes. However, these profound changes were mainly observed in the organic layer of the soil, and differences in the mineral soil were less detectable.

We observed a higher concentration of SOC under N the fertilisation treatment and a higher concentration of stable C in the organic layer, suggesting a potential role of N addition in soil C stabilization. This led us to reject the null hypothesis (no changes in C stocks after N addition) and focus on hypotheses 1–4, which aimed to pinpoint the phenomenon behind these increases in C stocks. In addition, in other studies and meta-analyses, N fertilisation mainly led to increased SOC (Lucander et al., 2021; Tang et al., 2023).

It has been proposed that the effect of N fertilisation on C stabilization depends on N availability in the ecosystem and thus on the ability to retain N (Lucander et al., 2021). In N-limited soils, N input can stimulate plant growth (Nohrstedt, 2001), however, in N-rich soils, it can potentially cause soil acidification (Lucas et al., 2011; De Vries et al., 2014) via elevated nitrification, leading to reduced decomposition of litter (Shen et al., 2021). Our first hypothesis considered this potential effect of N fertilisation on soil pH. We hypothesized that N fertilisation decreases pH and decelerates microbial biomass and activities leading to the accumulation of SOC. However, pH did not differ among our treatments, and microbial growth and enzymatic activities were higher under N fertilisation than in the control. Moreover, nitrate concentration was below the detection limit, although the concentration of ammonium was elevated in the organic layer in the N fertilisation treatment. Although a decrease in pH after N addition has been observed in other studies e.g., (Ye et al., 2018), this phenomenon has been mostly observed for N deposition rather than for N fertilisation (Tian and Niu, 2015). N deposition differs from N fertilisation in terms of N application time (constant for N deposition, one addition per couple of years for N fertilisation), doses and that N fertilisation is often combined with Ca addition to avoid acidifying effect (Lundin and Nilsson, 2014). Thus, we did not find confirmation for our first hypothesis and the increase in SOC due to N fertilisation cannot be explained by changes in pH and the subsequent consequences on microbial activities and decomposition rate.

Soil microbial community structure is driven by numerous factors including nutrient content (Lladó et al., 2018); thus, N enrichment has the potential to change microbial communities, particularly in N-limited ecosystems (Siegenthaler et al., 2022). Accordingly, our second hypothesis was based on the expectation that the fungal community structure would change owing to N enrichment. We hypothesized that N fertilisation increases SOC due to changes in fungal community structure, more precisely *via* the fungi not being able to decompose effectively polyphenolics, over polyphenolic decomposers which in turn decreases the release of polyphenolic–bound N, leading to SOC build–up. Contrary to hypothesis 2, N fertilisation did not cause an increase in polyphenolic concentrations (lignin and condensed tannins) and decrease of oxidoreductase activities. Recently it has been proposed that abundance of a group of ECM fungi, *Cortinarius acutus* s.l. producing Mn–peroxidases correlates negatively with SOC accumulation (Lindahl et al., 2021). More precisely, C storage of organic topsoil across Swedish boreal forests was found to be 1/3 lower in presence of *Cortinarius acutus* (Lindahl et al., 2021). These ectomycorrhizal fungi with potent decomposer capabilities diminished in abundance under elevated inorganic N (Bödeker et al., 2009, 2014; Jörgensen et al., 2022), which is in line with our observation of slight loss in relative abundance of *Cortinarius* after N fertilisation. Thus, we could expect lower activity of Mn-peroxidases and further higher concentration of polyphenolics under N fertilisation explaining SOC-build-up. However, another study from our experimental site have not found lower activity of Mn-peroxidases after N addition (Richy et al., 2024). Also other ECM fungi which are effective decomposers of polyphenolics, *Russula* and *Lactarius*, and saprotrophic fungi (Bödeker et al., 2009), did not change much in relative abundance with N fertilisation. Thus, as we did not

observe significant changes in fungal community structure (see PERMANOVA test Table S1) and we noticed only minor changes in relation of fungal decomposers, we could not find solid ground to confirm hypothesis 2a because of similar concentration of lignin and the lack of decelerated activity of oxidoreductases, including Mn peroxidases. Similarly, hypothesis 2b, thus increased microbial biomass due to higher CUE and reduced mineral C protection (Feng et al., 2022) has to be rejected as although we observed elevated microbial biomass, but MAOM-C also increased.

. Current methodologies do not allow for the separate measurement of fungal biomass across different fungal guilds. Methods based on DNA may provide relative abundances of fungal guilds but do not provide distinct information about functional guilds (Camenzind et al., 2024), thus caution should be made for differences in fungal community structure. However, our hypothesis 2 was based not only on fungal biomass and their guilds, but also on activity of specific enzymes and changes in soil chemistry, thus rejection of hypothesis 2 should still be valid.

N fertilisation in nutrient–limited ecosystems can increase plant growth, leading to elevated litter input (Mäkipää et al., 1998), feeding soil microorganisms and thus increasing microbial biomass, the source of microbial necromass. In line with this, our third hypothesis assumes a role for microbial necromass in SOC stabilization (Liang et al., 2019). We hypothesized that the SOC increase is derived from enhanced microbial necromass which was further stabilised in the soil. As a confirmation for hypothesis 3, we observed an elevated microbial necromass under N fertilisation in the organic and mineral layers. This microbial necromass was of fungal origin, which is in line with other studies suggesting that fungal necromass, rather than bacterial, builds up most of microbially–derived SOC (Clemmensen et al., 2013; Liang et al., 2019). Supporting this, fungal biomass, which in turn contributes to fungal necromass, was also higher under N fertilisation. Increased biomass of microorganisms was also reflected in the elevated $CO_2$ under the N fertilisation treatment; however, after normalization via C stocks, there was no significant difference in soil respiration. Higher fungal biomass and enzyme activities, with no significant increase in soil respiration, suggest increased C use efficiency (Manzoni et al., 2017) or a slower turnover rate of fungal mycelia (Hagenbo et al., 2017), which supports the accumulation of microbial necromass, and thus the build-up of SOC. MAOM-C slightly increased under N fertilisation, suggesting that fungal necromass could be stabilised on minerals, which is supported by a high positive correlation between MAOM–C and microbial necromass ($r = 0.695$, $p < 0.01$) and a significantly higher concentration of glucosamine (a marker of fungal necromass) detected in the MAOM fraction of the N fertilized treatment. A recent meta–analysis of N fertilisation experiments showed a positive correlation between MAOM-C and microbial necromass (Tang et al., 2023), supporting our findings. Increased plant litter input due to N fertilisation may fuel formation on MAOM directly (plant C bound to minerals) (Angst et al., 2021) or indirectly, as microorganisms feed on more abundant plant litter effecting in high microbial necromass production. Stabilization of fungal necromass under N treatment was likely not driven by the recently proposed plant–root tannins and fungal necromass interactions (Adamczyk et al., 2019a, 2019b) as soil tannin concentrations decreased under N fertilisation. However, the concentration of tannins in the soil under N fertilisation was sufficiently high to effectively interact with the fungal necromass to build up a stable SOC (Adamczyk et al., 2019b; Hupperts et al., 2025), thus this mechanism cannot be fully excluded. Our third hypothesis was confirmed and the increase in SOC due to N fertilisation by increased microbial necromass production and further stabilization in the soil appears to be the most plausible conclusion from our observations.

Though we measured also bacterial necromass, it was below detection limit in soil and only in MAOM fractions we detected very low amounts. This low bacterial necromass is not surprising, taking into account that boreal forests fungi may play dominant role due to low pH and high C content resulting in low bacterial abundance (Clemmensen et al., 2013). Moreover, fungi are able to decompose persistent C compounds, while bacteria are mainly associated with turnover of easily degradable substrates (Rousk et al., 2016), which are not abundant in boreal forest soil.

Our fourth hypothesis, thus changes in soil C stock as driven by variations in abundance and diversity of ground vegetation was not confirmed above and belowground, as vegetation survey did not show any differences, nor did the relative abundance of plant roots and abundance of ericoid mycorrhiza (Table 2). Although the effect of N fertilisation on abundance and diversity of plants have been shown to vary (Sullivan and Sullivan, 2018) the direction and severity of changes seem to be N-dose dependent (Jetsonen et al., 2024). In our experiment we have used low N dose, which could explain the lack of measurable changes in ground vegetation cover.

### 4.2. N fertilisation effect on plant metabolism

N fertilisation affected not only soil chemistry, including C pools, but also plant metabolism. The decreased concentration of tannins in the roots and tree needles after N fertilisation is in line with the carbon-nutrient balance hypothesis (Bryant et al., 1983) which states that low resource availability limits plant growth more than photosynthesis, and the plant allocates extra C that cannot be used for growth to the production of secondary metabolites such as tannins. Although the carbon–nutrient balance hypothesis has been heavily criticized (Hamilton et al., 2001), our study provides evidence that plant secondary metabolism may be decelerated by N fertilisation with unknown consequences for forest resilience. For example, N fertilized forests may be more vulnerable to beetle bark outbreaks and other plant pathogens, because tannins inhibit bark beetle infestation (Felicijan et al., 2016) and have anti–fungal activities (Anttila et al., 2013). Thus, the repercussions of decreased production of secondary compounds due to N fertilisation on forest resilience should be considered in future studies.

### 4.3. Comparison of our results with other N fertilisation studies

Recent meta-analyses aiming to elucidate the effect of N fertilisation on soil C have clearly shown that although both, POM and MAOM are elevated after N addition; however, especially less stable POM increase after N addition (Chen et al., 2018; Rocci et al., 2021; Tang et al., 2023). On the contrary, in our experiment especially MAOM pool was enriched after N fertilisation. In two meta-analyses which took into account N amount used (Tang et al., 2023; Wu et al., 2023) POM increase is enhanced with higher doses of N, on the contrary, MAOM is the highest with lower N doses. The amount of N used in our experiment (2 g N $m^{-2}$ $y^{-1}$) falls in low range of fertilisation, thus our results are in line with meta-analyses considering N dose. Moreover, as MAOM has greater microbially derived compounds than POM (Von Lützow et al., 2007; Williams et al., 2018), boreal forests, which are richer in microbial biomass comparing to temperate zone (Patoine et al., 2022) may especially promote formation of MAOM, which further supports our results. The reason for the decelerated MAOM formation under high N addition could emerge from weakened mineral protection due to higher concentration of understory in the soil (Feng et al., 2022). Oxalic acid (and other simple organic acids), released by plant roots and by microorganisms, liberate organic C compounds from protective associations with minerals (Keiluweit et al., 2015). Increase of oxalic acid concentration was mainly observed with medium to high N additions (Feng et al., 2022), not in the range of N addition used in our study.

Additional reasons for differences in MAOM increases between our study and others are timeframe of experiment (on average 10 years in meta-analyses, 60 years in our experiment) and ecosystem type,i.e., grasslands, croplands and forests under different climatic zones (e.g. temperate vs boreal). However, it seems to be that amount of added N could form a most convincing basis for these discrepancies.

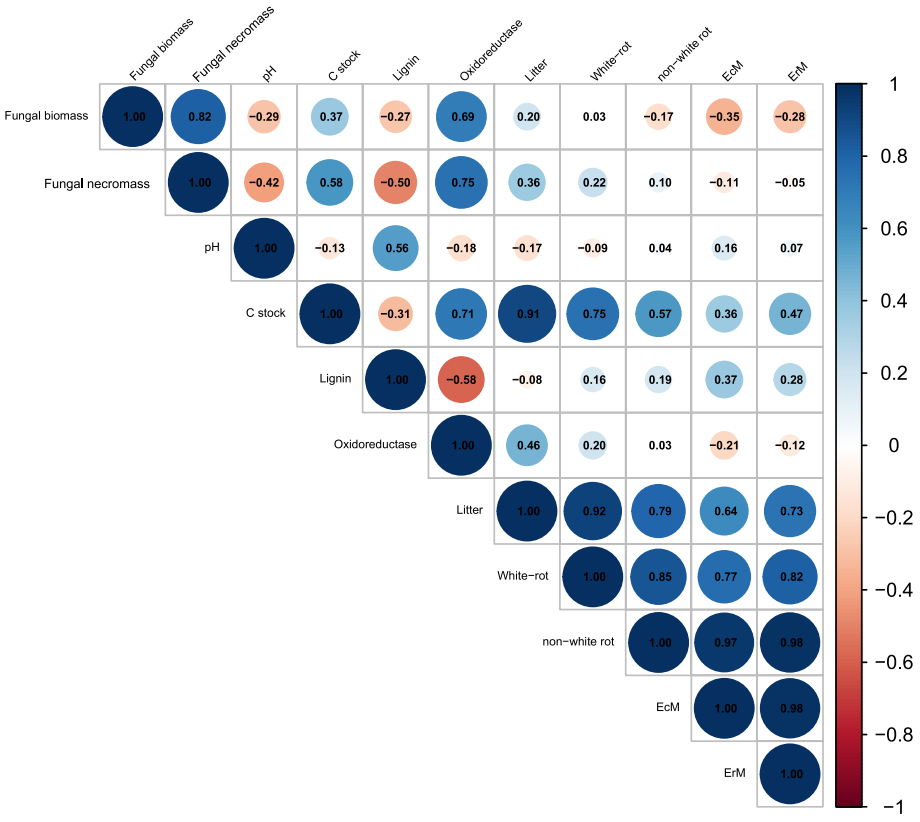


**Fig. 5.** Correlation plot (Spearman test), showing correlation coefficient between variables.

Although N fertilisation seems to be a beneficial forest management strategy to increase C pools, it has also some limitations related to emissions from inorganic fertilizer production and $N_2O$ emissions. Although Haber-Bosch reaction by which $N_2$ is converted to $NH_3$ emits around 3 kg $CO_2$ eq per kg of $NH_3$ (Osorio-Tejada et al., 2022), due to low doses of N used in our experiment, mitigation potential is decreased from 262 to 246g $CO_2$ $m^{-2}y^{-1}$ (including also elevated $N_2O$ emissions, equivalent to 9 g $CO_2$ $m^{-2}y^{-1}$), as calculated for the same study site (Tupek et al., 2024). Also in other work in boreal forests N fertilisation elevated $N_2O$ emissions, these changes were marginal compared to the reduced soil $CO_2$ emissions and elevated $CO_2$ uptake and sequestration into biomass and soil C pools (Håkansson et al., 2021; Öquist et al., 2024).

## 5. Conclusions

N fertilisation may have a multilevel effect on soil processes, plant and microbial communities and their metabolism. We observed higher content of C and N under the N fertilisation treatment and higher concentration of stable C pool in the soil organic layer. Our results jointly point to increased plant litter, fungal biomass and fungal necromass stabilised in the soil as mechanism of increased SOC pool rather than changes in fungal community composition and suppression of the release of polyphenolic–bound N or changes in pH. This conclusion is based on our set of hypotheses and no changes in community structure (Tables S1 and S2, Table 3) nor pH (Table 1). Moreover, such conclusion is supported via correlations between C stock and microbial biomass, necromass and plant litter (Fig. 5). Although our study supports the idea that employing N fertilisation to increase microbial necromass in persistent C pools could be considered an effective means to increase C stabilization in soil to help mitigate climate change, future studies should also consider the potential side–effects of N fertilisation. These include changes in the functioning of tree secondary metabolism, effects on forest resilience, and long–term effects on microbial community structure and activity. Moreover, N fertilisation in forest management aimed at increasing SOC stocks should adjust the level of N addition according to the N status of the soil, site–specific effects and plant and soil biodiversity to obtain the most effective treatments.

## CRediT authorship contribution statement

**Bartosz Adamczyk:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sylwia Adamczyk:** Methodology, Investigation, Formal analysis, Data curation. **Boris Tupek:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Qian Li:** Writing – review & editing, Visualization, Formal analysis. **Tijana Martinovic:** Writing – review & editing, Formal analysis. **Etienne Richy:** Writing – review & editing, Validation, Formal analysis. **Aleksi Lehtonen:** Writing – review & editing, Investigation, Funding acquisition, Conceptualization. **Petr Baldrian:** Writing – review & editing, Funding acquisition, Formal analysis, Conceptualization. **Raisa Mäkipää:** Writing – review & editing, Funding acquisition, Data curation, Conceptualization.

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Raisa Makipaa reports financial support was provided by EU Framework Programme for Research and Innovation Euratom. Bartosz adamczyk reports financial support was provided by Research Council of Finland. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2025.109917.

## Data availability

The data that support the findings of this study are available in the Supplementary Materials of this article and in Zenodo (https://doi.org/10.5281/zenodo.13837158, 10.5281/zenodo.13734692); The sequence data supporting the findings of this study are available in the NCBI database under the project number PRJNA1225681.").

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