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RESEARCH ARTICLE

Phosphorus limitation promotes soil carbon storage in a boreal forest exposed to long-term nitrogen fertilization

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Abstract

Forests play a crucial role in global carbon cycling by absorbing and storing significant amounts of atmospheric carbon dioxide. Although boreal forests contribute to approximately 45% of the total forest carbon sink, tree growth and soil carbon sequestration are constrained by nutrient availability. Here, we examine if long-term nutrient input enhances tree productivity and whether this leads to carbon storage or whether stimulated microbial decomposition of organic matter limits soil carbon accumulation. Over six decades, nitrogen, phosphorus, and calcium were supplied to a *Pinus sylvestris*-dominated boreal forest. We found that nitrogen fertilization alone or together with calcium and/or phosphorus increased tree biomass production by 50% and soil carbon sequestration by 65% compared to unfertilized plots. However, the nonlinear relationship observed between tree productivity and soil carbon stock across treatments suggests microbial regulation. When phosphorus was co-applied with nitrogen, it acidified the soil, increased fungal biomass, altered microbial community composition, and enhanced biopolymer degradation capabilities. While no evidence of competition between ectomycorrhizal and saprotrophic fungi has been observed, key functional groups with the potential to reduce carbon stocks were identified. In contrast, when nitrogen was added without phosphorus, it increased soil carbon sequestration because microbial activity was likely limited by phosphorus availability. In conclusion, the addition of nitrogen to boreal forests may contribute to global warming mitigation, but this effect is context dependent.

KEYWORDS
boreal forest ecosystem, fertilization, microbial community composition, microbial degradation, nutrient limitation, soil carbon storage, structural equation modeling, tree woody biomass

1 | INTRODUCTION

Forests play a key role in global carbon (C) cycling by absorbing and storing significant amounts of atmospheric carbon dioxide (CO_2). Spanning approximately 30% of the Earth's land area, forests store 45% of the terrestrial carbon, equivalent to ~7.6 Gt of CO_2 annually (Harris et al., 2021). Forest carbon is distributed between soils and vegetation and varies across latitudes due to differences in temperature, precipitation, and length of growing season (Baldrian et al., 2023). Tropical and boreal forests are the most widespread and represent the largest forest carbon pools (Pan et al., 2011). In tropical forests, aboveground and belowground carbon stocks are comparable, while boreal forests store carbon mainly in the soil, equivalent to the combined aboveground and belowground carbon stocks of tropical forests (Ameray et al., 2021). This is primarily due to the high rate of microbial decomposition in tropical forests and the slow microbial activities in boreal forests, limited by low temperatures and low nutrient concentrations (Malhi et al., 1999).

However, the future of carbon dynamics in boreal forests faces uncertainties due to global change. Rising temperatures and extended growing seasons are expected to enhance forest productivity (Gauthier et al., 2015) and increase carbon input into the soil. Additionally, higher atmospheric nitrogen deposition from human activities (Galloway et al., 2004) may reduce soil respiration by inhibiting microbial growth and altering microbial community composition (Janssens et al., 2010; Treseder, 2008; Zhang et al., 2018), potentially promoting soil carbon storage. Nevertheless, the accelerated microbial decomposition due to warming could increase CO_2 release from soil (Karhu et al., 2014; Treseder et al., 2016) and offset these gains. These changes may impact not only future carbon sequestration but also the current carbon stocks of boreal forests. Therefore, it is essential to understand the complex interplay between primary production, microbial decomposition, and nutrient availability to predict future carbon dynamics in these ecosystems.

Since boreal forests are nutrient limited, artificial nutrient addition can enhance both tree productivity and carbon storage. Increased nutrient availability from fertilization or deposition stimulates tree growth, leading to higher atmospheric carbon uptake, increased wood and litter formation, and greater carbon inputs into the soil (De Vries et al., 2009; Hyvönen et al., 2008; Jörgensen et al., 2022, Jörgensen et al., 2021; Maaroufi et al., 2015; Pregitzer et al., 2008). Additionally, a reduction in soil respiration following nitrogen addition has been observed, likely due to a decrease in microbial biomass or an alteration in microbial enzyme activity involved in organic matter (OM) decomposition (Craine et al., 2007; Janssens et al., 2010; Olsson et al., 2005; Zhang et al., 2018). Soil acidification resulting from nitrogen addition could also potentially contribute to carbon storage by negatively impacting microbial OM decomposition and soil respiration (Chen et al., 2016; Li et al., 2018). While numerous studies have investigated the impact of nutrient addition in forests, they often focus on short-term outcomes (Baldrian et al., 2022). However, since ecosystem responses are often nonlinear and change over time (Xing et al., 2022; Zheng et al., 2022), it is essential to conduct long-term studies that extend over the natural lifespan of forests, that is, at least several decades.

Despite the potential positive effects of nutrient addition on forest carbon storage, long-term artificial nutrient addition may disrupt the complex relationships between trees and soil microbial communities, potentially affecting soil carbon stocks. In boreal forests, conifer tree litter contains organic nitrogen forms inaccessible to plants (Korhonen et al., 2013). To overcome this limitation, plants establish symbiotic associations with mycorrhizal fungi, which release extracellular enzymes to extract organic nitrogen compounds and supply them to plants in exchange for carbohydrates (Chalot & Brun, 1998; Lindahl & Tunlid, 2015). These mycorrhizal fungi compete with free-living saprotrophs—major contributors to OM decomposition—for nutrients, slowing litter decomposition rates and potentially promoting long-term carbon sequestration (Gadgil & Gadgil, 1971; Orwin et al., 2011). By reducing the dependence of trees on mycorrhizal fungi for nutrient acquisition, fertilization could alter microbial community composition and decrease the competition between these two functional groups, leading to increased OM decomposition and limited carbon accumulation.

This study investigated the impact of long-term nutrient inputs on soil carbon storage in a nutrient-limited boreal forest located in Karstula, Finland (Figure 1a). Over six decades, nitrogen (N) and phosphorus (P) were repeatedly added to the forest (Figure 1b), along with calcium (Ca) to prevent potential soil acidification caused by nitrogen fertilization (Goulding, 2016). These nutrients were applied separately (N, P, Ca) or in combinations (NP, CaNP, CaN, CaP) and ecosystem responses were compared to unfertilized plots (CTRL). Our objectives were to confirm that (i) nutrient inputs increase tree productivity, and to elucidate (ii) whether this increase leads to soil carbon sequestration or (iii) whether shift in microbial community composition and stimulated OM decomposition limits soil carbon storage. Forest growth was monitored every 10 years from 1980 to 2015, with a final measurement in 2015, while geochemistry, microbial communities, biopolymer decomposition, and carbon stocks were analyzed in the topsoil after 62 years. To unravel the cascade effect of fertilization on contemporary carbon stocks, we employed structural equation modeling (SEM). We hypothesized that N addition would enhance tree productivity, increase the proportion of saprotrophs over mycorrhizal fungi, and promote OM decomposition.

2 | MATERIALS AND METHODS

2.1 | Study site and experimental design

The study site is a Scots pine (*Pinus sylvestris*) stand managed for 62 years, located in the municipality of Karstula, central Finland (62°54′43.343″ N; 24°34′16.021″ E). Understory vegetation was dominated by ericoid plants, including *Vacciunium vitis-idaea*, *Vaccinium myrtillus*, *Empetrum nigrum*, *Calluna vulgaris*, and abundances of herbs and grasses were very low. The soil texture at the

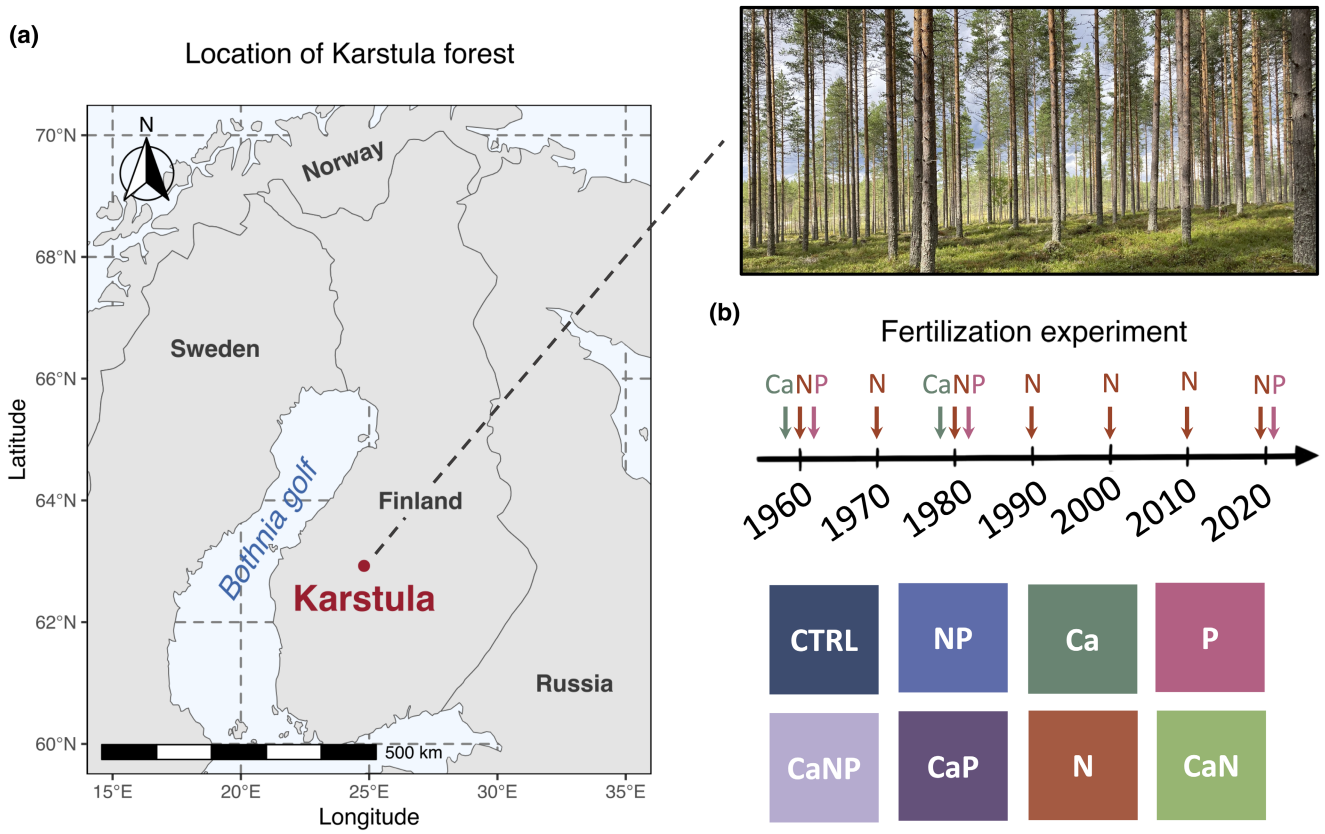

FIGURE 1 Study site location and experimental design. (a) The Karstula study site (62°54′43.343 ″N; 24°34′16.021 ″E) is a Scots pine (*Pinus sylvestris*) forest located in central Finland. (b) The factorial fertilization experiment was established in 1959 and continued until 2020. The experimental design comprised eight plots measuring 25 × 25 m, each corresponding to a treatment repeated in three sets (blocks). Plots were at least 10 m apart and randomized within each block and consisted of a control (CTRL, unfertilized stand), Ca, N, P, Ca + N, Ca + P, N + P, and Ca + N + P additions. Over the 62 years of the experiment, N, P and Ca were applied several times.

site was sandy loam. The mean temperature at 5 cm soil depth was 0.8°C in winter (November-April) and 10.3°C in summer (May–October), with a mean annual temperature of 5.9°C. The sandy soil was well-drained, with an annual mean volumetric soil water content of 0.28 m^3.

The factorial fertilization experiment was established in 1959 by the Finnish Forest Research Institute (now part of the Natural Resources Institute Finland). The site was composed of eight plots of 25 × 25 m size, each corresponding to a treatment repeated in three blocks ($n = 24$). The distance between plots was at least 10 m. Treatments were randomized within each set and consisted of control (CTRL, unfertilized stand), Ca, N, P, Ca + N, Ca + P, N + P, and Ca + N + P additions. During the experiment, quantities and forms of applied fertilizers varied according to recommended good practices. Between 1960 and 2020, N treatments were applied seven times, with each fertilized plot receiving a total of 1102 kg ha^{-1} of nitrogen over 60 years. The first application occurred in spring 1960, using ammonium sulfate (26% N) at a dose of 82 kg ha^{-1}. In 1970, urea (46% N) was applied at 120 kg ha^{-1}. Subsequent treatments in 1980 and 1990 used calcium nitrate (27% N, 6% Ca, 3% Mg) at 180 kg ha^{-1}. The final three applications in 2000, 2010, and 2020 used saltpeter (26% N, 3% Ca, 1% Mg, 0.02% B), with the dose maintained at 180 kg ha^{-1}. Ca addition (liming) was performed twice using 2000 kg ha^{-1} of dolomite powder in summer 1959 and 4000 kg ha^{-1} of dolomite in spring 1980. Phosphorus fertilizers were applied three times in summer 1959, spring 1980, and spring 2020. First, using 29 kg ha^{-1} of fine phosphate (Ca 36% and P 14%) once and then using 40 kg ha^{-1} of superphosphate (Ca 16% and P 20%) twice.

2.2 | Trees, ground vegetation, and litterfall survey

Trees were measured every 10 years from 1980 to 2010, with a final measurement in 2015. For each plot, the breast-height diameter (d1.3) of all trees was recorded. A permanent subset of sample trees, selected to represent different size categories, was measured for height using a hypsometer. The volume of these sample trees was calculated based on their breast-height diameter and height (Laasasenaho, 1982). These values were then used to estimate the height and volume of the remaining trees in each plot using a volume equation derived from the measured data. Tree characteristics were further analyzed with the KPL calculation program (Heinonen, 1994). Tree and root biomass were assessed using biomass models from Repola (2008, 2009). Tree and root growth were estimated by calculating the difference in biomass between consecutive measurements from 1980 to 2015.

2.3 | Soil sampling and chemical analyses

Soil sampling was conducted in September 2021 by collecting two composite samples per plot ($n=48$) up to the depth of 10 cm (including organic layer and topmost part of the mineral soil). Each composite sample consisted of five soil cores (diameter 4.5 cm) with a central core positioned 9 m along the plot diagonal from either the north or south corner of the plot and four additional cores located 2 m from the central core in all cardinal directions (Martinović et al., 2021). Soil was sieved through a 5-mm mesh to remove stones and roots. The sieved soil was mixed and about 45 mL of mixed soil was collected into a 50-mL plastic tube, freeze-dried, and conserved at −20°C. For ammonium (NH_4^+) and nitrate (NO_3^-) analyses, soil subsamples were transported to the laboratory at +4°C and immediately extracted with 1 M KCl. Extracts were centrifuged, filtered, and frozen at −20°C. Inorganic N was measured according to Hood-Nowotny et al. (2010). The assay for ammonium-N was based on a modified indophenol method and for nitrate-N on the reduction of nitrate by vanadium (III). Absorbance was measured with a microplate reader (Infinite M200, Tecan, Switzerland).

Dry mass content was measured after drying at 85°C, and pH was measured in distilled water (1:10, w:v). Total C and N content was measured using a CN analyzer (LECO, Michigan, USA). Available P was assessed in samples shaken with water (1:10) for 18 h, filtered through 0.22-µm membrane filter and measured with the malachite green method (Řezáčová et al., 2019). Carbon density was derived by multiplying carbon concentration by bulk density and subtracting the stone percentage. Bulk density and stone percentage were obtained from Saarsalmi et al. (2014). Samples for total nutrients (i.e., Al, Ca, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, P, Pb, S, Zn) were digested by the closed wet HNO_3-HCl digestion method in a microwave (CEM Mars 6) and the extract was analyzed by a iCAP 6500 DUO ICP-emission spectrometer (Thermo Scientific, United Kingdom) according to ISO 11466:1995(E). Furthermore, NH_4^+ and NO_3^- were quantified using a flow injection analyzer (Tecator 5042, Foss, Hillerød, Denmark), from soil samples collected in September 2022.

2.4 | DNA extraction, sequencing, and bioinformatic analysis

Total genomic DNA was extracted in triplicate from 0.25 g of freeze-dried soil using a modified Miller method (Sagova-Mareckova et al., 2008). DNA extracts were cleaned using the Geneclean Turbo Kit (MP Biomedicals) and pooled for PCR amplification. The fungal ITS2 region was amplified using gITS7 and ITS4 primers (Ihrmark et al., 2012) and the prokaryotic hypervariable V4 region of the 16S rRNA gene was amplified using 515F and 806R primers (Caporaso et al., 2011) with barcodes on both reverse and forward primers. After purification of the triplicate PCR products, a DNA sequencing library was produced using the TruSeq PCR-free Kit (Illumina) and sequenced by the MiSeq reagent kit v2 (Illumina) on an Illumina MiSeq instrument (Illumina, San Diego, CA, USA). Raw sequences were processed using the SEED2 v2.1.2 pipeline (Větrovský et al., 2018). Briefly, paired-end reads were merged using fastq-join (Aronesty, 2013), and sequences were filtered with a cut-off mean quality score of 30. The fungal ITS2 region was extracted using ITSx v1.0.11 (Bengtsson-Palme et al., 2013). Detected chimeric sequences were deleted, and the bacterial 16S rRNA gene or the fungal extracted ITS2 regions were clustered into operational taxonomic units (OTUs) at a 97% similarity level using UPARSE, a part of the USEARCH toolkit (Edgar, 2010). The most abundant sequence was selected as representative for each OTU, and their closest matches at the genus or species level were identified using BLASTn against Silva v138 (Quast et al., 2012) for bacteria or the Dynamic release of UNITE v9.0 (Nilsson et al., 2019) for fungi. Fungal taxa were further categorized into ecological groups based on Põlme et al. (2020). Sequences not classified as bacteria or fungi were removed from the dataset. The singletons were removed and the OTU tables were rarefied to a common sampling depth of 6834 sequences/sample for fungi and 9732 sequences/sample for bacteria using the function rarefy_even_depth() in *phyloseq* (McMurdie & Holmes, 2013).

2.5 | Quantification of microbial biomass and extracellular enzyme activities

Phospholipid fatty acids (PLFA) were extracted as described by Šnajdr et al. (2008) to estimate soil microbial biomass. Lipids were extracted using approximatively 1 g of dry soil with a mixture of chloroform:methanol:phosphate buffer (1:2:0.8) and separated by solid-phase extraction cartridges (HyperSep Silica SPE columns 200 mg/3 mL, Thermo Fisher Scientific). PLFA fractions were eluted using 2 mL of methanol. The chosen fractions were subjected to mild alkaline methanolysis, and the methyl esters of PLFA were analyzed using gas chromatography–mass spectrometry (GC–MS; 450-GC, 240-MS ion trap detector, Varian, Walnut Creek, CA). Fungal biomass in the polar PLFA fraction was quantified based on the content of 18:2ω6,9 (fungal PLFA), while bacterial biomass (total bacterial PLFA) was assessed by summing up i14:0, i15:0, a15:0, 16:1ω5, 16:1ω7, 16:1ω9, 10Me-16:0, i16:0, i17:0, a17:0, cy17:0, 17:0, 10Me-17:0, 18:1ω7, 10Me-18:0, cy19:0 (Actinobacteria 10Me-16:0, 10Me-17:0, 10Me-18:0, Gram-positive i14:0, i15:0, a15:0, i16:0, i17:0, a17:0, 10Me-16:0, 10Me-17:0, 10Me-18:0, and Gram-negative 16:1ω7, 16:1ω9, 18:1ω7, cy17:0, cy19:0).

Extracellular enzyme activities involved in biopolymer degradation were determined using enzyme assays following the method outlined by Štursová and Baldrian (2011). The activities of cellobiohydrolase, beta-galactosidase, beta-glucosidase, beta-xylosidase, lipase, N-acetylglucosaminidase (chitinase), alpha-glucosidase, and acid-phosphatase were assessed at pH 5.0 using 1:100 (w/v) sample slurries. Methylumbelliferone-based substrates were utilized, with an excitation wavelength of 355 nm and an emission wavelength of 460 nm. Quantification of enzyme products was conducted through standard curves encompassing a range of 4-methylumbelliferol concentrations within the same sample slurry. The manganese (Mn) peroxidase activity was assayed in soil extract following the product of the oxidative

coupling of 3–methyl–2–benzotiazolinon hydrazone and 3,3–dimetylaminobenzoic acid spectrophotometrically at 595 nm (Baldrian, 2009).

2.6 | Statistical analysis

All statistical analyses were conducted with R 4.2.2 (R Core Team, 2022). The Karstula forest location map was generated using the r *sf* package (Pebesma, 2018). To model the causal relationships between fertilization and carbon sequestration, we used SEM following the guidelines proposed in Grace et al. (2012). The first step of the SEM approach was to devise a metamodel (Figure S1), defined based on a priori theoretical knowledge and insights from the exploratory data analysis. In this metamodel, we considered that carbon accumulation to be mediated by drivers associated with forest productivity, biopolymer degradation, and microbial composition (Clemmensen et al., 2013). Variables were selected by respecting the ratio between the sample size and the number of free parameters (5 to 1, Bentler & Chou, 1987). As the experimentation was conducted within the same forest, our study model accounts for the main environmental parameters influencing tree productivity and biopolymer degradation (e.g., temperature, precipitation, solar radiation). Consequently, no environmental parameters were considered in the model.

We used root growth in the model because roots release carbon into the soil and influence microbial populations through rhizodeposition (Lynch & Whipps, 1991). We included the relative abundance of ectomycorrhizal (EcM) fungi in the model, because EcM plants dominate at this latitude (Soudzilovskaia et al., 2019). We used the relative abundance of the saprotroph and the EcM fungi based on metabarcoding data, since we were not able to discriminate them in the polar PLFA fraction. We implemented them separately because we predicted a negative effect of EcM fungi on saprotroph (Gadgil & Gadgil, 1971). In opposition, we used the Gram-positive (G+) to Gram-negative (G-) ratio since we had no hypothesis concerning a specific bacterial group, and predicted a positive influence of bacteria and saprotroph on biopolymer degradation (Mayer et al., 2021). Biopolymer degradations were estimated from seven extracellular enzyme activities (Mn peroxidase, alpha-glucosidase, beta-glucosidase, beta-xylosidase, beta-galactosidase, chitinase, and cellobiohydrolase), which were scaled and averaged to obtain a composite variable. We used this variable as a proxy of microbial decomposition and predicted a negative effect on carbon sequestration (Tao et al., 2023).

In the second step of the SEM approach, we used the maximum likelihood method to obtain a global estimation (Grace et al., 2012). Exploratory data analysis suggested the need to square-root transform (EcM fungi relative abundance) or ln-transform (G+:G− ratio, saprotroph relative abundance, carbon density) the response variables, to ensure linearity of relationships and the suitability of the global estimation method. Considering that the exogenous variable (predictor) was a categoric variable, the metamodel was fitted and tested using linear mixed-effects models with the function psem() in the R package *piecewisesem* (Lefcheck, 2016). As a result, the effect of fertilization on forest productivity, microbial composition and biopolymer degradation were tested by multiple regression, and the predicted values of fertilization were computed while holding the values of other variables at their mean. Marginal means represent the averages of these predictions, and particularly useful in that case because provide an estimated mean for each treatment. In addition, *piecewisesem* allowed to fit the model by incorporating a random effect blockID (number of parcels receiving the same treatment = 3) and replicate (number of replicates per parcel = 2). To determine the goodness of fit for piecewise structural equation models, we evaluated the conditional independence claims using the function dsep(). Next, we computed the Fisher's C statistic using the *p*-value obtained from the d-sep test (Fisher's C = 6.067, $p = .416$, df = 6), and computed the log-likelihood based χ^2 statistic ($\chi^2 = 9.875$, $p = .361$, df = 9). As Fisher's C statistic and χ^2 statistic fail to reject the model at *p*-value >.05, the final models were accepted.

Finally, we tested the impact of fertilization on soil chemistry, microbial biomass, and biopolymer degradation using linear mixed-effects models with the function lme() in the R package *nlme* (Pinheiro & Bates, 2000) using blockID as random effect. Variables were transformed prior the statistical test for non-normally distributed data. We compared carbon accumulation levels between unfertilized and fertilized plots by calculating the standardized effect size with the function escalc() in the R package *metafor* (Viechtbauer, 2010). Finally, five methods were implemented to identify fungal OTUs significantly enriched by nutrient addition (i.e., indicative species), including LEfSe, MetagenomeSeq, ALDEx, ANCOM, and ANCOMBC using the package *microbiomeMarker* (Cao et al., 2022). This analysis was performed on filtered data, and only OTUs with an abundance sum greater than 2% in more than 11% of the samples were kept. When appropriated, adapted microbial abundance normalization was applied (CPM for LEfSe, CSS for MetagenomeSeq, TSS for ANCOM). The p-values were adjusted using Holm method for ANCOM and ANCOMBC, and with the fdr method for ALDEx. The model ZIG was used for MetagonomeSeq and glm_anova for ALDEx. We then scrutinized fungal indicator species to identify those potentially influencing soil carbon stock (Hawkins et al., 2023). Specifically, we searched for lignin-oxidizing EcM fungi and melanized species (e.g., dark septate endophytes, *Cenococcum*), based on a literature review (Busk et al., 2017; Fernandez et al., 2016; Fernandez & Kennedy, 2018; Kohler et al., 2015; Miyauchi et al., 2020), searches on https://mycocosm.jgi.doe.gov and manual curation.

3 | RESULTS

3.1 | Fertilization of forest stands affects soil chemistry

The addition of nutrients significantly acidified the soil of CaN, N, and NP plots (pH ~4.7) compared to the unfertilized plots (pH 5.2) and increased soil water content in the CaN and NP plots

(Table S1). The C: N ratio remained similar between unfertilized and fertilized plots, as plots with elevated total nitrogen concentrations (i.e., CaNP, NP, N, and CaN) also exhibited higher carbon concentrations. Plots fertilized with CaN, NP, and CaNP exhibited higher ammonium concentrations, while nitrate concentrations were below the detection limit in all plots. Calcium concentrations were higher in CaNP plots than in unfertilized plots. Total phosphorus concentrations varied across treatments, with lowest concentration observed in CaN plots (200 mg/kg), approximately half of the concentration found in unfertilized plots (412 mg/kg). Available phosphorus was lowest in unfertilized plots and higher P fertilized plots (linear mixed-effects model, $p = .0015$), including CaP, CaNP, NP, and CaN plots. While plots fertilized with CaN and CaP exhibited lower aluminum and magnesium concentrations than the unfertilized plots, potassium concentrations were similar between fertilized and unfertilized plots (Table S1).

3.2 | Nitrogen input stimulates tree biomass and carbon storage

SEM was employed to investigate the relationship between long-term nutrient addition, tree productivity, and carbon sequestration, considering microbial communities and biopolymer degradation (Figure S1). The resulting refined model depicted a diagram of causal relationships comprising eight paths (Figure 2a), enhancing the likelihood of the metamodel. This refined model validated the predicted relationships between nitrogen fertilization, tree productivity, and carbon sequestration. Notably, treatments involving nitrogen (N, NP, CaNP, and CaN) resulted in greater root growth (Table S2). Within the final model, root growth along with the relative abundance of saprotrophic fungi accounted for 44% of the variability in carbon accumulation. Specifically, root growth (with a path coefficient of 0.54) emerged as a more important driver of carbon accumulation compared to the relative abundance of saprotrophic fungi (0.30, Figure 2a). This confirmed that nitrogen fertilization stimulated root growth, subsequently enhancing carbon input into the soil, and promoting carbon sequestration.

3.3 | Difference of carbon accumulation points to additional regulation

We conducted a comparative analysis to determine the nutrient combination maximizing forest productivity. Tree biomass (Figure 2b) and carbon accumulation (Figure 2c) clearly increased in plots fertilized with nitrogen (N, NP, CaNP, CaN) compared to plots fertilized without nitrogen (Ca, CaP, P) and unfertilized plots. Notably, nitrogen fertilization led to an average increase of 50% ± 10% in tree biomass between 1980 and 2015 (Table S3). Consistently, plots fertilized with nitrogen (N, CaNP, CaN, NP) showed an increase in carbon accumulation by 65% ± 24% compared to unfertilized plots after six decades. However, while the N, NP, CaNP, and CaN treatments significantly increased tree biomass by 40%, 44%, 44%, and 64%, respectively, the corresponding increase in carbon accumulation was 74%, 48%, 43% and 95% (Table S3). These results indicate that soil carbon stocks were not proportional to the increase in tree productivity. Carbon accumulation was only significantly higher than in unfertilized plots in the N and CaN plots (Figure 2d), indicating additional regulatory factors for carbon storage.

3.4 | Fertilization changes microbial community composition

Fertilization had no impact on bacterial biomass, which remained similar to unfertilized plots, except in P-fertilized plots where bacterial biomass was reduced (Figure S2a). However, fertilization altered the bacterial community composition, notably changing the proportions of G+ and G- bacteria (Figure S2b). The proportion of G+ increased relative to G- bacteria with the addition of P, CaN, N, and NP. Specifically, the relative abundance of Proteobacteria (G-) reached 35% in the P, Ca, CaP, and unfertilized plots; 33% in the CaN, N, and CaNP treatments; and 29% in the NP treatment (Figure 3a). Acidobacteriota (G-) followed a similar trend, prevailing in treatments without nitrogen (25% in P, Ca, CaP, and CTRL), decreasing to 17% in CaN, N, and CaNP treatments, and further to 13% in the NP treatment. In contrast, the relative abundance of Actinobacteria (G+) was approximately 18% in treatments without nitrogen, increased to 27% in the CaN, N, and CaNP treatments, and further to 38% in the NP treatment, consistent with their biomass variation (Figure S2c).

Unlike bacteria, fertilization impacted both the proportions of fungal functional groups and fungal abundance. Fungal biomass increased with the addition of CaN, N, and NP compared to unfertilized plots (Figure S2d). The relative abundances of saprotrophic and EcM fungi also changed due to fertilization. The proportion of saprotrophic fungi exceeded that of EcM fungi in the NP (2.09) and CaN (1.54) plots, was similar in the Ca (1.10) and P (0.96) plots, and was lower in the N (0.88), CTRL (0.84), CaP (0.70), and CaNP (0.69) plots (Figure 3b). Interestingly, a positive relationship between the relative abundance of saprotrophic fungi and carbon storage was found (Figure 2a). Specifically, plots with elevated carbon stock (e.g., CaN and NP) exhibited a high relative abundance of saprotrophs, while plots with low carbon stock (e.g., CTRL and P) had a low relative abundance of saprotrophs (Table S2).

3.5 | Specific fungal species positively responded to nitrogen addition

Of the 2718 fungal OTUs recovered in our study site, 87 were prevalent (i.e., indicative) in two treatments, and 299 were indicative of a single treatment (Table S4). Among the fungal indicative

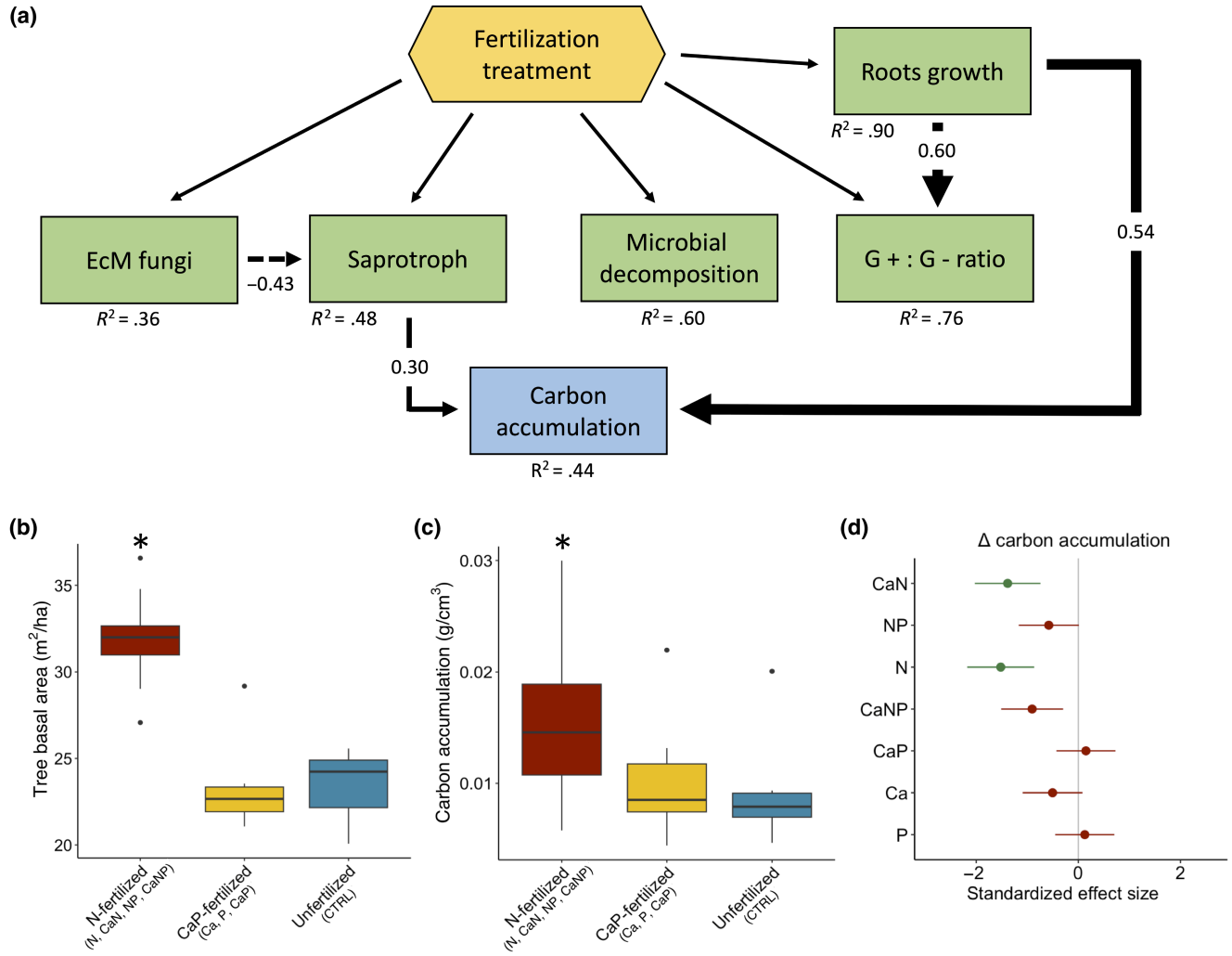

FIGURE 2 Impact of long-term nutrient input on tree productivity, microbial dynamics, and soil carbon sequestration. (a) Final accepted structural equation model (SEM) describing the effect of long-term fertilization on carbon accumulation (global fit parameters: $\chi^2 = 9.875$, $p = .361$, df = 9; Fisher's C = 6.067, $p = .416$, df = 6). The yellow and green frames indicate, respectively, distal and proximal drivers. The blue frame represents the response variable. The hexagons indicate exogenous variables and the rectangles indicate endogenous variables. Arrows represent the strength and direction of the relationships. Solid arrows indicate positive relationships, dashed arrows indicate negative ones, and their width is proportional to the strength of the effect size. Numbers adjacent to arrows are indicative of the standardized effect size of the relationship. The fertilization variable is a categoric variable, the estimated mean for roots growth, EcM fungi, saprotroph, bioplolymer degradation, and G+: G− ratio of each treatment are shown in Table S2. Biopolymer degradation represents a composite variable combining activity of seven extracellular enzymes (Mn peroxidase, alpha-glucosidase, beta-glucosidase, beta-xylosidase, beta-galactosidase, chitinase, and cellobiohydrolase). R^2 values denote the percentage of variance explained. (b) Variations in Scots pine biomass (tree basal area) and (c) carbon accumulation (carbon density) across nitrogen fertilized plots (N, CaN, NP, CaNP), calcium and phosphorus fertilized plots (Ca, P, CaP), and unfertilized plots (CTRL). Treatments marked with * are significantly different from CTRL, assessed via mixed-effects models accounting for the block design. (d) Difference in carbon accumulation between unfertilized plots and fertilized plots. Points represent standardized mean difference and error bars depict the standard errors. Positive values indicate lower carbon accumulation than in the unfertilized plot, and negative values denote higher carbon accumulation than in the unfertilized plot. Green and red dots indicate a significant and nonsignificant difference compared with unfertilized plots, respectively. Fertilization significantly increased carbon storage in CaN ($p = .031$) and N ($p = .020$) plots.

species, we identified 23 lignin-oxidizing EcM fungi, 41 molds, and 44 melanized fungi. Melanized fungi predominated in the CaN plots (Figure 3c), particularly *Cladophialophora* sp. (OTU0027), representing 5.5% of sequences in CaN (Table S4). Molds were prevalent in NP plots, specifically due to *Penicillium* sp. (OTU0004), accounting for 14.1% of the total fungal relative abundance in NP. Lignin-oxidizing EcM fungi (i.e., harboring class II peroxidases) dominated in nitrogen fertilized plots. In N plots, *Russula decolorans* (OTU0005), *Suillus variegatus* (OTU0046), and *Russula* sp. (OTU0021) represented 6.7%, 4%, and 2.9% of the total fungal relative abundance, respectively. *Russula* sp. (OTU0021) were also enriched in CaN plots, comprised 3.6% of the total fungal relative abundance. In CaNP, *Cortinarius* sp. (OTU0022) contributed 5.2% of the total fungal relative abundance (Figure 3c).

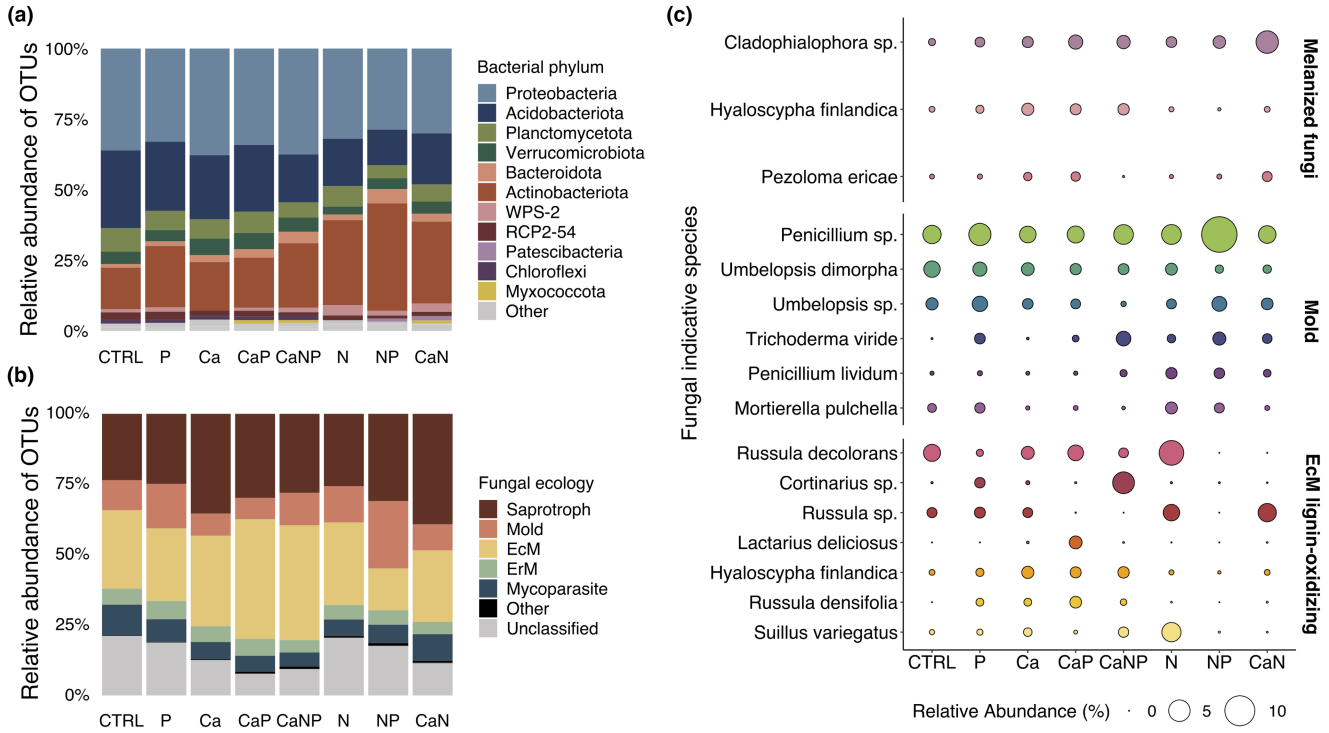

FIGURE 3 Response of microbial community to long-term nutrient addition. (a) Composition of bacterial communities in the treatments plotted at the phylum level. Bacterial phyla constituting less than 1% of the total are grouped as "Other." (b) Composition of fungal communities in the treatments categorized by their ecological groups. Fungal ecological groups comprising less than 1% of the total are grouped as "Other." (c) Relative abundance of melanized fungi, molds, and ectomycorrhizal (EcM) lignin-oxidizing indicative species with an average relative abundance >2%.

3.6 | Nitrogen input promotes biopolymer degradation

Nitrogen addition (CaN, NP, N, and CaNP) selected microbial communities with higher biopolymer degradation capabilities compared to unfertilized plots (Figure 4a). These microbial activities displayed similar patterns, except for Mn peroxidase activity (Figure 4b). Cellulose decomposition (beta-glucosidase) was approximately three times higher than hemicellulose (beta-xylosidase and beta-galactosidase) and fungal biomass decomposition (chitinase). Starch decomposition (alpha-glucosidase) and the hydrolysis of 1,4-β-D-glucosidic linkages in cellulose (cellobiohydrolase) were less pronounced (Figure S3). While phosphate solubilization (acid phosphatase) was generally low, it was nearly three times higher in NP plots than in unfertilized plots (Figure 4c) and positively correlated with phosphorus availability (linear mixed-effects model, $p = 0.0224$). Class II peroxidase (Mn peroxidase) activity was highest in plots fertilized with N and CaNP (Figure 4d), consistent with the increased proportion of lignin-oxidizing EcM fungi.

4 | DISCUSSION

In this study, we analyzed the response of a boreal forest to different fertilization combinations in order to quantify the effects of long-term nutrient addition on soil geochemistry, tree productivity, microbial dynamics, and soil carbon sequestration. Our results showed that N fertilization alone or in combination with Ca and/or P stimulated tree growth, resulting in a biomass increase of 50% ± 10% over just 35 years. This increase in tree productivity subsequently enhanced carbon input into the soil, promoting topsoil carbon accumulation by 65% ± 24% after six decades. This result aligns with previous findings demonstrating that N fertilization (Hyvönen et al., 2008; Jörgensen et al., 2021) and N deposition (De Vries et al., 2009; Maaroufi et al., 2015; Tipping et al., 2017) enhance the carbon stock in this ecosystem and confirm that nitrogen represents the primary limiting factor in boreal forests (Högberg et al., 2017). However, when N was applied alone or in combination with Ca and/or P, it also modified soil geochemistry, which significantly influenced microbial biomass, community composition, and OM decomposition. This had consequences on forest carbon dynamic, leading to differences in soil carbon sequestration between these treatments.

When P was co-applied with N, it significantly increased nutrient concentrations, stimulating tree productivity and root activity. This has probably led to greater inputs of OM in the form of root exudates and aboveground residues, expanding the pool of carbon sources available to microorganisms. The alleviation of nutrient limitations resulted in an increase in fungal biomass and favored copiotrophic microorganisms, such as saprotrophic fungi, *Penicillium* sp., and Actinobacteria. These microorganisms possess a wide range of hydrolytic enzymes, enabling to efficiently decompose complex

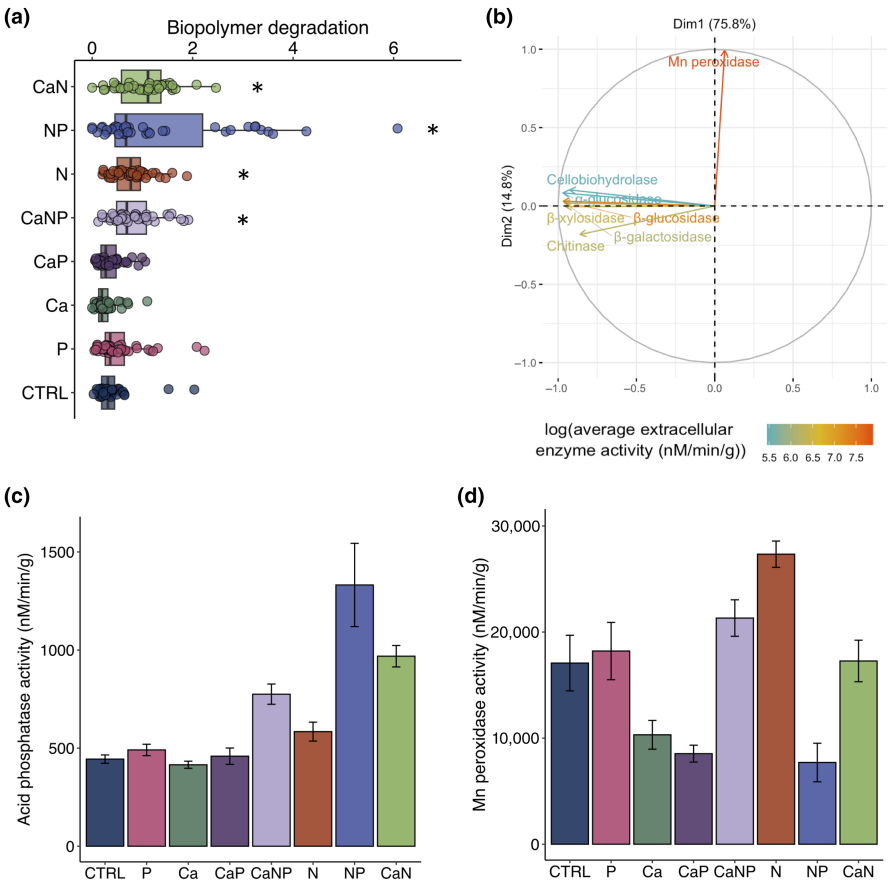

FIGURE 4 Biopolymer degradation and phosphate solubilization across treatments. (a) Biopolymer degradation across treatments, estimated from the activities of seven extracellular enzymes (Mn peroxidase, alpha-glucosidase, beta-glucosidase, beta-xylosidase, beta-galactosidase, chitinase, and cellobiohydrolase). These activities were scaled and averaged to obtain a composite variable, and * indicates treatments where biopolymer degradation was significantly higher from CTRL, assessed via mixed-effects models accounting for the block design. (b) Principal component analysis showing sample locations based on the activity of seven extracellular enzymes. Significant association of factors with the first PC axes is denoted as vectors. Colors represent the logarithm of the average extracellular enzyme activity. (c) Acid phosphatase activity and (d) Mn peroxidase activity in treatments ($n = 6$).

OM (Baldrian & Valášková, 2008; Eichlerová et al., 2015; Morin et al., 2012), energy-rich fresh litter (Baldrian et al., 2011; Picart et al., 2007), plant material (Bao et al., 2021; Richy et al., 2024) as well as wood residues (Tláskal & Baldrian, 2021). This shift in community composition certainly explains the elevated biopolymer degradation and the limited soil carbon stock observed in NP plots. Although these results do not fully align with previous findings, this discrepancy is probably due to differences between the length of the studies. Meta-analyses have shown that nitrogen addition generally reduces fungal biomass and microbial activity, thereby increasing soil carbon storage (Janssens et al., 2010; Zhang et al., 2018). However, these conclusions are often based on studies of less than 10-year duration, whereas the beneficial effects of fertilization on microbial biomass might only become evident after two decades or more (Geisseler & Scow, 2014). This underscores the intricate relationships between nutrient addition, microbial dynamics, and carbon storage, highlighting the need for long-term studies.

While we initially suspected that nutrient addition would reduce plant investment in fine roots and mycorrhizal fungi (Treseder, 2004), it was in the plots fertilized with CaNP that we observed the highest proportion of EcM fungi. Moreover, no evidence of competition between EcM and saprotrophic fungi increasing carbon accumulation was found. We found in plots dominated by saprotrophs higher carbon stocks than in plots dominated by EcM fungi. However, the CaNP plots exhibited a significant proportion of *Cortinarius* sp., an EcM fungal species likely limiting soil carbon storage in boreal forests (Bödeker et al., 2014; Clemmensen et al., 2015; Lindahl et al., 2021). These *Cortinarius* species are among the EcM fungi that have retained oxidative capacities (i.e., class II peroxidase genes) inherited from their white-rot saprotrophic ancestors (Miyauchi et al., 2020), allowing them to oxidize and decompose phenolic macromolecules such as lignin and humic acids. By degrading recalcitrant biopolymers, lignin-oxidizing EcM fungi release carbon that was previously locked in this complex OM into simpler organic molecules, which can then be mineralized into CO_2 by soil microorganisms and increase soil respiration. Since the degradation of recalcitrant OM is an energy-intensive process, EcM fungi benefit from the carbohydrates provided by trees, giving them a competitive advantage over saprotrophs that rely solely on OM decomposition. Taking together, this suggests that the presence of key functional groups is responsible for the limited carbon stock in NP and CaNP plots.

The two treatments exhibiting the highest carbon stock were the CaN and N plots. We found in CaN plots a high proportion of melanized fungi, which have been previously identified as positive contributors to soil carbon storage (Clemmensen et al., 2015). Some mycorrhizal fungi produce melanized mycelium, composed of phenolic (or indolic) monomers, which form complex, large, and irregular macromolecular structures. These compounds lead to a higher proportion of necromass resistant to decomposition, which, being preserved in humus, promotes carbon storage (Fernandez & Kennedy, 2018). However, since *Cladophialophora* sp. is a dark septate endophyte saprotroph that remains poorly studied in natural

environments (Chang et al., 2023), concluding that *Cladophialophora* melanization has a positive feedback on carbon accumulation at Karstula is challenging. Nonetheless, soil acidification due to nutrient addition offered an alternative explanation for the elevated carbon stock. Nitrogen addition induces in soil acidification by promoting the leaching of base cations and increasing the concentration of acid-producing ions such as aluminum (Tian & Niu, 2015). At intermediate pH levels (4.5–7.5), this acidification process is primarily buffered by base cations such as calcium, magnesium, and potassium, which can result in nutrient deficiencies and increased aluminum toxicity. Although potassium and calcium concentrations were not affected by fertilization, the CaN plots exhibited a significant decrease in magnesium and aluminum compared to unfertilized plots. Therefore, it is unlikely that aluminum toxicity affected microbial activity, but the reduction in magnesium concentrations may have limited microbial processes, potentially contributing to the increased soil carbon storage in CaN plots.

The common factor in the CaN and N treatments was the addition of nitrogen without phosphorus, which appears to be the critical determinant for long-term carbon sequestration. Nitrogen addition alone or with Ca significantly increased both tree biomass and soil carbon storage. In contrast, when P was added alone or with Ca, tree biomass remained similar to unfertilized plots, but soil carbon accumulation was lower (Figure 2d), indicating faster OM decomposition in CaP and P plots. These results are in agreement with previous findings showing that phosphorus is not limiting for tree growth in boreal forests (Du et al., 2020), and suggest that P addition stimulates microbial degradation, thereby limiting long-term carbon sequestration. This pattern can be explained by the different C:N:P ratios between trees and microorganisms. Scots pine has a typical C:N:P ratio of 900-10-1 (Skonieczna et al., 2014), while it is about 60-7-1 in soil microbial biomass (Cleveland & Liptzin, 2007), meaning that tree need less phosphorus than microorganisms per carbon unit for biomass production. This is because trees invest resources in structural components (i.e., wood), which have a lower phosphorus content than microbial biomass, while microorganisms have high surface-to-volume ratios and rapid growth rates, requiring more phosphorus-rich cellular components for biomass synthesis. Hence, we propose that nitrogen addition promotes tree growth which increases carbon input into the soil, but as microbial activity is limited by phosphorus availability, this leads to long-term soil carbon sequestration. By mediating the effects of nitrogen input, phosphorus limitation seems to play a positive role on soil carbon storage in boreal forests.

At a global scale, forests play a crucial role in climate regulation by sequestering atmospheric CO_2. The optimization of forest productivity has the potential to convert a significant portion of the CO_2 emitted by anthropogenic activities into soil carbon stocks and mitigate global warming. We show that N input holds promise for increasing carbon stocks in both tree biomass and boreal forest soils. While implementing a method similar to our study on a large spatial scale pose economic and ecological challenges, analogous outcomes could be expected following long-term nitrogen deposition. Notably, models have consistently demonstrated that N deposition tends to enhance carbon stock in the tree biomass of nitrogen-limited boreal forests (Schulte-Uebbing et al., 2022). Furthermore, our results underscore that N addition increases soil carbon sequestration in boreal forests over time. Consequently, we recommend integrating the soil phosphorus concentrations alongside nitrogen deposition quantities (Maaroufi et al., 2015) to refine predictive models of forest carbon balance. Such comprehensive modeling approaches will offer a better insight into the complex dynamics of carbon sequestration in forest ecosystems.

AUTHOR CONTRIBUTIONS

Etienne Richy: Conceptualization; data curation; formal analysis; investigation; methodology; visualization; writing – original draft; writing – review and editing. **Tania Fort:** Data curation; formal analysis; writing – review and editing. **Inaki Odriozola:** Formal analysis; methodology; writing – review and editing. **Petr Kohout:** Investigation; writing – review and editing. **Florian Barbi:** Investigation; writing – review and editing. **Tijana Martinovic:** Validation; writing – review and editing. **Boris Tupek:** Formal analysis; writing – review and editing. **Bartosz Adamczyk:** Formal analysis; writing – review and editing. **Aleksi Lehtonen:** Data curation; funding acquisition; writing – review and editing. **Raisa Mäkipää:** Data curation; funding acquisition; writing – review and editing. **Petr Baldrian:** Conceptualization; funding acquisition; methodology; project administration; supervision; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The sequence data that support the findings of this study are openly available from NCBI under project number PRJNA1111134. The data that support the findings of this study are openly available in Zenodo at https://doi.org/10.5281/zenodo.13734692. The R scripts for structural equation modeling and fungal indicator species analysis are also openly available in Zenodo at https://doi.org/10.5281/zenodo.13742776.

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SUPPORTING INFORMATION

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