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Plant-Soil Properties Associated with Nitrogen Mineralization: Effect of Conversion of Natural Secondary Forests to Larch Plantations in a Headwater Catchment in Northeast China

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Abstract: To understand the relative importance of plant community composition and plant-induced soil properties on N transformations, the soil N mineralization, ammonification and nitrification characteristics of natural secondary forests (*Quercus mongolica-Juglans mandshurica* forest: QJF, and *Quercus mongolica-Populus davidiana* forest: QPF) and the adjacent larch plantations (*Larix kaempferi* forest*:* LF1 and LF2) were studied during the growing season. All of the forest types showed seasonal dynamics of N mineralization rates. The total cumulative N mineralization was significantly higher in QPF (73.51 kg hm^{-2}) than in LF1 (65.64 kg hm^{-2}) and LF2 (67.51 kg hm^{-2}) ($p < 0.05$). The total cumulative nitrification from May to November was significantly higher in QJF (65.16 kg hm^{-2}) and QPF (64.87 kg hm^{-2}) than in LF1 (52.62 kg hm^{-2}) and FL2 (54.17 kg hm^{-2}) ($p < 0.05$). Based on the variation partitioning, independent soil properties were the primary determinants of the N transformations (13.5%). Independent climate conditions explained 5.6% of the variations, while plant variations explained 3.2% of the variations in N transformations. We concluded that different forest types with various plant community compositions have different influences on the litterfall quantity and quality and the nutrient availability, and these differences interact with seasonal climate conditions that in turn drive the differences in N mineralization.

Keywords: soil nitrogen mineralization; plant-soil interactions; resin core method; forest conversion; headwater catchment

1. Introduction

Nitrogen (N) is an essential element for the growth of organisms and the productivity of forest ecosystems [1]. In headwater catchments, soil N mineralization of organic matter plays an important role in determining soil N availability, primary productivity and N losses from soil to stream, thus contributing to ground water contamination and the pollution of the water environment [1–4]. Factors affecting the temporal and spatial patterns of soil N dynamics have been well documented [5–7]. Numerous previous studies and practices have shown that seasonal changes in N mineralization result in patterns with the highest mineralization rates in the summer and the lowest rates in the winter, which appears to follow seasonal patterns of temperature and precipitation [8–11]. Soil temperature, moisture, and precipitation patterns are important drivers of soil N transformations, and each of these seasonal climate conditions may have different impacts on various forest types [12]. Forest types with varying plant communities may have different influences on the N cycle due to differences in the physiology, morphology, nutrient requirements, and life histories of various plant species [13–15].

There are several approaches of studying the N mineralization for different forest types, but they generally do not consider plant community compositions and species diversity. Conversion of natural forests to plantations often leads to considerable losses of plant species and consequently a reduction in the diversity of litter species compositions and the amount of litter production, which affects soil nutrient availability and N transformation [16]. Larch (*Larix kaempferi*) plantations are the most widespread forests in northeastern China, but their ecological impacts receive little attention. Therefore, understanding the mechanisms underlying the effects of natural forests converted into plantations on N mineralization of organic matter in headwater catchments is useful for forest management and structure regulation, and can thus help minimize N exports to aquatic ecosystems.

There are three dominant processes between plant communities and soil properties that could explain the mechanisms underlying the effects of natural forests converted into plantations on N mineralization of organic matter. First, changes in plant community composition could influence soil N mineralization via affecting soil nutrient availability, e.g., total nitrogen (TN), soil organic carbon (SOC), C:N ratio, and dissolved organic carbon (DOC), since tree species exhibit differences in the quality of plant material and chemical compounds which significantly affect organic matter input and decomposition. Grime (1998) found that a community dominated by plants with high nitrogen concentrations would likely have positive effects on N mineralization rates [17]. Second, productivity could also influence N mineralization because approximately 50–60% of plant-assimilated N in deciduous forest is annually returned to the soil via litterfall [18]. Denton (1999) and Mikola (2000) found that greater inputs of plant material could increase N mineralization rates because soil microbial biomass and activity have been shown to respond to increased nitrogen and carbon resources [19,20]. Third, plant diversity could also affect N mineralization rates because a more diverse array of plant material entering the soil through leaf litter, fine root production and root turnover could affect N mineralization rates by providing a consistent long-term supply of organic nitrogen as the qualities of plant material decomposed at various rates [21]. These three attributes of the interactions between plant communities and soil processes may simultaneously affect N mineralization. Temperature and precipitation changes are likely to influence N mineralization by altering factors like those discussed above. For example, an increase in temperature can enhance microbial activities and increase the rates of litter decomposition, which, as a result, can change the N mineralization rate. Different plant community compositions have different substrate inputs, soil chemistry and microbial activity, and such differences may contribute unequally to the soil N mineralization; therefore, the N mineralization processes in different forest types are likely to respond differently to seasonal changes in temperature and precipitation.

To understand the relative importance of plant community composition and plant-induced soil property effects of forest conversion on N transformation patterns, organic N mineralization, nitrification and ammonification were investigated in natural secondary forests and the adjacent larch plantations in the headwater catchment of the Taizi River in China. This study aimed to (1) investigate and compare seasonal N mineralization rates under field conditions in natural secondary forests and the adjacent larch plantations; and (2) assess the extent to which N mineralization rates could be explained by the plant-soil properties that are associated with plant community compositions and seasonal climate conditions in temperature and precipitation.

2. Materials and Methods

2.1. Study Area

The Laotudingzi National Nature Reserve (124°41′13″–125°05′15″ E; 41°11′11″–41°21′34″ N) is situated in the headwater catchment of the Taizi River in Liaoning Province, China. The area has a temperate monsoon climate, with mean annual temperature of 6.2 °C and a mean annual rainfall of 778 mm, of which 60–65% falls between June and August. During the study period, the temperature and precipitation largely followed this long-term seasonal pattern. Due to the cold weather during the

long winter in Northeast China, soil freezing occurred from November to early April. The average growing season is approximately 215 frost-free days. The air temperature and precipitation from January to December 2014 was measured at a weather station close to the experimental site (Figure 1).

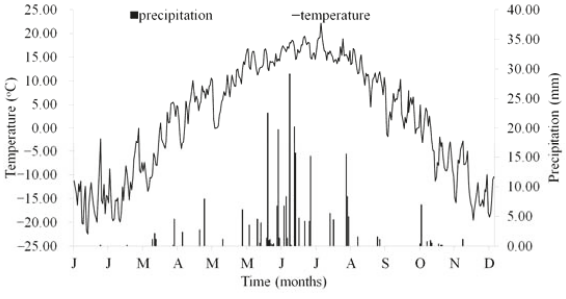

Figure 1. Seasonal dynamics of air temperature and precipitation during 1 year from January to December 2014 in Laotudingzi National Nature Reserve.

The study area had been primarily covered by broadleaf Korean pine forests until the 1930s and thereafter subjected to unregulated timber removal for decades. Massive controlled burns where used in the early 1950s for clearing out the original forest. Since then, the study site has been progressively covered by a naturally regenerated secondary forest. The natural secondary forests consisted of *Quercus mongolica*, *Juglans mandshurica*, *Populus davidiana*, *Acer mono Maxim*, *Phellodendron amurense*, *Fraxinus mandshurica*, *Pinus koraiensis*, *Betula platyphylla* and *Tilia amurensis*. At the beginning of the forest succession, some patches of the natural secondary forests were cleared and replaced by 3-year-old larch (*Larix kaempferi*) seedlings. The larch plantations contain *Larix kaempferi*, *Phellodendron amurense*, *Quercus mongolica*, *Juglans mandshurica* and *Fraxinus mandshurica*.

In this study, two larch plantations sites (LF1 and LF2) and two natural secondary forests (QJF and QPF) were selected. Three independently fixed 20 × 20 m plots were randomly selected at each site. The soil is a typical brown forest soil (classified as Udalfs according to the second edition of USDA soil taxonomy) and with depth 20 to 40 cm. Detailed data on the stands, plots and samples are given in Table 1.

Table 1. Main characteristics of LF1, LF2, QJF and QPF stands.

Item	LF1	LF2	QJF	QPF
Representative plants	*Larix kaempferi*	*Larix kaempferi*	*Quercus mongolica* *Juglans mandshurica*	*Quercus mongolica* *Populus davidiana*
Slope (°)	18	18	16	18
Elevation (m)	656	684	672	663
Forest age (a)	43	43	43	46
No. of tree species	3	8	12	10
Tree density (stems/hm^2)	150	410	383	312
Canopy density	0.6	0.8	0.9	0.9
Diameter at breast height (cm)	24.57 ± 5.98	24.82 ± 3.74	24.53 ± 9.73	25.37 ± 8.00
Tree height (m)	20.16 ± 2.44	20.22 ± 1.53	19.00 ± 3.56	19.88 ± 3.90

2.2. Variables Assessed

2.2.1. Vegetation Survey

All individual trees ≥1 cm in diameter at breast height (DBH) were tallied and recorded by species name, tree height, DBH and canopy density at each plot. Within each plot three sub-plots of 1 × 1 m were laid for herbs, and the number of species, number of individuals per species and

coverage in the three sub-plots were recorded. In each sub-plot, forest floor litter, including leaf litter, senesced branches, and bark were collected. We divided the litter layer into two sub-layers: the L layer (undecomposed litter, consisting of litter with clear recognizable structure lying loosely on the forest floor surface) and the F/H layer (mixture of partly decomposed litters where plant remains are partially decomposed by biological activity but with plant morphology still recognizable and humus without recognizable plant structures and a fine granular morphology) [22]. After litter materials were collected, the above and below ground parts of all herbs were harvested through destructive sampling from these sub-plots. To account for the annual litterfall, three samples were randomly collected in each plot every month using a 0.5 m^2 litterfall traps from May to November. Then, all samples were weighed and oven-dried at 65 °C to measure the dry mass and evaporated water content.

The Shannon Weaver Diversity Index was used to measure species diversity [23], and Margalef's Index was used to estimate species richness [24].

Shannon Weaver Diversity Index (H'):

$$\exp(H') = \sum_{i=1}^{R} p_i \ln p_i \quad (1)$$

where pi = species proportion, R = total number of species types.

Margalef's Index (MI):

$$MI = \frac{(s-1)}{\ln N} \quad (2)$$

where S = total number of species, N = total number of individuals.

We estimated the biomass of foliage, branches, stems, roots and total tree using an allometric equation relating each biomass component to the diameter at breast height (DBH), respectively. This allometric equation established by Wang (2006) [25].

$$\log_{10}^{B} = a + b(\log_{10}^{DBH}) \quad (3)$$

where B is biomass component, a and b are regression coefficients. The total biomass for trees in each plot was calculated by the biomass of total tree species in the plots.

2.2.2. Soil Sampling and Incubation

The experiment was conducted in May to November 2014 using a modified resin core technique in situ [26–29], similar to the methods of Raison (1987) [30] and Hübner (1991) [31]. At every experimental site, five sampling points were randomly allocated to the replication plots on the first sampling date of 18 May. After the litter and above-ground vegetation was removed, a PVC tube (12 cm long, 5 cm diameter) sharpened in advance was driven 10 cm into the ground to collect the soil core, which was used to determine the initial NO_3^--N, NH_4^+-N and mineral N (NH_4^+-N and NO_3^--N) concentrations and other soil properties; another identical tube was driven 12 cm into the ground to confine the soil core, with soil structure undamaged, and then the bottom 2 cm of soil was removed, and a resin bag with 10 g anion and cation exchange resin beads (717# and 732# produced by the Huizhi resin Plant of Shanghai) tied into a nylon stocking was placed in the bottom of the PVC tube. The PVC tube containing the soil core and resin was inserted back into its original position and then incubated in situ for one month. At the end of the incubation period, the soil core and resin were removed to determine NO_3^--N, NH_4^+-N and mineral N (NH_4^+-N and NO_3^--N) concentrations. This was repeated until the experiment ended on 16 November.

2.2.3. Soil Chemical Properties

The collected tubes and soil samples were stored and extracted within 24 h. The resin bags were washed with distilled water and air-dried. The soil samples were homogenized and sieved through a 2-mm screen. For the determination of the soil mineral N (NH_4^+-N and NO_3^--N), the soil was

shaken with 2 M KCl for 1 h on a 250-rpm shaking table [32]. For the determination of resin NH_4^+-N and NO_3^--N, the resin bags were shaken with 2 M KCl for 12 h on a 250-rpm shaking table and, then the suspension was filtered. The concentrations of NH_4^+-N and NO_3^--N in the extracts were determined by colorimetric methods using a segmented flow injection analyser (Skalar Autoanalyzer SAN^{++}, The Netherlands). The soil microbial biomass C (MBC) and soil microbial biomass N (MBN) were determined by a chloroform fumigation-extraction method [33,34]. Extracts from the fumigated and unfumigated soils (25 g fresh soil) were taken with 50 mL 0.5 M L^{-1} K_2SO_4 for 30 min and filtered. The organic C and N concentrations in the extracts were measured by a dichromate oxidation method and a $K_2S_2O_8$ oxidation method, respectively. MBC and MBN were calculated from the differences in the K_2SO_4-extractable C or N concentration between the fumigated and unfumigated soils divided by the efficiency factors for MBC or MBN (K_C = 0.38; K_N = 0.45, respectively). K_2SO_4-extractable DOC value was also used as a proxy for the soil available DOC concentration [35]. The soil samples were air-dried and then used for analyses of the soil pH, TN and SOC. The soil pH was determined with a glass electrode (water:soil = 2.5:1). The SOC was analysed using the H_2SO_4-$K_2Cr_2O_7$ oxidation rapid titration method. The TN was measured by the Kjeldahl acid-digestion method. The soil bulk density was measured using the core method. The soil temperature was measured with a thermometer inserted in the soil to a depth of 10 cm.

Nitrogen mineralized was calculated as follows [32]:

$$N_{nit} = [NO_3^- - N]_{sc} + [NO_3^- - N]_{res} - [NO_3^- - N]_{in} \quad (4)$$

$$N_{amm} = [NH_4^+ - N]_{sc} + [NH_4^+ - N]_{res} - [NH_4^+ - N]_{in} \quad (5)$$

$$N_{\min} = N_{nit} + N_{amm} \quad (6)$$

where N_{nit}, N_{amm}, and $N_{\min}$ are the net nitrification, ammonification and N mineralization, respectively; $[NO_3^-\text{-}N]_{sc}$ and $[NO_3^-\text{-}N]_{in}$ are the mean concentrations of nitrate N in soil core at the end and beginning of each one month incubation period, respectively; $[NH_4^+\text{-}N]_{sc}$ and $[NH_4^+\text{-}N]_{in}$ are the mean concentrations of ammonium N at the end and beginning of incubation period in soil core, respectively; $[NO_3^-\text{-}N]_{res}$ and $[NH_4^+\text{-}N]_{res}$ are the mean concentrations of NH_4^+-N and NO_3^--N in the resin at the end of the incubation period.

2.3. Statistical Analysis

One way analysis of variance (ANOVA) was performed to test the significance of differences in the forest cover characteristics and soil properties, and the least significant difference values (LSD) were calculated at a significance level of $p < 0.05$. The N availability and N mineralization data were tested using the repeated measures analysis of variance (RM-ANOVA). The forest types served as between-subject factors, and months were within-subject factors. The Pearson correlation was used to examine the correlations of mineralization rates with the plant community composition. All statistical analyses were performed with SPSS 14.0 (SPSS Inc., Chicago, IL, USA).

Redundancy analysis (RDA) was used to explore the association of N transformations with environmental properties, using the net organic N mineralization, ammonification and nitrification rates as response variables and the environmental factors as explanatory variables. Before the actual analyses, soil N transformations data were analysed using detrended correspondence analysis (DCA) to determine whether linear or unimodal methods would be appropriate in the analyses [36]. As the eigenvalue was 0.111, redundancy analysis (RDA) was applied. We split the environmental dataset into three groups: climate, plant, and soil variables. In order to avoid high multicollinearity in the following analytical steps we removed within-group correlations of $|r| > 0.7$ (Spearman, $p < 0.05$) by exclusion of variables. By this procedure, selected variables as well as all uncorrelated variables were included in the final matrices. Climate variables include precipitation and temperature; plant variables include biomass of foliage, stems for trees, litter mass for F+H layer, annual litterfall, H' for tree layer

and *H′* for herb layer; soil variables include SOC, DOC, MBC and soil pH. In order to assess the contribution of the three variable groups on N transformation, the variation partitioning method led to the identification of six fractions: independent soil (a), climate (b), plant (c), and joint effects of soil and climate (ab), soil and plant (ac), climate and plant (ac). All multivariate analyses were performed with CANOCO 4.5 [37].

3. Results

3.1. Seasonal Soil Mineral N

NO_3^--N, NH_4^+-N and mineral N contents and dynamics are shown in Figure 2. Generally, the NO_3^--N and mineral N contents in the QJF and QPF plots were higher than those in the LF1 and LF2 plots, and NH_4^+-N contents in the LF2 plots were higher than the QJF plots (Table 2, Figure 2). The mean NO_3^--N contents across the month were 8.87, 7.78, 10.73 and 12.46 mg kg^{-1} in LF1, LF2, QJF and QPF, respectively. The mean NH_4^+-N contents for the LF1, LF2, QJF and QPF were 5.37, 5.73, 4.01 and 5.25 mg kg^{-1}, respectively, and the mean mineral N contents were 14.1, 13.51, 14.74 and 17.71 mg kg^{-1}, respectively. The concentrations of both mineral N and NO_3^--N displayed a distinct seasonal pattern, with generally high concentrations in June and July and low concentrations in August and September (Figure 2). In LF1 and LF2 the maximum soil NH_4^+-N concentration occurred in May, with a second peak value in August. In QJF and QPF, soil NH_4^+-N concentration was higher in May than other months. The ratio of NO_3^--N to NH_4^+-N was approximately 1.79:1 during the growing season, so NO_3^--N was the dominant form of mineral N.

Table 2. Summary of repeated measures ANOVA results on the nitrate N, ammonium N, mineral N, net nitrification rate (N_{nit}), net ammonium rate (N_{amm}) and net mineralization rate (N_{min}).

Factor	NO_3^--N	NH_4^+-N	Mineral N	N_{nit}	N_{amm}	N_{min}
Forest types	54.44 ***	30.31 **	37.54 ***	14.57 **	16.86 **	4.03
Months	94.10 **	263.11 ***	81.71 **	56.26 **	164.12 ***	88.80 ***
Forest types × Months	2.35	15.65 ***	3.54 **	1.02	10.30 ***	1.08

** $p < 0.01$, *** $p < 0.001$.

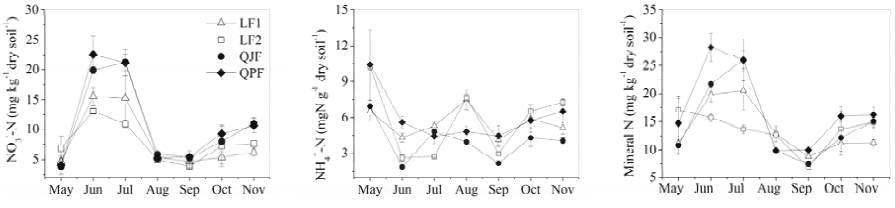

Figure 2. Dynamics of NO_3^--N (**a**) and NH_4^+-N (**b**) and inorganic N (**c**) concentrations in LF1, LF2, QJF and QPF (means ± SE, $n = 3$).

3.2. Net N Mineralization of Organic Matter

The net nitrification rates were significantly different among the months and forest types, but were not different in forest types × months interaction (Table 2). The net nitrification displayed strong seasonal dynamics, with highest rate during the period from June to July and the lowest rate during the period from October to November in LF2 plots (Table 2, Figure 3). In the LF1, QJF and QPF plots, the highest values of net nitrification were observed in the period from May to June. Furthermore, the net N nitrification rates of the QJF and QPF plots were significantly higher than LF2 plots from May

to June, and the QPF plots were significantly higher than LF1 from September to October. The mean net N nitrification rates were 8.77, 9.03, 10.86 and 10.81 mg kg^{-1} $month^{-1}$ for LF1, LF2 QJF and QPF, respectively.

The net ammonification rates were significantly different among the months and forest types, and their interactions (Table 2). The net ammonification rate was significantly lower than the rates of nitrification in all of the forest types ($p < 0.01$, Figure 3). In LF1, LF2 and QPF, the net N ammonification rate significantly increased during the periods of May to August and decreased during August to September. In QJF the net N ammonification rate significantly increased during the periods of May to June and decreased during July to September. The mean net N ammonification rates were 2.17, 1.82, 1.13 and 1.44 mg kg^{-1} $month^{-1}$ for LF1, LF2, QJF and QPF, respectively.

The mean net organic N mineralization rates were 10.94, 11.05, 11.25 and 12.25 mg kg^{-1} $month^{-1}$ for LF1, LF2, QJF and QPF, respectively. The net N mineralization displayed strong seasonal dynamics; the temporal variations in the net N mineralization rates were similar to those observed for net nitrification, with the highest rates during the period from June to July and the lowest rates during the period from August to September in all the plots (Figure 3). The net N mineralization rates of the LF1, QJF and QPF plots were significantly higher than LF2 plots from May to June, and the LF2 plots were significantly higher than QJF and QPF from July to August, and the LF2 plots were significantly higher than LF1 from September to October.

The total cumulative nitrification from May to November was significantly higher in QJF and QPF than in LF1 and FL2 ($p < 0.05$, Figure 4a). However, the total cumulative ammonification was lower in QJF and QPF than in LF1 and FL2 ($p < 0.05$, Figure 4b). The total cumulative organic N mineralization was significantly higher in QPF than in LF1 and FL2 ($p < 0.05$, Figure 4c), but QJF was not different with other forest types.

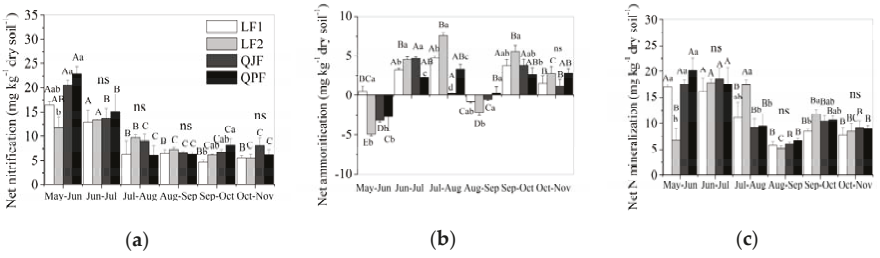

Figure 3. Rates of nitrification (**a**), ammonification (**b**) and net N mineralization (**c**) at one-month intervals (means ± SE, $n = 3$). Different lowercase letters indicate significant differences among forest types; different uppercase letters indicate significant differences among months (adjusted $p < 0.05$).

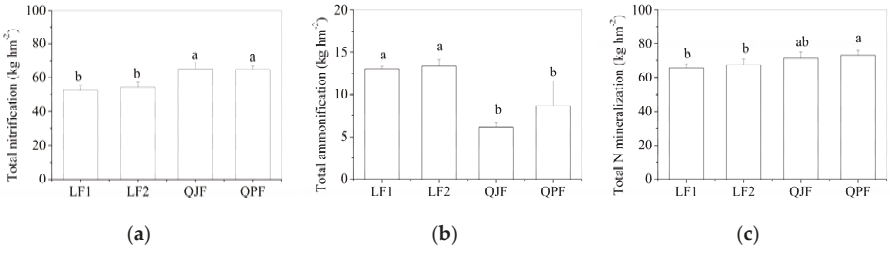

Figure 4. Differences of soil total cumulative nitrification (**a**), ammonification (**b**) and N mineralization (**c**) in various forest types from May to November. Different letters indicate significant differences among forest types (adjusted $p < 0.05$, $n = 3$).

3.3. Plant and Soil Properties

Total biomass estimates of trees in LF2, QJF and QPF were statistically higher than LF1 (Table 3, $p < 0.05$). The biomass recorded in leaves, branches and roots were higher in the QJF than the LF1. The total biomass and below ground biomass for herb layer was not statistically different among forest types (Table 3). Forest floor litter total mass and F+H layer litter mass were highest in the LF2 ($p < 0.05$), and litter mass in L layer was not statistically different among forest types (Table 3). Annual litterfall mass was higher in the QJF and QPF than the LF1 and LF2 (Table 3, $p < 0.05$). The H' and MI were higher in the QJF and QPF than the LF1 and LF2 for tree layer. In herb layer the H' and MI were higher in QPF than other forest types (Table 3, $p < 0.05$). The concentrations of SOC were higher in the QJF than the LF2 (Table 4, $p < 0.05$). The concentrations of TN were higher in the QPF than the LF2 ($p < 0.05$). The MBC concentrations were higher in QPF and QJF than LF1 and LF2 (Table 4, $p < 0.05$). The MBC:MBN ratios were higher in QJF than in LF1 and LF2 (Table 4, $p < 0.05$). The MBN concentrations, C:N ratio, DOC and pH were not statistically different among forest types.

Table 3. Vegetation properties in different forest types (means ± SE, $n = 3$).

Item	LF1	LF2	QJF	QPF
	Biomass for tree (t/hm^2)			
Foliage	0.71 ± 0.07c	2.31 ± 0.43b	3.05 ± 0.88a	1.96 ± 0.23b
Branches	4.87 ± 0.60b	16.28 ± 3.31ab	34.30 ± 18.5a	15.10 ± 2.55ab
Stems	31.20 ± 1.27c	90.66 ± 8.67a	83.43 ± 25.88ab	75.70 ± 10.52b
Roots	7.29 ± 0.39c	21.79 ± 2.69ab	25.20 ± 7.87a	18.24 ± 2.32b
Total	44.06 ± 2.33c	131.03 + 15.04a	145.98 ± 51.57a	111.01 ± 15.29b
	Biomass for herb (t/hm^2)			
Above ground	0.93 ± 0.57c	1.57 ± 0.21a	1.11 ± 0.47b	0.96 ± 0.39c
Below ground	1.81 ± 0.49a	2.36 ± 0.59a	2.80 ± 1.04a	2.27 ± 0.75a
total	2.75 ± 1.06a	3.93 ± 0.66a	3.91 ± 1.46a	3.23 ± 0.99a
	Forest floor litter mass (t/hm^2)			
L layer	0.82 ± 0.16a	0.87 ± 0.14a	0.77 ± 0.18a	0.74 ± 0.23a
F+H layer	3.73 ± 1.62b	4.46 ± 0.92a	2.64 ± 0.50b	2.42 ± 0.42b
Total	4.53 ± 1.76b	5.33 ± 1.05a	3.14 ± 0.63b	3.61 ± 0.64b
	Annual litterfall mass (kg/hm^2)			
	30.67 ± 1.53c	44.64 ± 9.16b	61.33 ± 10.21a	62.13 ± 2.64a
	H' and MI for tree layer			
H'	0.42 ± 0.07d	0.89 ± 0.20c	1.31 ± 0.03b	2.16 ± 0.25a
MI	0.53 ± 0.07b	0.63 ± 0.27b	1.72 ± 0.24a	1.76 ± 0.18a
	H' and MI for herb layer			
H'	2.28 ± 0.13b	2.07 ± 0.21b	1.99 ± 0.29b	2.89 ± 0.54a
MI	4.11 ± 0.38ab	4.04 ± 0.96b	3.9 ± 0.53b	4.93 ± 0.42a

Different letters indicate significant differences among forest types (adjusted $p < 0.05$).

Table 4. Mean soil properties from May to November in different forest types (means ± SE, $n = 21$).

Item	LF1	LF2	QJF	QPF
SOC (g/kg dry soil)	60.69 ± 6.97ab	59.17 ± 11.94b	66.52 ± 9.04a	63.22 ± 5.24ab
TN (g/kg dry soil)	4.99 ± 0.70ab	4.46 ± 1.02b	4.90 ± 1.57ab	5.26 ± 0.85a
C: N	12.47 ± 2.91a	10.35 ± 5.42a	10.40 ± 5.33a	12.27 ± 1.94a
MBC (mg/kg dry soil)	403.79 ± 13.33b	406.81 ± 27.18b	539.28 ± 47.51a	543.83 ± 15.99a
MBN (mg/kg dry soil)	167.66 ± 38.29a	169.62 ± 38.80a	176.52 ± 40.51a	183.30 ± 30.83a
MBC: MBN	2.54 ± 0.63b	2.51 ± 0.52b	3.15 ± 0.90a	2.97 ± 0.66ab
DOC (mg/kg dry soil)	129.24 ± 23.05a	138.07 ± 50.85a	128.89 ± 43.84a	135.15 ± 40.89a
Soil pH	6.39 ± 0.24a	6.37 ± 0.24a	6.67 ± 0.03a	6.41 ± 0.31a

Different letters indicate significant differences among forest types (adjusted $p < 0.05$).

3.4. Relationship of Soil Organic N Mineralization, Ammonification and Nitrification to Climate, Plant and Soil Variables

3.4.1. Precipitation and Temperature Effects on Net Organic N Mineralization and Nitrification Rates

The net organic N mineralization and nitrification rates in all of the forest types were positively correlated with the precipitation (Figure 5a,c). The net nitrification rates were significantly and positively correlated with precipitation (Figure 5a, $p < 0.01$). In addition, the nitrification rates were positively and significantly correlated with the soil temperature in the LF2 ($p < 0.01$), but not in the LF1, QJF and QPF (Figure 5b). The mineralization rates were significantly and positively correlated with precipitation (Figure 5c, $p < 0.01$). In contrast, the mineralization rates were not significantly correlated with the soil temperature in any forest (Figure 5d).

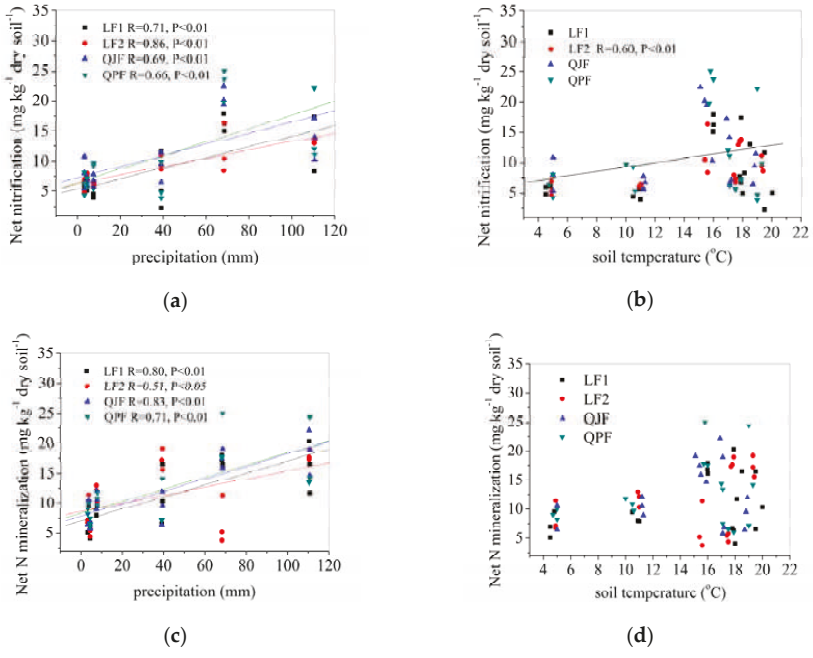

Figure 5. Correlations for nitrification rates to precipitation (**a**) and soil temperature (**b**); correlations for N mineralization rates to precipitation (**c**) and soil temperature (**d**); the precipitation was amount of precipitation per one month and soil temperature was the average for one month.

3.4.2. Correlations between Soil Properties and Organic N Mineralization, Ammonification and Nitrification

The soil net nitrification rate was significantly and positively correlated with the concentrations of MBC ($R = 0.59$, $p < 0.01$), and negatively correlated with soil pH (Figure 6d, $R = -0.71$, $p < 0.01$). There was an increasing trend in soil net nitrification rate with SOC ($R = 0.41$, $p < 0.01$) and DOC ($R = 0.42$, $p < 0.01$) (Figure 6a–d). There was no correlation between the soil net nitrification rate and the TN, MBN and C: N ratio (data not shown). The soil net mineralization rate was significantly and positively correlated with the MBC (Figure 6e, $R = 0.51$, $p < 0.01$). The soil net organic mineralization rate was significantly and negatively correlated with soil pH (Figure 6g, $R = -0.62$, $p < 0.01$). There was no correlation between the soil net N mineralization and the SOC, TN, MBN and C:N ratio (data not shown).

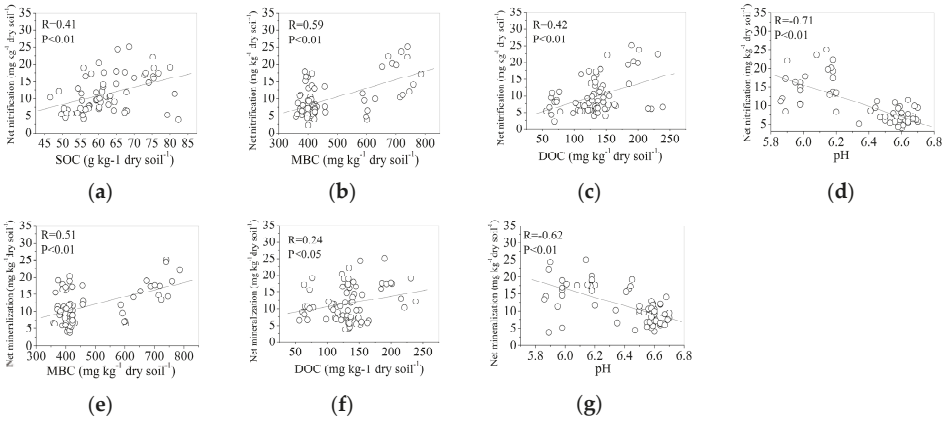

Figure 6. Correlations between net nitrification rates to SOC (**a**), MBC (**b**), DOC (**c**), and pH (**d**), and correlations between net N mineralization rates to MBC (**e**), DOC (**f**), and pH (**g**); the SOC, TN, MBC, DOC and MBC were determined in the beginning of each one-month incubation period, and the data represent the whole experimental period (from May to November) among forest types (n = 72).

3.4.3. Relationship between Vegetation Parameters and N Mineralization of Organic Matter

The Pearson correlation was conducted to estimate the relationship between the vegetation parameters and N mineralization of organic matter (Table 5). The results show that soil net nitrification was positively correlated with annual litterfall mass ($p < 0.01$), total foliage biomass ($p < 0.05$), branches biomass ($p < 0.05$), root biomass ($p < 0.01$), H' ($p < 0.01$) and MI ($p < 0.01$) for tree layer. Net ammonification was negatively correlated with annual litterfall mass ($p < 0.01$), total foliage biomass ($p < 0.05$), branches biomass ($p < 0.05$), H' ($p < 0.05$) and MI ($p < 0.01$) for tree layer. Net organic N mineralization was positively correlated with annual litterfall mass ($p < 0.01$), total foliage biomass ($p < 0.05$), H' ($p < 0.01$) and MI ($p < 0.01$) for tree layer and H′ for herb layer ($p < 0.05$).

Table 5. Pearson's correlation coefficients (r) between plant variables and soil N mineralization (n = 12).

Item	Above Ground	Below Ground	L	F + H	Annual Literfall	Foliage	Branches	Stems	Roots	H'_{tree}	MI_{tree}	H'_{herb}	MI_{herb}
N_{nit}	−0.09	0.53	0.20	−0.32	0.88 **	0.68 *	0.60 *	0.55	0.63 **	0.79 **	0.89 **	0.27	0.42
N_{amm}	0.26	−0.31	0.15	0.51	−0.79 **	−0.61 *	−0.59 *	−0.38	−0.52	−0.65 *	−0.87 *	0.10	−0.17
N_{min}	−0.02	0.57	0.41	−0.14	0.74 **	0.55 *	0.46	0.51	0.53	0.71 **	0.72 **	0.52 *	0.53

* $p < 0.05$, ** $p < 0.01$.

3.5. Multivariate Analysis (Redundancy Analysis)

To investigate possible relationships between N mineralization of organic matter and environmental variables across forest types and seasons, we performed a Redundancy Analysis (RDA), including organic N mineralization, ammonification, nitrification rates, and the relative climate, plant, and soil variables (Figure 7). A total of 57.6% of variations in seasonal net N mineralization, ammonification, nitrification rates were explained by 13 selected environmental variables. The first two RDA axes explained 49.9% and 7.7% of data variations. Soil nitrification rates were affected by the temperature, precipitation, MBC, H' for tree layer, annual litterfall, total litterfall mass of F + H layer, total foliage and roots biomass for trees, DOC and SOC. Soil N mineralization rates were affected by the temperature, precipitation, MBC and DOC concentration and soil pH. Soil ammonification rates were affected by the total litterfall mass of F + H layer and pH.

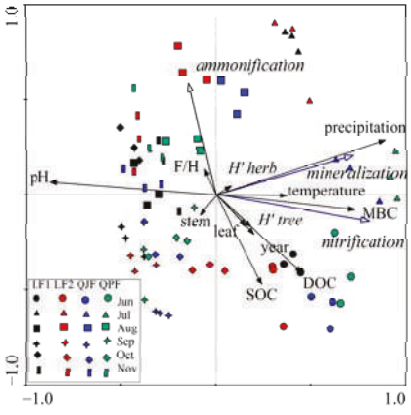

Figure 7. Redundancy analysis using net N transformations data as response variables and climate, plant and soil properties as explanatory variables.

3.6. Variation Partitioning

In order to characterize the relative importance of the broad factors of classification to the soil N mineralization of organic matter in different forest types, variation partitioning was computed independently using climate, soil properties and plant parameters as predictor variables (Figure 8). Results from RDA showed that variables could explain 57.6% of variability in soil N mineralization of organic matter. Both the independent soil (a) and total soil variables (a + ab + ac) accounted for the largest contribution to the variations in soil N mineralization (13.5% and 48.8%), while the independent climatic (b) and total climatic (b + ab + bc) variables were of secondary importance (5.6% and 41.4%). We found 35.8% shared variations of N mineralization explained by soil and/or climatic variables (Figure 7). However, the plant and soil had a negative shared variation of joint fractions (−0.4%).

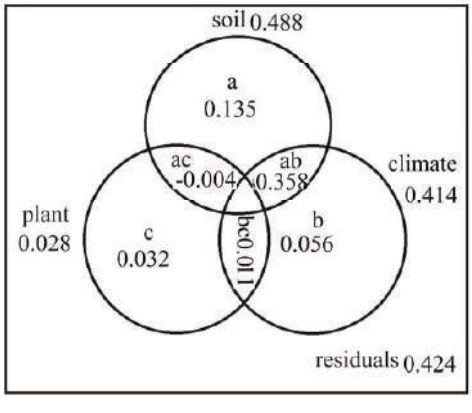

Figure 8. Variation partitioning of N transformations using the matrices of soil variables, plant variables and climate variables. Variables a, b and c denote the independent effects of soil, climate and plant, respectively. Variables ab, ac, bc denote the joint effects of soil and climate, soil and plant, climate and plant, respectively.

4. Discussion

The ranges of net N mineralization of organic matter were different among forest types. Here all of the forest types in this study are considered similar in community structure and historical soil conditions. As such, differences in present soil N mineralization patterns may reflect the impacts of the shifts in plant species composition and plant-induced soil properties on N transformation processes [38,39]. Our results indicate that there were considerable variations among forest types in the plant diversity for tree layer, annual litterfall mass, biomass recorded in leaves, branches, stems and roots, and aboveground biomass for herbs. Surface soil properties were also different among forest types in SOC, TN, MBC and MBC: MBN ratio. We suggest that shifts in plant species composition and resulting plant diversity, forest biomass and soil properties are the most probable explanations for the soil N mineralization patterns of natural secondary forest converted into larch plantations.

4.1. The Effects of Plant on N Mineralization

Our results showed that the two natural secondary forests had higher levels of soil nitrification rates than the two larch plantations, while, net ammonification rates were lower in natural secondary forests than in larch plantations, which could be attributed to the following reasons. Firstly, changes of tree species composition could influence the qualities of litterfall, which may significantly alter the available soil nutrients. Previous work has shown that coniferous tree species typically provide a lower quality of litter material (lower N contents and higher C:N ratio) and a slower litter decomposition, which contribute to a poor soil nutrient level [16,40], and consequently reduced the rate of N cycling [41]. In our study sites, the annual litterfall mass were lower in LF1 and LF2 than QJF and QPF, but the forest floor litter mass was not lower, which indicated the litter decomposition rate was slower in in LF1 and LF2 than QJF and QPF. Secondly, conversion of natural secondary forests to plantations could affect forest biomass productions, which influence the quantities of litterfall. Koutika (2014) found that the higher biomass production may increase soil nitrogen through an enhanced production of litter [42]. In our study, the biomass amounts recorded in leaves, branches, and roots were significantly higher in QJF than LF1 and LF2, thus the annual litterfall mass was higher in QJF than in LF1 and LF2. Our result also indicated soil N mineralization and nitrification rates were positively correlated with the foliage biomass, and the annual litterfall mass. These results suggested that the relatively lower quantities and qualities of litter and slower litter decomposition of larch plantations made N mineralization and nitrification rates decrease after conversion from natural secondary forests. Thirdly, N transformations could also be affected by plant diversity. It has been suggested a more diverse array of plant compositions entering the soil through leaf litterfall could enhance nitrification rates by providing a consistent long-term supply of organic nitrogen as the different qualities of plant material break down at different rates over time. In our study, nitrification rate was positively correlated with plant diversity and richness of tree layer. These results are consistent with previous studies [21,43,44] which also detected a positive plant diversity effect on nitrification.

4.2. The Effects of Soil Properties on N Mineralization

As expected, soil properties were closely correlated with soil organic matter mineralization. Previous studies have shown that soils with high nutrient availabilities could have high N mineralization rates [45,46]. As a biological process, soil N mineralization of organic matter is mainly determined by substrate availabilities and microbial activities [47,48]. We observed the net N mineralization and nitrification rate was positively correlated with MBC, and there was an increasing trend in soil net nitrification rate with SOC and DOC. Soil C and N pools provide available substrates and energy to stimulate microbial activity and which increase the N mineralization rates [39,49]. In our study, MBC concentrations were significantly higher in the QJF and QPF than the LF1 and LF2, the SOC concentrations were significantly higher in the QJF than LF2, TN were significantly higher in the QPF than LF2, consequently, the nitrification was higher in the QJF and QPF than the LF1 and

LF2, the cumulative mineralization was higher in QPF than the LF1 and LF2. Some studies have shown that soil pH is an important factor of soil N mineralization during conversion of broad-leaved forests to coniferous forests. Plantations with coniferous species can produce acidic leaf litters and root exudation of H^+, which in turn lower soil pH and affect micro-fungi activities [50,51]. In our study, soil pH was negatively correlated with net N mineralization and nitrification rate, however, soil pH was not significantly different among forest types, which indicted factors other than pH might have restricted soil N transformation. In this study, we considered the substrate availabilities and microbial activities to be the important factor for N mineralization in organic matter during the forest conversion of natural secondary forests to larch plantations.

4.3. Seasonality Effects on Soil N Transformation Patterns

Seasonal dynamics of temperature and precipitation played an important role in controlling N transformations [11,52]. The present study showed that net nitrification and N mineralization rates were higher from June to July and lower from August to September, whereas the net ammonification rates were higher from July to August and lower from May to June, which can be explained by the seasonal dynamics of temperature and water availability. Soil N mineralization involved biological processes that are moisture and temperature dependent [11,53]. The seasonal changes of temperature and moisture directly control soil microbial activity, which directly influences soil N transformations. In our study, the regression analysis showed that the rates of N mineralization and nitrification had a significantly positive relationship with the precipitation for each forest, while a significant relationship between the net N nitrification and temperature was only found in the LF2. The relationships between the N mineralization rate and precipitation were much stronger than those between the N mineralization rate and temperature, suggesting that precipitation had the major effect on the net N mineralization and nitrification rates in our research region.

4.4. Interaction of Climate, Plant and Soil Variables on N Transformation

In this study, we found a dominant effect of soil properties on N transformations (48.8%). This result is consistent with those of previous studies [10]. Interestingly, the plant variations explained a relatively small fraction in N transformation (2.8%), and the plant and soil had a negative shared variation. This indicated that the interaction effect of plant and soil on N transformation was larger than the summation of independent soil and plant variables. This result can be explained by taking into consideration previous studies wherein plant variables have significant effects on soil properties and which were simultaneously influenced by soil properties, indicating that plant and soil have mutual promoted effects [54]. Climate was also a strong predictor variable of N transformation [8,9]. Many other studies found temperature and precipitation to be the main constraining factors for N transformation [10,11]. In our study, the climate accounted for the secondary contribution to the variations in N mineralization. Overall, these three variations should not be considered mutually exclusive; we may expect each to contribute to the explanation of the potential effects of seasonal dynamics in N mineralization of natural secondary forest converted into larch plantations. In northeast China, plantation/secondary forest landscapes account for the largest proportion of forest areas, and this kind of plantation has been progressively increased throughout the nation. However, we did not study the latitude and longitude, and global climate change effect on N transformation due to limited study sites. Therefore, it is necessary to make a further study on plantation/secondary forest soil N mineralization with a wider study area including latitudinal and longitudinal differences, and varied climatic regions.

5. Conclusions

Our study compared seasonal dynamics of soil N mineralization of organic matter between the natural secondary forests and larch plantations and assessed which could be explained by the plant-soil properties that were associated with forest conversion and seasonal climate conditions.

We demonstrated that plant diversity, litterfall quantity and quality and the soil nutrient availability varied considerably during the forest conversion, which made N mineralization different between the natural forests and plantations. Soil properties were the primary determinant of the N mineralization, and climate conditions also contributed to N mineralization significantly, whereas plant variations were a tertiary contribution to N mineralization. Our results indicated that the larch plantations reduced plant diversity, litter quantity and quality and soil fertility as well as N transformation rate.

Author Contributions: Q.W., F.L., X.R. and Z.F. conceived, designed and installed the experiment; Q.W. and Z.F. were responsible for field work; Q.W. performed laboratory and data analysis under F.L. and X.R. supervision; Q.W. and F.L. wrote the manuscript with contributions from the other authors. All authors approved the final version of the manuscript.

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Thinning Treatments Reduce Deep Soil Carbon and Nitrogen Stocks in a Coastal Pacific Northwest Forest †

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Abstract: Forests provide valuable ecosystem and societal services, including the sequestration of carbon (C) from the atmosphere. Management practices can impact both soil C and nitrogen (N) cycling. This study examines soil organic C (SOC) and N responses to thinning and fertilization treatments. Soil was sampled at an intensively managed Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) plantation in north-western Oregon, USA. Management regimes—thinning, fertilization plus thinning, and no (control) treatment—were randomly assigned to nine 0.2-ha plots established in 1989 in a juvenile stand. Prior to harvest, forest floor and soil bulk density and chemical analysis samples were collected by depth to 150 cm. During a single rotation of ~40 years, thinning treatments significantly reduced SOC and N stocks by 25% and 27%, respectively, compared to no treatment. Most of this loss occurred in deeper soil layers (below ~20 cm). Fertilization plus thinning treatments also reduced SOC and N stocks, but not significantly. Across all management regimes, deeper soil layers comprised the majority of SOC and N stocks. This study shows that: (1) accurately quantifying and comparing SOC and N stocks requires sampling deep soil; and (2) forest management can substantially impact both surface and deep SOC and N stocks on decadal timescales.

Keywords: soil organic carbon; carbon sequestration; nitrogen; deep soil; forest floor; forest management; fertilization; thinning; fixed depth; equivalent soil mass

1. Introduction

The world's forests are an important terrestrial carbon (C) sink, sequestering as much as 30% (~2 Pg C y^{-1}) of annual global anthropogenic CO_2 emissions between 1990 and 2007 [1,2]. In addition to their importance in the global C cycle, forests provide many other valuable ecosystem and societal services. Forest-management practices can enhance or reduce the ability of a given forest stand to act as a C sink and provide these services [3,4]. Since approximately two-thirds of forests are managed [1], understanding how forest-management practices affect the global C cycle and the capacity to sustainably produce natural resources is a high priority.

While much research has been conducted regarding the aboveground effects of forest management, comparably little is known about the effects belowground [5]. Soils comprise the majority of the terrestrial C stock [6] and account for ~85%, 60%, and 50% of the total C stock in boreal forest,

temperate forest, and tropical rainforest ecosystems, respectively [3,7]. Therefore, determining the fate of forest soil organic C (SOC) in response to management is an essential part of understanding climate-carbon feedbacks and changes in forest ecosystem C budgets. Gains or losses in SOC affect numerous soil properties essential to maintaining beneficial ecosystem services and productive forest stands, including the water- and nutrient-holding capacity of the soil [8].

In Pacific Northwest forest ecosystems, nitrogen (N) is often the primary limiting nutrient [9–11]. The fate of soil N in response to forest management is thus a key concern in this region. In general, N fertilization stimulates biomass production, but the effects on SOC and other soil-nutrient dynamics are variable and highly site dependent due to complex interactions between soil properties, microorganisms and vegetation [12]. Increases in soil N have the potential to enhance aboveground biomass growth and forest productivity, as well as to increase SOC and the retention of other nutrients in the soil through a combination of increased belowground biomass and delayed root decomposition [13].

In a recent meta-analysis, James and Harrison [14] found that harvesting reduced soil C by an average of ~11% globally. Significant losses in both the litter layer (O horizon) and the mineral soil were observed, with particularly large losses in very deep soil (60–100+ cm in depth) compared to more superficial soil. Interestingly, thinning treatments resulted in greater losses of mineral soil C than clear-cut harvesting by a difference of ~9% [14]. Although this may seem counterintuitive, thinning treatments can lead to less SOC accumulation over time due to reduced root C inputs (from reduced root biomass) [15], and they can also affect numerous soil properties that enhance microbial metabolic activity or encourage increased leaching and export of SOC and other soil nutrients. Mechanisms that can lead to SOC and N losses due to thinning include: (1) increased soil temperature; (2) microbial stimulation or priming; (3) nitrate leaching; and (4) groundwater-level rise.

1.1. Increased Soil Temperature

Decreased radiation interception by trees due to thinning treatments can result in soil temperature increases [16]. Several studies examining the upper 10 cm of soil have shown post-thinning increases in soil temperature ranging from 1–6 °C when compared to reference stands [16–18]. Soil temperature also tends to increase with thinning intensity [16,17]. Along with increases in soil temperature, Cheng et al. [17] measured an increase in soil respiration ranging from ~8% to 20% and increasing with thinning intensity. Hicks Pries et al. [19] found that mineral soil respiration in a temperate forest ecosystem increased by 34–37% to a depth of 100 cm when subjected to 4 °C warming, with soil below 15 cm contributing to ~50% of the total respiration. Forest harvest has been observed to increase mean soil temperature and mean daily soil temperature flux by ~3 °C and 5 °C, respectively, at 10 cm in depth and by ~2 °C and 3 °C, respectively, at 100 cm in depth [20]. Any increase in SOC decomposition rates also increases microbial demand for N, as microorganisms require about a 24:1 C:N ratio during organic matter decomposition [21]. Competition for available N in the soil environment is increased in particular during the decomposition of organic matter with a higher C:N ratio, such as coarse woody debris left as slash on the forest floor post-thinning, and can lead to N scavenging as plants and microorganisms compete for this essential nutrient [21,22].

1.2. Microbial Priming

Microbial priming is a mechanism by which potential energetic barriers to SOC decomposition are alleviated by the introduction of fresh C compounds [23]. Similarly, higher rates of nitrification and additional nitrate can result in increased SOC decomposition and dissolved organic carbon (DOC) production by alleviating microbial nutrient limitations [24,25]. The priming phenomenon is particularly relevant in deeper soil layers where SOC is often thousands to tens of thousands of years old [23,26]. When the environmental conditions under which deep SOC accumulated change, such as through the addition of fresh C compounds, this SOC is vulnerable to decomposition [19,23,26–28]. Removing trees results in decreased transpiration and rain interception, and in turn can lead to

increased DOC flux and transport to deeper soil layers, which is driven largely by rain events and new inputs of organic matter [29,30]. Organic matter left on-site post-thinning (e.g., roots and slash) substantially increases soil C inputs and the potential for priming effects. While forest floor fresh C inputs are often mineralized in the litter layer [26,30], C inputs due to the mineralization of root biomass can persist for many years following the harvest of trees [31]. Post-thinning increases in channels of decaying roots, particularly coarse roots, could also create preferential flow paths for DOC transport. Along these pathways, DOC has fewer opportunities for abiotic and biotic interactions, potentially introducing large amounts of fresh DOC to deeper soil layers [32]. As preferential pathways have been shown to have greater SOC concentrations and microbial biomass than the surrounding bulk soil [33], mineralization rates in these pathways are likely enhanced [34], which may lead to increased SOC and N losses from the adjacent bulk soil.

1.3. Nitrate Leaching

Several studies have observed increased leaching or export of nitrate (and other forms of N) following forest harvest under logging residues [35–39]. Rosen and Lundmark-Thelin [39] attributed this phenomenon to a combination of reduced N uptake by roots and increased mineralization of the litter layer. Fertilized forest stands may be particularly vulnerable to nitrate leaching under slash left on the forest floor post-thinning due to increased N availability. High rates of nitrification, nitrate leaching, and increased soil acidity due to fertilization can negatively affect soil quality through the priming of SOC and through the co-leaching of other nutrients such as calcium and magnesium [40].

1.4. Groundwater-Level Rise

The removal of trees via thinning treatments or harvest reduces transpiration, which can result in groundwater-level rise and potential increases in the export of DOC and various forms of N [37,41]. Laudon et al. [41] observed a >70% increase in DOC export from harvested compared to unharvested sites one year post-harvest. They attributed this increase in DOC export primarily to a raised groundwater level contacting more superficial soil layers that have higher DOC (and N) concentrations. Depending on the original height of the water table prior to the removal of trees, substantial portions of deeper SOC and N could be especially vulnerable to export and loss via this mechanism.

1.5. Summary and Objectives

Jandl et al. [12] concluded that, in general, forest thinning increases the stability of a stand at the expense of SOC stocks. However, other studies have found no difference in SOC stocks post-thinning [5]. In a long-term reforestation study in the subtropical south-eastern USA. Mobley et al. [42] found that thinning treatments reduced SOC and N stocks in particular in deeper soil layers where losses exceeded new inputs. Although deeper soil is often ignored in short- and even long-term studies, the observation of deep SOC losses on decadal timescales due to management or land-use change is not uncommon [42–44]. Considering that most SOC is contained in deeper soil layers (below ~20 cm) [6,45,46], and most trees root deeper than 100 cm [47], studying deep-soil nutrient dynamics is essential to understanding forest-management effects on SOC stocks. Unfortunately, soils are often sampled to 20 cm or less and are rarely sampled below 100 cm [4,6,14,27,42,45,48].

The objective of this study was to determine the responses of SOC and N to thinning and fertilization plus thinning treatments of varying intensity, and to understand how any observed response differed vertically in the soil profile to a depth of more than 100 cm. Both the fixed-depth and mass-based approaches were used to quantify and compare SOC and N stocks. We found that thinning treatments substantially reduced both SOC and N stocks, particularly in deeper soil layers, highlighting that forest-management practices can affect both surface and deep SOC and N stocks on decadal timescales, and that accurately quantifying and comparing SOC and N stocks requires sampling deep soil.

2. Materials and Methods

Soil was sampled at an intensively managed Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) plantation in north-western Oregon, USA (Figure 1, inset). The plantation was planted on commercial forest land in 1977 with 2-year-old Douglas-fir seedlings and was ≥90% Douglas-fir throughout the ~40-year rotation. The previous plantation, also Douglas-fir, underwent uniform treatment and harvest. Square plots were established in 1989, the boundaries of which were designed to maximize uniformity within and between plots. Management regimes—thinning (T_{trt}), fertilization plus thinning (FT_{trt}), and no (control) treatment (C_{trt})—were randomly assigned to nine 0.2-ha plots spanning a total area of ~5 ha (Figure 1). Three plots received thinning treatments; three plots received fertilization plus thinning treatments of varying intensity; and three plots received no (control) treatment. Additionally, the initial trees per hectare were systematically reduced to one-half or one-fourth on randomly assigned plots (Table 1). The removed trees were left on the forest floor. Fertilized plots received 224 kg N ha^{-1} as urea every 4 years starting in 1989 for a total of 1120 kg N ha^{-1} over 16 years. Thinning treatments were based on Curtis' [49] relative stand density. The stems of thinned trees were either removed or left on the forest floor, depending on the size of the trees at the time of treatment. Slash from trees was consistently left on the forest floor, even when stems were removed. Landform across all plots was nearly level to gently sloping, with an average slope of 10% and a maximum slope of <30%. The climate is characterized by cool, wet winters and warm, dry summers. Mean annual precipitation from 1981 to 2010 was ~220 cm, and mean annual temperature was 9 °C, with an annual maximum and minimum temperature of 14 °C and 4 °C, respectively [50]. Elevation ranged from 620 m to 660 m above sea level. Soil was moderately well drained with a low mean rock fragment content by sample weight (<2% fine to medium gravel). The soil sampled was an older, clayey soil (a Palehumult, closely resembling the Cumley series), making it reasonably uniform and an ideal soil for sampling to compare forest-management treatments.

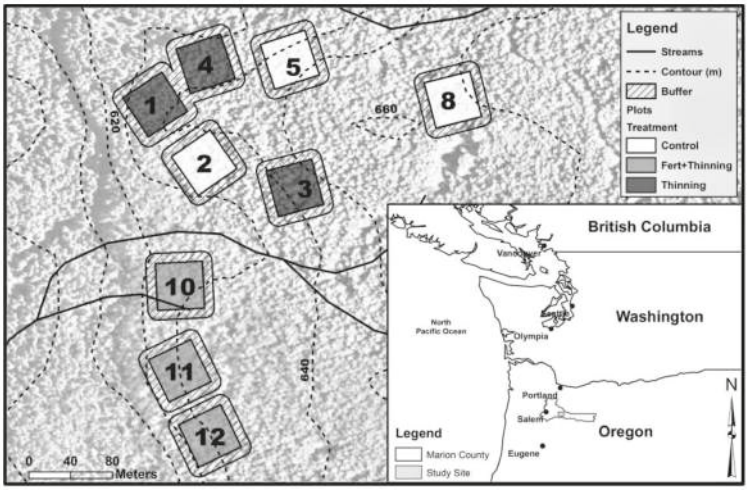

Figure 1. Location of study site (inset) and layout of treatment plots. The site coordinates are 44.87417, −122.566 (Latitude/Longitude decimal degrees). Fert, fertilized.

Table 1. Summary of plot treatments. The plantation was planted in 1977 with 2-year-old Douglas-fir seedlings. Plots are each 0.2-ha and span a total area of ~5 ha. Fert, fertilized.

Plot	Initial Trees (ha^{-1})	Treatment Group	Fertilized [§]	Thinning Treatment	Thinning Year	Stems Left on Forest Floor [#]
1	1117	Thinning	No	RD55→RD35	2001	Yes
2	297 [†]	Control	No	None	-	-
3	558 [‡]	Thinning	No	RD55→RD35	2011	No
4	1181	Thinning	No	RD55→RD35	1999	Yes
5	1196	Control	No	None	-	-
8	554 [‡]	Control	No	None	-	-
10	1240	Fert + Thinning	Yes	RD55→RD35 RD55→RD40	1997 2005	Yes No
11	558 [‡]	Fert + Thinning	Yes	RD55→RD35	2009	No
12	311 [†]	Fert + Thinning	Yes	None	-	-

[†] initial trees per hectare were reduced to one-fourth in 1989; stems and slash left on forest floor. [‡] initial trees per hectare were reduced to one-half in 1989; stems and slash left on forest floor. [§] fertilized plots received 224 kg N ha^{-1} as urea every four years starting in 1989 for a total of 1120 kg N ha^{-1} over 16 years. RD = Curtis's [49] relative density. [#] slash consistently left on forest floor, even when tree stems were removed.

Three soil pits per plot were excavated with a shovel to 100 cm or 150 cm. Major genetic horizons, soil colors, textures and structures were identified, and roots and stone content were recorded (Table 2). Soil bulk density and chemical analysis samples were collected in the late summer and early fall of 2015, immediately preceding harvest. Soil was collected from all nine plots over (three) two-week time periods to minimize any differences in weather conditions between plots during the sampling timeframe. Samples were collected randomly from within the middle of soil depth layers 0–10, 10–20, 20–50, 50–100, and 100–150 cm. One forest floor sample was collected from a randomly placed 20 × 30 cm quadrat nearby each soil pit. All soil samples were analyzed separately, and repeated measurements within a plot and soil depth layer were subsequently averaged to account for within-plot variation. Soil bulk density and chemical analysis samples were collected using a 5.4-cm diameter hammer-core, as well as using clod and excavation (irregular hole, water replacement) methods. Repeated measurements within the same plot and soil depth layer across methods were analyzed separately to adjust for differences between the methods using regression (wherein the excavation method was used as the standard). A detailed description of soil-sampling techniques, laboratory methods, and regression equations is found in Gross and Harrison [51]. All soil samples collected in the field were sealed in plastic bags, returned to the laboratory within 48 h, and stored at 3 °C until analysis.

Soil subsamples used in elemental analysis were taken from bulk density samples to avoid potential biases, as SOC concentrations and bulk density are not independent variables [52]. Air-dried samples were sieved to <4.75 mm (rather than to <2 mm) to avoid discarding a meaningful portion of SOC [53,54]. The >4.75 mm fraction was weighed and the volume determined by displacement of water in a graduated cylinder. Litter layer samples were weighed, air-dried to a constant weight, reweighed, and ground to less than ~0.5 mm. Representative subsamples of litter layer and <4.75 mm mineral soil fractions were ground with a mortar and pestle and analyzed for total C and N concentrations (g kg^{-1}) using an automated elemental analyzer (Perkin-Elmer 2400, PerkinElmer, Waltham, MA, USA). Approximately 20% of the samples were run twice to verify the precision of the analysis, and quality control samples of known C concentration were run every 10 samples. The average of samples run twice was used for analysis. Due to a lack of carbonates measured in the region [55] and strongly acid soils (pH < 6), total C concentrations are equated to organic C [56].

Table 2. Typical soil profile description. Resembles Cumley series (a Palehumult). Average slope of 10%. Mean elevation ~640 m above sea level. Gr, gravel (0.2–7.5 cm); Cb, cobbles (7.5–25 cm); St, stones (>25 cm); YR, yellow-red.

Horizons	Depth (cm)	Color (moist)	Texture	Structure	Roots	Rock (%)
Oi	3.5–0					Gr < 2% Cb < 5% St < 5%
A	0–15	10 YR 3/3 dark brown	Sandy clay loam	Medium to coarse granular, weak to moderate	Many fine, medium, and coarse	Gr < 2% Cb < 5% St < 5%
BA	15–30	10YR 3/4 dark yellowish brown	Sandy clay	Medium to coarse subangular blocky, moderate	Many fine and medium; few coarse	Gr < 2% Cb < 5% St < 5%
Bt1	30–80	5YR 4/4 reddish brown	Sandy clay to clay	Coarse to very coarse subangular blocky, moderate to strong	Common fine, few medium; very few coarse	Gr < 2% Cb < 5% St < 5%
Bt2	80–150+	10YR 4/4 dark yellowish brown	Sandy clay	Coarse subangular blocky, moderate	Few fine and medium; very few coarse	Gr < 2% Cb < 5% St < 5%

Soil pH was measured in a 1:1 (deionized H_2O, mL; soil, g) mix for mineral soil and a 4:1 mix for the litter layer with a digital pH meter (Model PC-700, Oakton Inst., Vernon Hills, IL, USA). Soil mixtures were stirred and left to stand undisturbed for at least 30 min to allow homogenization before pH was measured. The volume of field-moist clods [57] was determined by the paraffin wax method and displacement of water. Oven-dry weights for the clod method were determined by drying the clods in the oven at 105 °C for at least 48 h. Subsamples of each core and excavation sample were dried for at least 48 h at 105 °C, and oven-dry weights were determined by applying conversions to the air-dried weights. Bulk density was calculated according to:

$$\text{Bulk density} = \left(\frac{\text{oven dry sample weight, g} - \text{rock fragment weight, g } (> 4.75 \text{ mm})}{\text{soil volume, cm}^3 \text{ (solids + pores)} - \text{rock fragment volume, cm}^3 (> 4.75 \text{ mm})}\right), \quad (1)$$

Soil organic C and N stocks were determined using the fixed-depth equations:

$$\text{Mg SOC ha}^{-1} = \left(\frac{\text{mg SOC}}{\text{g soil}}\right)\left(\frac{\text{g soil}}{\text{cm}^3 \text{ soil}}\right)\left(\frac{\text{cm}}{1}\right)\left(\frac{\text{Mg}}{10^9 \text{ mg}}\right)\left(\frac{10^8 \text{ cm}^2}{\text{ha}}\right), \quad (2)$$

and,

$$\text{kg N ha}^{-1} = \left(\frac{\text{mg N}}{\text{g soil}}\right)\left(\frac{\text{g soil}}{\text{cm}^3 \text{ soil}}\right)\left(\frac{\text{cm}}{1}\right)\left(\frac{\text{kg}}{10^6 \text{ mg}}\right)\left(\frac{10^8 \text{ cm}^2}{\text{ha}}\right), \quad (3)$$

where mg SOC g soil^{-1} is SOC concentration, mg N g soil^{-1} is N concentration, g soil cm soil^{-3} is bulk density, and cm is soil layer thickness. The mass-based approach according to the procedure of Wendt and Hauser [58] was also used to estimate SOC and N stocks. For this approach, soil-sample mass for each depth layer ($M_{SAMPLE(DL)}$, g) and method was calculated according to:

$$M_{SAMPLE(DL)} = \pi\left(\frac{5.4 \text{ cm}}{2}\right)^2 \times \left(\frac{\text{soil layer thickness, cm}}{1}\right) \times \text{bulk density}, \quad (4)$$

where 5.4 cm is the inside diameter of the core. Soil-sample masses, SOC and N concentrations, the inside diameter of the core, and the number of cores per sample were subsequently input into the web-accessible spreadsheet [59] created by Wendt and Hauser [58], which fits a cubic spline function to model the relationship between cumulative areal soil mass and cumulative SOC mass. Reference mass layers were set using the lowest mean soil mass across treatments for each soil depth layer as recommended by Lee et al. [60] for systems in which the initial conditions (e.g., SOC or bulk density) are not available.

In this study, both the fixed-depth and mass-based approaches used to calculate SOC and N stocks replace the volume of the >4.75 mm fraction with fine soil (<4.75 mm). Multiple studies have found that this method has the potential to overestimate [61] or underestimate [27,54,62–64] SOC and N stocks. However, these errors appear to be limited to rocky soils [54,61,62,64]. Because the soil sampled in the current study was generally non-rocky (<2% fine to medium gravel content by weight), this method is unlikely to cause substantial biases in SOC and N stock estimates.

Sampling in the middle of a soil depth layer likely underestimates SOC and N concentrations and overestimates the bulk density of soil above the sample, while resulting in errors in the opposite direction concerning soil below the sample (i.e., overestimating SOC and N concentrations and underestimating bulk density). An assumption of this sampling method is that the errors tend to have a canceling effect, giving an accurate estimate of SOC and N stocks in the given layer. This assumption applies to all calculations of mineral SOC and N stocks in this study. As the entire depth of the litter layer was sampled, this assumption does not apply to the litter layer. Additionally, litter layer SOC and N stocks were only determined using Equations (2) and (3).

Total aboveground and root C sequestration were determined using the biomass ratio equation and parameters provided by Table 6 in Jenkins et al. [65]. For a given plot, the quadratic mean diameter (QMD) was substituted for diameter at breast height (DBH). Total aboveground biomass (TAB) for each plot was calculated according to:

$$\text{TAB Mg ha}^{-1} = \frac{\left(\frac{450\text{ kg}}{\text{m}^3}\right)\left(\frac{\text{stand volume, m}^3}{\text{ha}}\right)\left(\frac{\text{Mg}}{1000\text{ kg}}\right)}{e^{\left(-0.3737-\left(\frac{1.8055}{\text{QMD,cm}}\right)\right)}}, \quad (5)$$

where 450 kg m^{-3} is the density of Douglas-fir [66], and the denominator is the biomass ratio equation for softwood-stem wood. The sum of the removed and final aboveground biomass was used for TAB for thinned plots to represent total aboveground and root C sequestration. A ratio of 0.512, which is the average C concentration (g C g^{-1}) for Douglas-fir trees in the Pacific Coast and Rocky Mountain regions [67], was used to convert total mass to C mass. Belowground C stocks were determined by summing the SOC stock (calculated using the mass-based approach) for a given plot with total root C for the same plot. Total C stocks were determined by summing the above- and belowground C stocks for a given plot. Merchantable volume for thinned plots was calculated by including removed volume only if the given volume was commercially thinned.

Analysis of variance (ANOVA) was used to determine whether treatment affected various soil properties and SOC and N stocks. When significant differences were detected at $p < 0.1$, Tukey's honest significant difference (HSD) post-hoc tests were conducted to compare the means. A significance level of 0.1 was chosen (rather than 0.05, for example) to reduce the chance of false negatives [68], as forest-management effects on soil properties or nutrient stocks can have lasting impacts on soil health and future productivity. Relationships between numerical variables such as SOC and N concentrations were determined using linear regression. Data met the assumptions of the analyses performed and no data transformations were necessary. Data were analyzed using R studio [69].

3. Results

3.1. Soil Bulk Density and Organic Carbon Concentrations

In the mineral soil layers, bulk density was consistently highest for T_{trt} at all depths and lowest for C_{trt} to a depth of 100 cm (Figure 2). In the upper 50 cm, this pattern was inversely related to SOC concentrations, with C_{trt} having the highest SOC concentrations and T_{trt} having the lowest (Figure 2). Soil organic C concentration was a significant ($p < 0.1$) predictor of bulk density in the mineral soil, the two variables following a quadratic relationship (Figure 3). However, a large amount of variation occurred where SOC concentrations were below ~15 g SOC/kg soil, which corresponded to depths below 50 cm. When the relationship between SOC concentration and bulk density was

analyzed by depths 0–50 cm and 50–150 cm, the former explained slightly more of the variation in bulk density values than the whole mineral soil (i.e., 0–150 cm in depth), while the latter was not significant (Figure 3). Soil organic C concentration and bulk density were not significantly related in the litter layer (Figure 3). Bulk density for T_{trt} was significantly (Tukey's HSD, $\alpha = 0.1$) higher than C_{trt} in the upper 20 cm of mineral soil and significantly higher than FT_{trt} in the 10–20 cm depth layer. C_{trt} had a significantly higher SOC concentration compared to both T_{trt} and FT_{trt} in the upper 20 cm of mineral soil, as well as in the 100–150 cm depth layer compared to FT_{trt}. No significant differences between treatment means were observed for bulk density or SOC concentration in the litter layer.

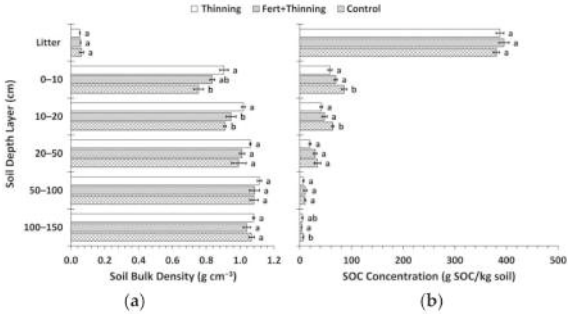

Figure 2. (**a**) Mean soil bulk density by treatment; (**b**) mean soil organic carbon (SOC) concentration by treatment. Sample size for all treatments and soil depth layers is three. Error bars represent ± one standard error. Means within each soil depth layer accompanied by the same letter (i.e., "a" or "b") are not significantly different (Tukey's honest significant difference (HSD), $\alpha = 0.1$). Fert, fertilized.

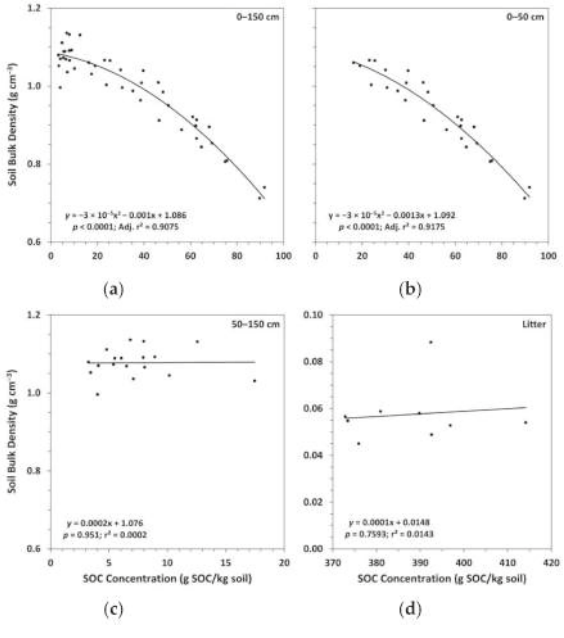

Figure 3. Soil bulk density versus soil organic carbon (SOC) concentration for: (**a**) the whole mineral soil ($n = 45$); (**b**) the 0–50 cm soil depth layers ($n = 27$); (**c**) the 50–150 cm soil depth layers ($n = 18$); (**d**) the litter layer ($n = 9$).

3.2. Soil Nitrogen Concentrations, Carbon to Nitrogen Ratios, and pH

Trends in soil N concentrations between treatments in the mineral soil followed a similar pattern as for SOC concentrations, although a few additional means were found to be significantly different (Figure 4). C_{trt} had a significantly higher soil N concentration compared to T_{trt} at all depths except the 50–100 cm depth layer. FT_{trt} also had a significantly higher soil N concentration compared to T_{trt} in the 0–10 cm depth layer. Compared to FT_{trt}, C_{trt} had a significantly higher soil N concentration in the upper 20 cm of mineral soil and in the 100–150 cm depth layer. Notably, in the litter layer, T_{trt} had a significantly lower soil N concentration compared to the other two treatments. No significant differences in the C:N ratio (SOC concentration/N concentration) in the mineral soil were observed (Figure 4). In the litter layer, T_{trt} had a significantly higher C:N ratio compared to C_{trt} and FT_{trt} (39, 32, and 33, respectively). Surface soil layers (0–20 cm in depth) had a mean C:N ratio of ~24 across all treatments, while deeper soil layers had normalized mean C:N ratios of 18, 19, and 17 for T_{trt}, FT_{trt}, and C_{trt}, respectively. Soil organic C concentration was a significant predictor of N concentration in the mineral soil, the two variables being positively related, and explained much of the variation in N concentration (r^2 = 0.9886) (Figure 5). These two variables were not significantly related in the litter layer (Figure 5).

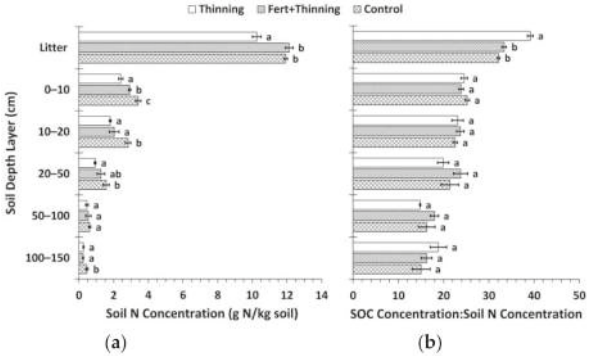

Figure 4. (**a**) Mean soil nitrogen (N) concentration by treatment; (**b**) mean soil organic carbon (SOC) concentration to N concentration by treatment. Sample size for all treatments and soil depth layers is three. Error bars represent ± one standard error. Means within each soil depth layer accompanied by the same letter (i.e., "a" or "b") are not significantly different (Tukey's HSD, α = 0.1). Fert, fertilized.

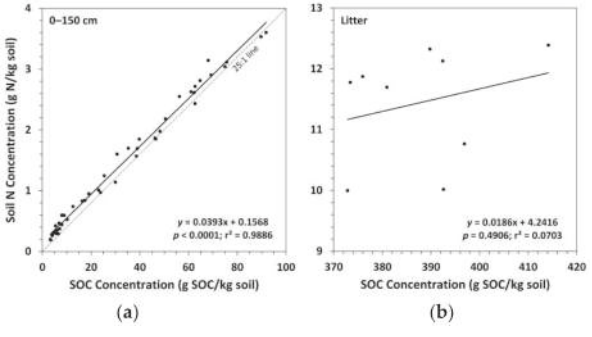

Figure 5. Soil nitrogen (N) concentration versus soil organic carbon (SOC) concentration for: (**a**) the whole mineral soil (n = 45); (**b**) the litter layer (n = 9). Dashed 25:1 line added to provide a reference for the C:N ratio for the whole mineral soil.

Soil was strongly acid across all treatments. Normalized by depth, mean soil pH values for the mineral soil for T_{trt}, FT_{trt}, and C_{trt} were 5.05, 4.98, and 5.01, respectively. In the litter layer and upper 100 cm of mineral soil, FT_{trt} had consistently lower pH values than the other two treatments (Figure 6). However, this difference was significant only in the 0–10 cm depth layer between FT_{trt} and T_{trt}.

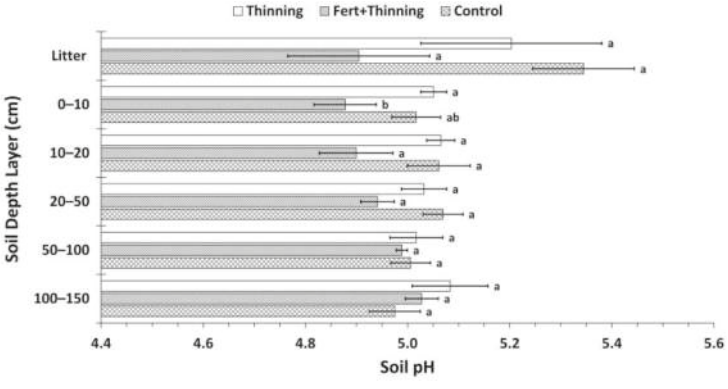

Figure 6. Mean soil pH by treatment. Sample size for all treatments and soil depth layers is three. Error bars represent ± one standard error. Means within each soil depth layer accompanied by the same letter (i.e., "a" or "b") are not significantly different (Tukey's HSD, $\alpha = 0.1$). Fert, fertilized.

3.3. Soil Organic Carbon and Nitrogen Stocks

Calculated using the fixed-depth approach, SOC and N stocks in the mineral soil were consistently highest for C_{trt} at all depths (Figure 7). Compared to T_{trt}, these differences were significant above 50 cm for SOC stocks and at all depths except the 50–100 cm soil depth layer for soil N stocks. These differences were significant in the 10–20 and 100–150 cm soil depth layers for both SOC and N stocks compared to FT_{trt}. Across all treatments, the majority of SOC and N stocks were below 20 cm ($\geq$55% and >60%, respectively) to a depth of 150 cm. FT_{trt} had a significantly greater litter layer mean SOC stock compared to C_{trt} and a significantly greater litter layer mean N stock compared to both C_{trt} and T_{trt}. Mean litter layer thickness was similar for FT_{trt} and T_{trt} (4.0 ± 0.3 and 3.8 ± 0.1 cm, respectively) and was considerably (though not significantly) thicker for these two treatments than for C_{trt} (3.1 ± 0.6 cm). Cumulative SOC stocks were significantly less at all depths below the litter layer for T_{trt} compared to C_{trt}, with a difference of 28% to a depth of 150 cm (Figure 7). The portion of this difference that occurred below 20 cm was 72%. FT_{trt} SOC stock to 150 cm in depth approximated the average of the other two treatments and was not significantly different from either. Cumulative N stocks followed similar trends (Figure 7). T_{trt} had 29% less soil N than C_{trt} to a depth of 150 cm, with 76% of this difference occurring below 20 cm.

The mass-based estimation of SOC and N stocks yielded similar results to the fixed-depth approach (Figure 8). Total differences in SOC and N stocks between C_{trt} and T_{trt} were 25% and 27%, respectively, slightly less than for the fixed-depth approach. The portion of these differences that occurred in deeper soil below ~20 cm was 56% and 64%, respectively.

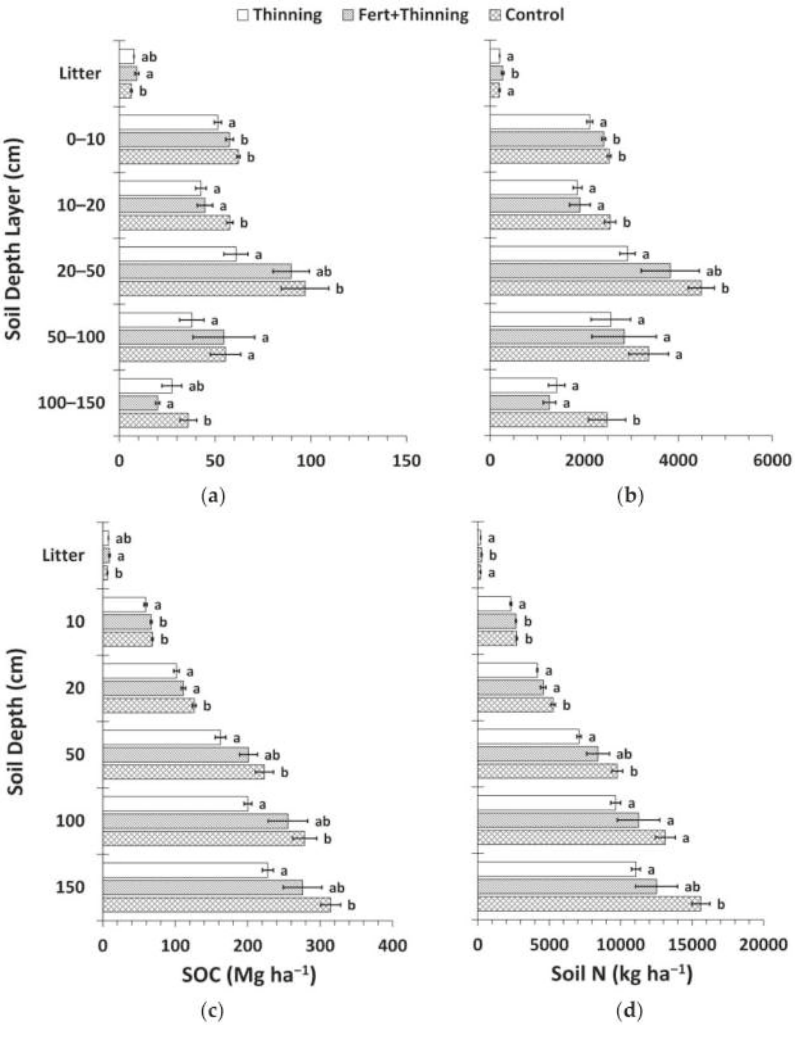

Figure 7. Mean soil organic carbon (SOC) and nitrogen (N) stocks by treatment calculated using the fixed-depth approach (Equations (2) and (3)). (**a**) Mean SOC stock by soil depth layer; (**b**) mean soil N stock by soil depth layer; (**c**) cumulative mean SOC stock; (**d**) cumulative mean soil N stock. Sample size for all treatments and soil depth layers is three. Error bars represent ± one standard error. Means within each soil depth layer accompanied by the same letter (i.e., "a" or "b") are not significantly different (Tukey's HSD, $\alpha = 0.1$). Fert, fertilized.

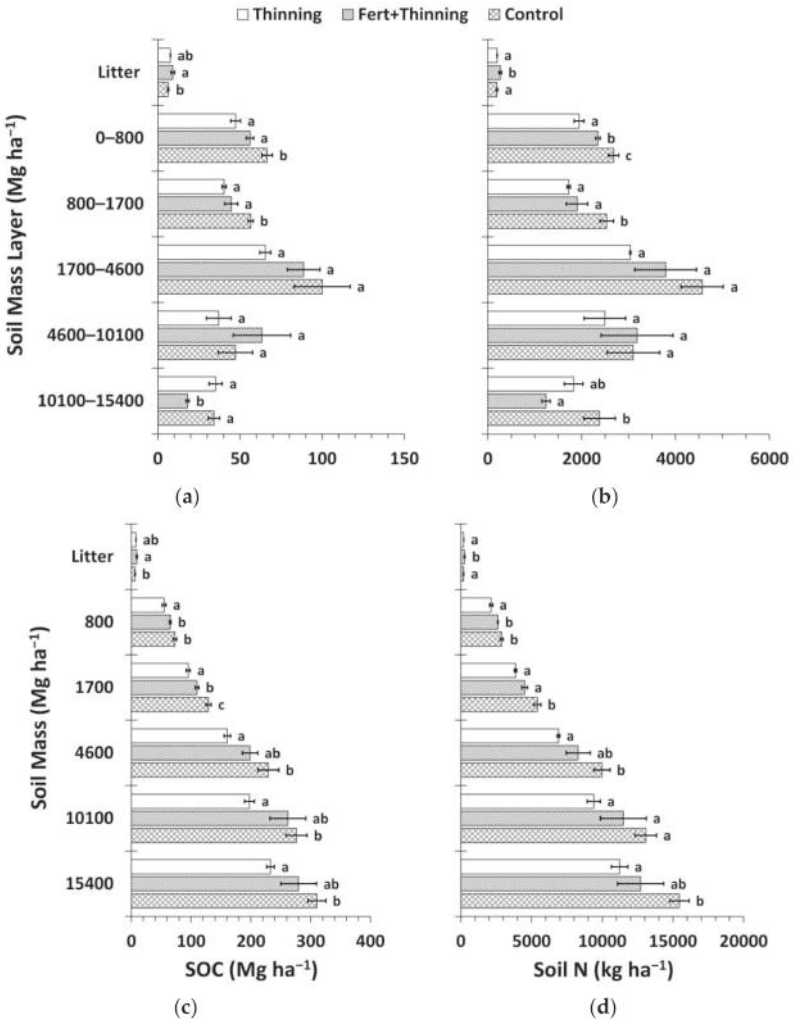

Figure 8. Mean soil organic carbon (SOC) and nitrogen (N) stocks by treatment calculated using the mass-based approach (Equation (4) and web-accessible spreadsheet [59] created by Wendt and Hauser [58]). (**a**) Mean SOC stock by soil depth layer; (**b**) mean soil N stock by soil depth layer; (**c**) cumulative mean SOC stock; (**d**) cumulative mean soil N stock. Sample size for all treatments and soil depth layers is three. Error bars represent ± one standard error. Means within each soil depth layer accompanied by the same letter (i.e., "a" or "b") are not significantly different (Tukey's HSD, $\alpha = 0.1$). Fert, fertilized.

3.4. Carbon Stocks and Sequestration

Total and belowground C stocks followed the same pattern as SOC stocks, with C_{trt} and T_{trt} having the highest and lowest C stocks, respectively (Figure 9). Trends in aboveground and root C sequestration were the opposite, with C_{trt} and T_{trt} having the lowest and highest C stocks, respectively. However, none of these differences were significant. Approximately half or more of total C stocks were contained in the soil across treatments (47%, 54%, and 58% for T_{trt}, FT_{trt}, and C_{trt}, respectively).

Initial trees per hectare was a significant predictor of aboveground (p = 0.0031; r^2 = 0.7363), root (p = 0.0026; r^2 = 0.7477), and total C stocks (p = 0.0479; r^2 = 0.4503) and was positively related to these three variables. There was no relationship between initial trees per hectare and SOC stock (p = 0.5513; r^2 = 0.053). Soil organic C stock was a significant predictor of total C stock, the two variables being positively related, and explained more of the variation in total C stock than initial trees per hectare (p = 0.0041; r^2 = 0.7143).

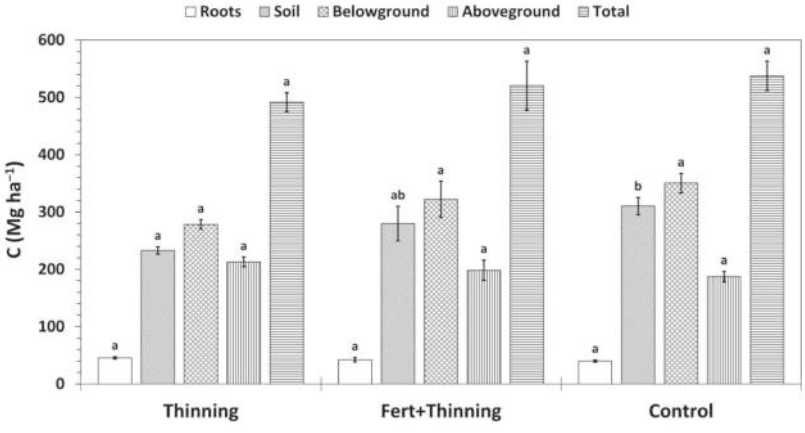

Figure 9. Mean carbon (C) stocks by treatment. Total C stocks are the sum of above- and belowground C stocks, where aboveground and root C stocks include the biomass of thinned trees, and belowground C stocks are the sum of root C and soil organic C (calculated using the mass-based approach). Sample size for all treatment groups and categories is three. Error bars represent ± one standard error. Means within each category accompanied by the same letter (i.e., "a" or "b") are not significantly different (Tukey's HSD, α = 0.1). Fert, fertilized.

3.5. Merchantable Volume

Merchantable volume, calculated in thousands of board-feet per acre (as 9.8-m long logs to a 15-cm top diameter), was significantly greater for FT_{trt} than T_{trt}, a difference of ~11% (41.1 ± 0.4, 45.6 ± 1.3, and 43.0 ± 1.2 for T_{trt}, FT_{trt}, and C_{trt}, respectively). No other variable (e.g., SOC or N stock, initial trees per hectare, and DBH) was significantly related to merchantable volume. Final mean DBH (cm) was greatest for FT_{trt} and lowest for T_{trt}, but these differences were not significant (13.0 ± 0.9, 16.2 ± 1.4, and 14.0 ± 1.6 for T_{trt}, FT_{trt}, and C_{trt}, respectively). Initial trees per hectare was a significant predictor of DBH, the two variables being negatively related (p = 0.0024; r^2 = 0.7529).

4. Discussion

It is important to first determine which approach, fixed-depth or mass-based, provided a more accurate quantification and comparison of SOC and N stocks. While the fixed-depth and mass-based approaches resulted in similar conclusions overall, the mass-based approach appears to have better represented the degree of change along the vertical soil profile by comparing equal soil masses and eliminating bulk density as a factor. Because bulk density was significantly higher for T_{trt} than C_{trt} above 20 cm, the mass-based approach resulted in greater SOC and N stock differences between these two treatments in the surface soil layers compared to the fixed-depth approach. Significant differences in bulk density observed between treatments were most likely the result of changes in SOC concentrations. This conclusion is supported by the lack of heavy equipment used during thinning treatments (i.e., a lack of soil-compacting operations), as well as the fact that significant differences in bulk density between treatments coincided with significant differences in SOC concentrations and

occurred in soil layers where SOC concentration was a significant predictor of bulk density. In order to account and correct for changes in bulk density with time (that have not resulted from soil erosion or deposition), the mass-based approach is increasingly recommended for SOC and other soil-nutrient inventories [58,60,70–73]. Soil organic C and N stocks quantified using the mass-based approach and compared among equal soil masses will be considered the more accurate account in the current study, as this process removed changes in bulk density as a confounding variable. Further discussion will refer to the mass-based approach and mass-based calculations of SOC and N stocks.

Litter layer SOC and N stock differences between FT_{trt} and C_{trt} primarily resulted from the greater thickness of FT_{trt} litter layer compared to C_{trt}, rather than from differences in SOC and N concentrations. This greater litter layer thickness may have been due to increased N availability post-fertilization treatments and thus increased understory biomass and turnover [12]. The significantly lower litter layer soil N concentration of T_{trt} compared to the other two treatments likely resulted from forest floor organic matter additions with high C:N ratios (such as coarse woody debris left as slash on the forest floor) and subsequent microbial N scavenging in this layer [21,22]. High microbial demand for N during organic-matter decomposition for FT_{trt} post-thinning was likely compensated for by the addition of N via fertilization treatments.

In the mineral soil, SOC and N stock differences between the treatments were due to differences in SOC and N concentrations. Cumulatively, T_{trt} and FT_{trt} contained 77.6 and 30.5 Mg ha^{-1} less SOC, respectively, than C_{trt} to a depth of ~150 cm. The difference between T_{trt} and C_{trt} SOC stocks was significant and occurred over a shorter post-treatment timeframe (~11 years) than the difference observed between FT_{trt} and C_{trt}. Assuming equivalent SOC stocks pre-treatment, the rate of post-treatment SOC loss for T_{trt} was ~700 g C m^{-2} y^{-1} compared to C_{trt}. The typical range of SOC accumulation rates in temperate forest soils is ~2 to 70 g C m^{-2} y^{-1} [74], with an average rate of 34 g C m^{-2} y^{-1}. Raich and Schlesinger [75] reported a mean soil respiration rate for temperate coniferous forests of 681 g C m^{-2} y^{-1}. However, rates ranging between ~950 and 1750 g C m^{-2} y^{-1} have been measured [19,20,76]. Using average C flux rates, we estimate a potential T_{trt} post-treatment respiration rate of ~1350 g C m^{-2} y^{-1}, which falls within the published range of soil respiration rates for temperate coniferous forests. Of course, other mechanisms of SOC (and N) loss, such as increased leaching and export, could also help explain observed differences in SOC and N stocks between treatments.

Decreased radiation interception by trees post-thinning could have increased soil temperatures, enhancing microbial metabolic activity and potentially accounting for some of the loss of SOC and N from T_{trt} compared to C_{trt} [16–20]. Additionally, greater bulk density in the surface soil layers of T_{trt} compared to C_{trt} could have increased heat-transfer rates to deeper soil layers [20,21] where the majority of the SOC stock was contained and also lost. Fresh C inputs and the creation of preferential flow paths due to the mineralization of root biomass, which can persist for many years following the harvest of trees [31], and increases in DOC flux due to additional organic matter inputs and decreased transpiration and rain interception [29,30] could have increased SOC decomposition via priming post-thinning [23–25,32–34]. The soil sampled in the current study may be particularly vulnerable to rapid SOC decomposition when subjected to changing environmental conditions and potential priming effects, as it is an older soil that likely developed and accrued SOC over hundreds of thousands to millions of years under relatively stable conditions [19,23–28,77].

In the surface soil layers (0–20 cm in depth), which contain the majority of root [78] and microbial [79] biomass, the mean C:N ratio across treatments was ~24, suggesting tight N cycling and the potential for N scavenging, particularly after the addition of high C:N slash to the forest floor post-thinning [21,22]. However, nitrate leaching, which has been observed to increase under slash left on the forest floor [35–39], is also a possible mechanism of N loss and SOC loss via priming [24,25]. FT_{trt} may have been particularly vulnerable to nitrate leaching post-thinning treatments due to reduced N uptake by roots coinciding with N fertilization. The lower pH in the upper 100 cm of mineral soil and litter layer of FT_{trt} compared to the other two treatments indicates that a considerable portion of nitrate

resulting from the nitrification of urea potentially was not taken up by plants, causing the net addition of one proton (H^+) to the soil solution per urea compound [80]. Nitrate leaching would have been generally promoted by the high precipitation in the region studied. Notably, several of the excavated pits had redoximorphic features as high as 75 cm in depth, indicating a relatively high or perched water table. A previous study conducted at the same site identified soil features (massive, clay cemented) below 3 m that could result in a perched water table [81]. As Douglas-fir roots commonly extend to at least 3 m in depth [47], thinning treatments may have sufficiently reduced transpiration to allow a local rise in groundwater level, particularly during the wet season. This rise would have enabled the groundwater to contact more superficial soil layers that have higher DOC and N concentrations, potentially increasing the export of DOC and various forms of N [37,41].

Although SOC stocks were significantly greater for C_{trt} than T_{trt}, differences in belowground and total C stocks between these two treatments were diluted due to T_{trt} having greater aboveground and root C stocks, which increased with initial trees per hectare. On average, T_{trt} had substantially higher initial trees per hectare than C_{trt} (952 and 682 trees ha^{-1}, respectively). Interestingly, there was no relationship between SOC stock and initial trees per hectare. This is somewhat counterintuitive when thinning treatments, which did affect SOC stocks, were carried out similarly within about a decade of the reductions in initial trees per hectare. However, several key differences may explain this phenomenon. While thinning treatments occurred after crown closure, thus exposing previously shaded and covered areas, reductions in initial trees per hectare occurred prior to crown closure and would not have drastically changed soil conditions. More substantial understory cover prior to crown closure likely insulated the soil from significant temperature changes and increased N uptake following reductions in initial trees per hectare, decreasing nitrate leaching and helping to retain N on-site [82,83]. At this early stage in stand and plant development, leaves and roots would have had a lower C:N ratio [84], reducing the potential for N scavenging during the decomposition of organic matter left on-site. Additionally, roots would have been less dense and rooted less deeply in the soil, likely decreasing DOC flux and the potential for priming effects compared to thinning treatments implemented years later. Differences in soil microbial communities can also lead to differences in SOC and nutrient dynamics. Smith et al. [85] found that soil microbial communities differed between younger and older forests in a study examining forests aged 20 years and older. Although initial trees per hectare were reduced at the site in the current study when the stand was aged <15 years, differences in ground and soil conditions—and thus differences in microbial communities—would likely be greater between juvenile and adult stands than between younger and older adult stands. The lack of relationship between SOC stock and initial trees per hectare suggests that potentially lower SOC accumulation over time due to reduced root C inputs (from reduced root biomass) post-thinning did not play a substantial role in decreasing the SOC stock of T_{trt} compared to C_{trt}.

Despite greater merchantable volume for FT_{trt} compared to C_{trt}, any net monetary gains would have been minimal due to the additional expenses of fertilization and thinning treatments. T_{trt} resulted in the least financial gain over the length of the rotation (i.e., it had the lowest merchantable volume in addition to incurring thinning expenses) and reduced soil quality and nutrient stocks for the succeeding rotation. On the other hand, reducing the initial trees per hectare prior to crown closure did not affect SOC and N stocks and the variation in total C stock was explained more by SOC stock (71%) than by initial trees per hectare (45%). Therefore, the typically low-cost practice of reducing initial trees per hectare could provide benefits such as increased stand stability, health, and DBH, while potentially avoiding negative effects such as reducing SOC and N stocks.

5. Conclusions

This long-term study shows that forest-management practices can affect both surface and deep SOC and N stocks on decadal timescales. Thinning treatments reduced SOC and N stocks by 25% and 27%, respectively, with most of this loss occurring below ~20 cm to a depth of ~150 cm. Changing the soil environment by affecting the ecosystem, C inputs, roots, and soil properties can increase

SOC decomposition, N mineralization, and SOC and N export. In this study, a combination of these factors and their complex interactions likely resulted in the observed decreases in SOC and N stocks post-thinning. Although thinning treatments had a negative effect on SOC and N stocks, reducing the initial trees per hectare prior to crown closure is a low-cost alternative practice that could provide the stand benefits associated with thinning a more mature stand without decreasing soil fertility and, potentially, the productivity of future stands. Additional field studies should examine the mechanisms behind SOC and N losses post-thinning (particularly in deeper soil layers) and the effects on stand and soil dynamics of reducing the initial trees per hectare at various intensities and stages prior to crown closure. As the majority of SOC and N stocks are contained in deeper soil layers, accurately assessing SOC and N budgets and comparing changes over time requires sampling soil deeper than 20 cm.

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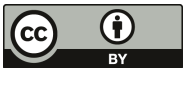

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Diversity and Enzyme Activity of Ectomycorrhizal Fungal Communities Following Nitrogen Fertilization in an Urban-Adjacent Pine Plantation

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Abstract: Rapid economic development and accelerated urbanization in China has resulted in widespread atmospheric nitrogen (N) deposition. One consequence of N deposition is the alteration of mycorrhizal symbioses that are critical for plant resource acquisition (nitrogen, N, phosphorus, P, water). In this study, we characterized the diversity, composition, and functioning of ectomycorrhizal (ECM) fungal communities in an urban-adjacent *Pinus elliottii* plantation under ambient N deposition (~24 kg N ha^{-1} $year^{-1}$), and following N fertilization (low N, 50 kg N ha^{-1} $year^{-1}$; high N, 300 kg N ha^{-1} $year^{-1}$). ECM functioning was expressed as the potential activities of extracellular enzymes required for organic N (protease), P (phosphomonoesterase), and recalcitrant polymers (phenol oxidase). Despite high ambient N deposition, ECM community composition shifted under experimental N fertilization, and those changes were linked to disparate levels of soil minerals (P, K) and organic matter (but not N), a decline in acid phosphatase (AP), and an increase in phenol oxidase (PO) potential activities. Based on enzyme stoichiometry, medium-smooth exploration type ECM species invested more in C acquisition (PO) relative to P (AP) following high N fertilization than other exploration types. ECM species with hydrophilic mantles also showed higher enzymatic PO:AP ratios than taxa with hydrophobic mantles. Our findings add to the accumulating evidence that shifts in ECM community composition and taxa specialized in organic C, N, and P degradation could modulate the soil nutrient cycling in forests exposed to chronic elevated N input.

Keywords: extracellular enzymes; hyphal exploration strategy; China; atmospheric nitrogen deposition; *Russula*

1. Introduction

Atmospheric nitrogen (N) deposition has more than doubled the inputs of N into many forest systems. One consequence of N deposition has been the increase in forest productivity [1]. Another is the alteration of soil microbial communities, and especially mycorrhizal communities [2–4]. Most forest trees, such as Pinaceae, depend on symbioses with ectomycorrhizal fungi (ECM) for resource uptake (nitrogen, N, phosphorus, P, water) from soil, and different ECM taxa appear to specialize in various forms of organic resources (reviewed in Lilleskov et al. [5]). As a result, mycorrhizal diversity may underpin many forest ecosystem services including nutrient cycling and water use efficiency. Although

studies have widely demonstrated declines in ECM diversity and changes in ECM community composition following N-enrichment [3,6–12], the extent to which these changes influence the functional capacity of ECM is less well understood [13–16]. In this study, we examined the diversity and functioning in ECM communities under ambient N deposition and following N fertilization in an urban-adjacent *Pinus elliottii* plantation.

Enzymatic activities comprise one type of ECM functional trait that can be directly linked to ecosystem nutrient cycling [17]. Most ECM fungal taxa produce a diversity of extracellular and cell wall-bound hydrolytic and oxidative enzymes that mobilize the release of smaller organic molecules (potential C, N, or P sources) from soil organic matter (SOM) [17], including polyphenol–protein complexes. Studies have revealed substantial interspecific differences in ECM enzymatic activities [18–28]. Such differences can be predicted in part by ECM life history strategies. Key among these is the abundance and morphology of external hyphae among ECM taxa, also referred to as "hyphal exploration strategy" [29]. Each exploration type can vary in its capacity for enzymatic mobilization, uptake, and transfer of nutrients to the host. For example, long-distance exploration types form extensive networks of hyphae and rhizomorphs, are typically abundant in N-limited soils, and appear to be specialized in N-acquisition from complex organic substrates. Conversely, contact-types are more frequently detected in mineral soils, show lower proteolytic capabilities, and access inorganic N sources that are more readily assimilated [6,29]. Differences in ECM taxa and their exploration strategies could therefore have an impact on tree nutrition through changes in their morphological and functional (enzymatic) traits.

Biotic (host C allocation) and abiotic (climate, soil nutrients, pH) factors can also influence ECM enzyme activities. Ectomycorrhizal fungi may respond to shortages in host C allocation by up-regulating the activity of enzymes used to obtain labile carbohydrates [13], while changes in the relative availabilities of N and P are known to modify the activity of extracellular N- and P-mobilizing enzymes. For example, N fertilization could accelerate the degradation of easily decomposable litter and reduce the activity of extracellular ECM enzymes targeting recalcitrant litter with high levels of lignin and complex organic forms of N [24–28]. Both outcomes may reflect the stimulation or repression of different sets of enzymes. In addition, the activity of P-mobilizing enzymes has been shown to increase following N fertilization as a way to offset plant P demand [7]. However, neutral and negative effects have also been noted [7]. Any changes in the activity of these enzymes may reflect alterations in the ECM community and the physiological functioning of their constituent species. Such shifts, in concert with declines in ECM root colonization following N fertilization [30], could feedback to impact plant nutrient uptake.

Much of our knowledge of N enrichment effects on ECM communities has been obtained from studies in North American and European forests. However, forests in China have also experienced increasing inputs of anthropogenic N deposition owing to rapid economic development, urbanization, and intensified agricultural activities [31–34]. In the forests of south-central China, dry N deposition contributes ~24 kg N ha^{-1} $year^{-1}$ (as NH_4-N) derived from power generation, traffic, and intensive fertilizer applications. In this region, forest plantations are comprised of a fast-growing non-native pine (*Pinus elliottii*, slash pine) that was planted to ameliorate land degradation. Although ECM fungi are critical for the growth and nutrition of *Pinus* species, it is unclear how interactions between N enrichment and a non-native pine could feedback to alter ECM communities and their ecosystem function in soil nutrient dynamics [35,36].

In this study, we examined the link between the ECM community structure and functioning under ambient N-deposition and following N fertilization in a *Pinus elliottii* (slash pine) plantation. To put our study in context with previous research, we first examined the effect of ambient N deposition and N fertilization on soil fertility and ECM community composition, diversity, and root colonization. Next, we tested the capacity of ECM fungal colonized root tips to produce an oxidative enzyme involved in the degradation of recalcitrant plant residues (phenol oxidase), and hydrolytic enzymes for organic N (protease) and P (phosphomonoesterase) mobilization. We used these results to address two questions:

(1) Are there parallel shifts in ECM fungal community structure and functioning with increasing N availability?; (2) Are there ECM fungus species-specific differences in N and P enzyme activity, and if so, do these changes reflect soil fertility or other factors (e.g., hyphal exploration)?

2. Materials and Methods

2.1. Study Site

Our study was undertaken in 35-year-old slash pine (*P. elliottii*) stands at the Hunan Forest Botanic Garden (113°02′03′ E, 28°06′07′ N), Changsha city, Hunan Province, China. The climate is typical subtropical humid monsoon with a mean annual temperature of 17.4 °C and annual precipitation of 1549 mm, most of which occurs between April and October. The soil is classed as an Alliti–Udic Ferrosol (equivalent to Acrisol; IUSS Working Group WRB, 2006), which is generally a clay loam red soil developed from slate and shale parent rock. These soils are acidic (pH = 4.14–4.21 [31]) with deficiencies of SOM and P, and high levels of Fe and Mn (Table 1).

Nine plots (each 10 m × 10 m) enclosing at least five pine trees were established in June 2010 using a completely randomized design. A 3 m buffer zone was installed around each plot to prevent N fertilizer contamination among plots. Three plots were randomly allocated to each of three N fertilizer levels: control (ambient N, no fertilization), low (50 kg N ha^{-1} $year^{-1}$), or high nitrogen (300 kg N ha^{-1} $year^{-1}$). The fertilization rates represent the expected input from N deposition in the near future (low N), as well the potential long-term cumulative N inputs from atmospheric deposition (high N [31–34]). Nitrogen fertilization treatments were applied twice a year (January, June) for three years as a solution of NH_4NO_3 uniformly sprayed across the plot. Control plots were sprayed with a similar volume of deionized water.

Table 1. Mean levels of soil nutrients in control and N-fertilized plots. Data represents mean with the standard error in parentheses.

	N Fertilization Level		
Soil nutrient	Control (n = 36)	Low (n = 36)	High (n = 36)
Organic matter (g kg^{-1})	26 (2) ab	21 (2) b	28 (2) a
Total N (g kg^{-1})	1.34 (0.1) a	1.26 (0.1) a	1.48 (0.1) a
Organic C:N	12 (1) a	10 (1) a	12 (2) a
Available N (μg g^{-1} soil)	26 (1.9) a	25 (1.3) a	27 (1.7) a
Total P (μg g^{-1} soil)	110 (4) b	122 (5) a	115 (4) b
Available P (μg g^{-1} soil)	3.5 (0.1) c	4.6 (0.2) a	3.0 (0.1) b
N:P	12 (1) a	11 (1) a	13 (1) a
K (μg g^{-1} soil)	69 (6.8) a	64 (2.7) a	41 (1.8) b
Ca (μg g^{-1} soil)	193 (18) a	257 (15) a	216 (16) a
Mg (μg g^{-1} soil)	896 (22) b	1051 (20) a	977 (23) a
Fe (μg g^{-1} soil)	15,986 (266) b	16,273 (140) b	17,034 (169) a
Mn (μg g^{-1} soil)	80 (7) b	101 (6) a	114 (6) a
CEC (cation exchange capacity)	20 (1.6) a	18 (1.2) a	19 (1.8) a

Means within rows with the same letter do not differ significantly at $p < 0.05$ by Tukey's Honestly Significant Difference (HSD) test.

2.2. Sample Collection

Three slash pine trees in each plot were sampled for ECM fungi in August 2013. Four soil cores (10 cm diameter, 15 cm deep), representing one core from each of the cardinal directions, were collected for each tree (total n = 12 cores per plot). Soil cores were placed in individual plastic bags and then stored at 4 °C until processing (within 7 days). A sub-sample of soil from each core was sieved to 2 mm and analyzed for soil N, P, K, Ca, Mg, Fe, Mn, and organic matter (OM, organic C × 1.724) at the National Engineering Laboratory for Applied Technology of Forestry and Ecology in South China. Analytical methods are detailed in supporting materials (Supplementary S1). Soil cation exchange

capacity (CEC) was calculated as the sum of exchangeable cations (K, Ca, Mg) on an equivalent basis. The remaining soil in each core was sieved over 2 and 0.25 mm sieves. Fine roots collected on each sieve were gently washed to remove adhering soil and pooled for each tree. To quantify ECM root colonization, six roots (~10 cm long) per tree were randomly selected and examined by counting ECM root tip numbers. Every root tip was examined under 40× magnification for the presence of ECM colonization (i.e., turgid, swollen root tips with a well-developed mantle), and then sorted into morphological categories based on mantle color and texture, and the morphology of external hyphae on the root tip [5,29] and the publicly available database DEEMY (http://www.deemy.de/).

2.3. Enzyme Assays

Three extracellular enzymes: acid phosphatase (AP, EC 3.1.3.2), protease (PRO, EC 3.4.23), and phenol oxidase (PO, EC 1.14.18.1) were assayed. The enzyme substrates were 5 mM p-NP (*p*-nitrophenyl phosphate) for AP; 25 mM L-DOPA (L-3, 4-dihydroxyphenylalanine) for PO; and a general proteolytic substrate, Azocoll® (<50 mesh, Calbiochem-Behring Corp. La Jolla, CA, USA) for PRO [37]. All substrates were prepared in 50 mM sodium acetate-acetic acid buffer (pH 5.0, Sigma Chemical, Co. St. Louis, MO, USA). Root tip enzyme activity was assayed using the high-throughput microplate method described by Pritsch et al. [20], with one modification. Instead of a porous microplate, we constructed strips of eight plastic microscopy capsules (diameter 5 mm, height 15 mm) that were perforated at the base (Leica catalog no. 16702738); each strip of capsules fit into eight wells of a 96-well microplate.

For each classified morphotype per tree (generally 3–5 morphotypes), seven active root tips of the same diameter were selected and trimmed to 4 mm length, and one root tip was then placed in an individual capsule. Using ECM root tips with trimmed ends may have introduced intracellular enzymes into the analyses. However, hydrolytic (e.g., AP) and oxidative (e.g., PO) enzymes tend to be unaffected by cell lysis [38], and intracellular phosphatase tends to have an alkaline pH optima [39]. Non-colonized roots were not assayed for enzyme activity, as these frequently host saprophytic fungi with similar enzyme capacities as ECM [37].

Strips of capsules containing root tips were incubated in microplate wells each containing 100 µL of an individual substrate for 1 h at 37 °C (AP, PO) or two-hours (PRO) in the dark. As a control, root tips were bathed in buffer for each incubation step. At the end of each incubation period, capsules were removed from the microplate, and enzyme reactions terminated by the addition of 100 µL of sterile water (PRO, PO) or 100 µL NaOH (pH > 10, 0.2 M; AP) to each well, and absorbance measured at 405 nm (AP), 520 nm (PRO), or 450 nm (PO). After the assays were completed, each root tip was removed from the well, rinsed in deionized water, and three root tips of each morphotype per tree were frozen at −80 °C for later molecular identification, while the remaining root tips were dried to constant weight (65 °C). All measured enzyme activities were calculated per gram dry weight root per hour, and averaged among the weighted root tips of the assay group.

2.4. Identification of ECM Fungi on Root Tips

DNA from ECM root tips was extracted using DNeasy Plant Mini Kit (Qiagen SA, Coutaboeuf, France) following the manufacturer's instructions. Genomic DNA was amplified using the ITS1-F/ITS4 primer pair [40,41], after which the PCR products were visualized by gel electrophoresis. Samples with single bands were prepared for sequencing using ITS-4 and Big Dye Terminator Kit (Applied Biosystems, Foster City, CA, USA), and analyzed on an Applied Biosystems 3130xl Sequencer. PCR products with multiple bands were cloned using TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). Successfully cloned colonies were amplified using primer pair M13F/M13R, screened using gel electrophoresis for the appropriate sized PCR products, and sequenced.

Sequences were manually aligned and edited in CodonCode Aligner 4.2.4 (CodonCode, Co. Centerville, MA, USA) and sequence homologies determined using the Basic Local Alignment Search Tool algorithm (BLAST v2.2.29 [42]) or the UNITE database v7 [43] for operational taxonomic unit

(OTU) clustering. A root tip sample was considered a species match to a database taxon if their sequences had 97% or greater similarity and were aligned over at least 450 base pairs. If no match could be made, a taxonomic placement was made by aligning the sample sequence with representative sequences of fungi from the major ECM clades. The same criteria for BLAST species matching were used to assign a taxonomic identity. Sequences from this study have been deposited in NCBI with access numbers KP866117–KP866136.

2.5. Data Analyses

All analyses—except species richness and diversity estimates—were completed in R 3.1.2 (R Project for Statistical Computing; http://www.R-project.org) with the "vegan" package [44]. Differences in the levels of soil nutrients between each treatment were analyzed using a one-way analysis of variance (ANOVA), followed by pairwise comparisons using the Tukey's Honestly Significant Difference (HSD) test. Data sets were cube root transformed before ANOVA to meet the assumptions of normality. Estimates of ECM species richness (Mao Tau, $Chao_2$, $Jackknife_2$) and diversity (Shannon-Wiener; Simpson) were calculated for each treatment in EstimateS using 50 randomizations with replacement [45]. Differences in ECM species richness and diversity and ECM root colonization among treatments were analyzed using one-way ANOVA and Tukey's HSD.

The effect of N fertilization on ECM community composition was tested using non-metric multidimensional scaling (NMDS) using Bray–Curtis dissimilarities followed by permutational analysis of variance (PERMANOVA) to test for ECM compositional differences between treatments (999 permutations). We then used vector fitting to the NMDS ordination to determine the effects of soil nutrients; significance values were generated using 999 random permutations. The effect of N fertilization on ECM community enzyme activity was similarly analyzed using NMDS and PERMANOVA.

Potential enzyme activities were used to calculate the relative contribution of each ECM species to community enzyme activity as well as changes in enzyme stoichiometry (also known as enzyme acquisition ratios) between N fertilization treatments. The relative contribution (RC) of each ECM species to community enzyme activity in each N fertilization treatment was calculated as

$$(\text{activity}_{species} \times \text{root tip abundance}_{species})/\text{total activity of the ECM community}$$

where *species* represents an individual ECM species, and total activity of the ECM community was calculated as [13,46]

$$\sum(\text{activity} \times \text{root tip abundance})_{species}.$$

Enzyme data were also used to calculate enzyme stoichiometry (also known as enzyme acquisition ratios) in each N fertilization treatment as PRO:AP, PO:PRO, and PO:AP.

Differences in potential enzyme activity (AP, PRO, PO) and stoichiometry (PRO:AP, PO:PRO, PO:AP) between N fertilization treatments and between ECM exploration and mantle types in response to N fertilization treatments were analyzed using mixed-effect ANOVA with N-treatments (control, low N, high N) and mantle type (hydrophilic, hydrophobic) or exploration type (contact, short-, and medium-distance) [5,29] as fixed effects and plots as random effects. ANOVA were followed by comparisons using Tukey's Honestly Significant Difference (HSD) test for significant variables. Relationships between the enzyme activity and ECM root colonization or soil factors were tested using Spearman's r correlation test. The relative enzyme activity in each ECM species was compared and analyzed against the community average in each enzyme and N fertilization level using *t*-tests. Data sets were square root (enzyme potential) or arcsine square root transformed (root colonization, enzyme ratios) before analyses to meet the assumptions of normality.

3. Results

3.1. Soil Fertility

Nitrogen fertilization resulted in significant increases in levels of Mn and Mg, and declines in available P and K relative to control plots. Levels of soil OM and Fe were highest in the high N fertilization treatment (Table 1). However, there was no significant effect of N fertilization on total or available soil N, C:N, total P, Ca, or CEC (18–20 ± 1.5 Meq).

3.2. ECM Community Composition and Diversity

Root tips (742; Control: 294; Low N: 224; High N: 224) were sorted into morphological groups, and from these tips, and we submitted 318 root tips for molecular analysis and successfully recovered 350 sequences, including several clones from double bands PCR products. Using a 97% sequence similarity cut-off, we identified 257 sequences representing 24 unique OTUs (hereafter referred to as species) that were ECM (Table 2). The remaining fungi were taxa traditionally considered as saprotrophic (*Paecilomyces, Sphaeropsis, Penicillium*) or of uncertain mycorrhizal status (e.g., *Basidiodendron*, *Mycena*).

Members of the Helotiales (Ascomycota) and Thelephoraceae (Basidiomycota) dominated the ECM community. Many ECM species were detected in both the control and N-fertilized plots, including *Tylospora* (Atheliaceae), *Lactarius* (Russulaceae), and members of the Ascomycota (e.g., *Helotiales* spp.). Nine ECM fungi were absent in control plots (e.g., *Scleroderma*) while an additional three taxa were N-sensitive and recovered only in the control plots (e.g., *Cenococcum* sp. 2). Levels of species richness and diversity did not differ significantly between N-fertilized and control plots (Table 3). Trees in Control (42 ± 9%) and Low N plots (44 ± 10%) showed significantly higher levels of ECM root colonization relative to high N plots (21 ± 6%; $p = 0.039$).

NMDS showed that ECM communities from N fertilization treatments were separated from one another in ordination space (Figure 1a; $p < 0.05$ for PERMANOVA among all three treatments). ECM community composition was significantly correlated with mineral nutrients (Mg, K, P) and organic C, as these resources decreased (mineral nutrients) or increased significantly (soil organic matter) in high N plots (Table 1).

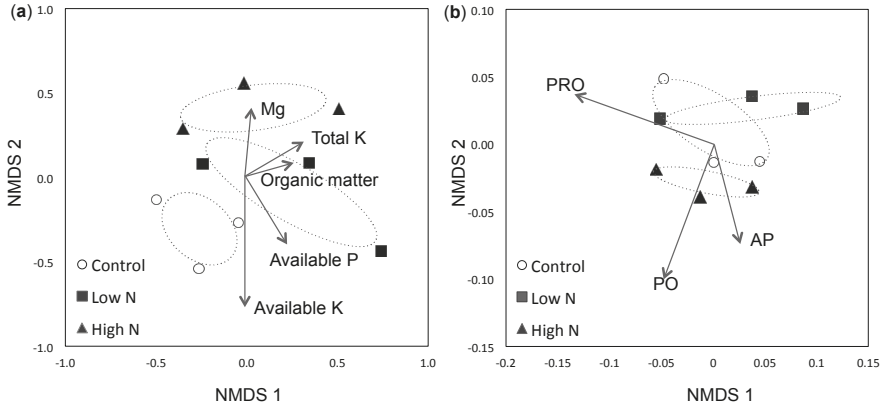

Figure 1. Non-metric multidimensional scaling (NMDS) ordination of ectomycorrhizal (ECM) fungal communities in control and N-fertilized plots based on: (**a**) the ECM root tip community, (**b**) the activity of extracellular enzymes. Each point represents the fungal community composition in each plot. Significant environmental variables are shown: P-phosphorus; K-potassium; Mg-magnesium. AP: acid phosphatase; PO: phenol oxidase; PRO: protease.

Table 2. Identification of ectomycorrhizal fungal operational taxonomic unit (OTU)s associated with *Pinus elliottii* growing at the study site in Hunan botanic garden, China. Species for group level enzyme analysis were tagged in bold font.

OTU	Accession Number [a]	Closest Blast Match in Genbank [b]	Query/Aligned length (bp) (similarity %) [c]	Closest UNITE Species Match	No. Of root tips/Frequency [d]		
					Control	Low N	High N
***Tylospora* sp.**	KP866117	HM189733 Corticiaceae sp. BB-2010	632/637 (99)	SH192265.07FU	29/2	13/1	21/3
***Atheliaceae* sp.**	KP866118	AB839405 Uncultured ECM fungus	475/512 (93)	SH193510.07FU	0	1/1	0
***Cenococcum* sp.1**	KP866119	JQ347051 Uncultured *Cenococcum*	567/571 (99)	SH214459.07FU	7/1	28/2	0
***Cenococcum* sp.2**	KP866120	JX456699 Uncultured fungus	486/497 (98)	SH214466.07FU	14/3	0	0
***Helotiales* sp.1**	KP866121	KF007259 Uncultured ECM fungus	608/618 (98)	SH214286.07FU	18/4	24/5	40/6
***Helotiales* sp.2**	KP866122	AB571492 Uncultured ECM fungus	637/639 (99)	SH023418.07FU	16/2	7/1	15/2
***Helotiales* sp.3**	KP866123	AB769894 Uncultured Helotiales	550/551 (99)	SH201717.07FU	14/2	7/1	27/2
Helotiales sp.4	KP866124	HM208727 Fungal sp. Phylum141	560/562 (99)	SH196495.07FU	1/1	0	0
Helotiales sp.5	-	FN397286 Uncultured fungus	499/536 (93)	SH211375.07FU	0	0	1/1
Lactifluus parvigerardii	KP866125	JF975641 *Lactifluus parvigerardii* XHW-2011	571/574 (99)	SH012454.07FU	7/1	7/1	7/1
Phialocephala fortinii	KP866127	KF313098 *Phialocephala* sp. YJM2013	562/564 (99)	SH204999.07FU	1/1	0	2/1
***Russula* sp.1**	KP866128	JX457011 Uncultured fungus	683/696 (98)	SH017121.07FU	0	7/1	7/1
Russula virescens	KP866129	KM373243 *Russula crustosa*	669/716 (93)	SH179774.07FU	0	1/1	0
***Russula* sp.2**	KP866130	AB597671 Fungal sp. JK-02M	580/582 (99)	SH017122.07FU	0	7/1	0
Scleroderma yunnanense	KP866131	JQ639046 *Scleroderma yunnanense* XEX-2012	584/588 (100)	SH189277.07FU	1/1	0	1/1
Scleroderma citrinum	KP866132	AB769913 Uncultured *Scleroderma citrinum*	561/569 (99)	SH008294.07FU	0	0	1/1
Sebacinaceae sp.	KP866133	KF000673 Uncultured *Sebacina* clone	628/657 (96)	SH214656.07FU	0	0	1/1
Thelephora terrestris	KP866134	KJ938034 Uncultured fungus	677/706 (96)	SH184510.07FU	7/1	14/2	0
***Tomentella* sp.1**	KP866135	AB769927 Uncultured Thelephoraceae	663/665 (99)	SH177859.07FU	14/2	7/1	0
***Tomentella* sp.2**	KP866136	JX456648 Uncultured fungus	705/706 (99)	SH189353.07FU	37/5	2/1	7/1
Ascomycota sp.1	-	EF619719 Uncultured Orbiliaceae	525/612 (86)	SH015725.07FU	2/2	0	1/1
Ascomycota sp.2	-	KP323399 Uncultured fungus	208/226 (92)	SH469383.07FU	2/2	0	0
Ascomycota sp.3	-	KP689247 Ascomycota sp.	618/625 (99)	SH181934.07FU	0	1/1	3/3
Meliniomyces sp.	-	FJ440931 Uncultured ectomycorrhiza	553/574 (96)	SH181081.07FU	0	2/1	0

[a] Accession numbers of sequences from this study deposited in NCBI; -, sequence not deposited. [b] Closest matched BLAST results with informative species and genera. [c] Similarity values were computed from the percent match between the portion of the query aligned and its reference sequence. [d] Frequency refers to presence/absence of ECM in each focal tree and treatment (n = 9 trees per treatment).

Table 3. Estimators of operational taxonomic unit (OTU) richness and diversity of ECM fungi in different treatments. Data represents mean with the standard error in parentheses per plot; n = 3 (3).

Sites	Rarified Species Richness		Estimators of Expected Total Species Richness		Diversity Indices	
	Mao Tau	Mao Tau (50 runs mean)	Chao 2	Jackknife 2	Shannon's H'	Simpson's 1/D
Control	5.67 (1.67)	6.41 (1.58)	8.54 (1.20)	8.78 (2.19)	2.79 (0.96)	5.89 (1.31)
Low N	4.33 (1.45)	4.5 (1.08)	5.1 (1.24)	6.28 (1.53)	1.35 (0.25)	4.06 (0.98)
High N	4.33 (0.67)	5.1 (0.37)	5.99 (0.32)	6.80 (0.12)	2.84 (1.40)	4.59 (0.43)
p [a]	0.729	0.508	0.119	0.52	0.519	0.444

[a] p-value for effect of fertilizer treatment.

3.3. Extracellular Enzyme Activity

Although we measured enzyme activities in 742 individual ECM root tips, we used results from only those ECM taxa with well-supported molecular identities in the statistical and comparative analyses. Consequently, we used the results from 426 root tips (Control: 166; Low N: 126; High N: 134), which represented eight ECM fungal taxa. These taxa were recovered in sufficient numbers across all treatments so that at least three individual root tips of each species from each treatment could be assayed (Table 2, species names in bold). Overall, High N fertilization resulted in a significant increase in PO activity (p = 0.004 for ANOVA) and decrease in AP activity (p = 0.035 for ANOVA), but had no significant effect on PRO activity (p = 0.795 for ANOVA) (Figure 2).

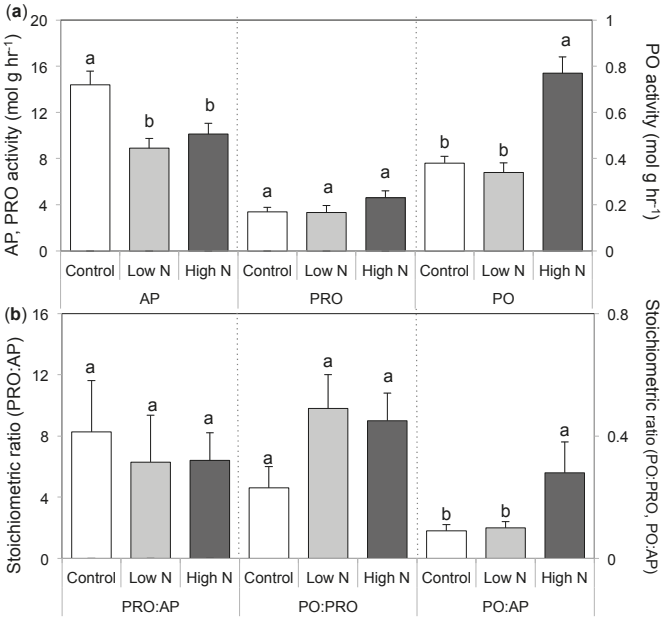

Figure 2. Mean levels of (**a**) acid phosphatase, protease, and phenol oxidase activity; and (**b**) enzymatic stoichiometric PRO:AP, PO:PRO, and PO:AP in ectomycorrhizal communities in control and N-fertilized plots. Vertical bars indicate the standard error of the mean; for each enzyme (or ratio), columns with the same letter do not differ significantly at $p < 0.05$ based on Tukey's HSD test.

Potential AP and PO activity were correlated with levels of soil P and K. Potential AP activity was negatively correlated with total ($r = -0.086$, $p = 0.023$) and available soil P ($r = -0.077$, $p = 0.044$), and total K ($r = -0.082$, $p = 0.030$). Potential PO activity was positively correlated with soil P ($r = 0.154$, $p < 0.001$) and available ($r = 0.109$, $p = 0.004$) and total K ($r = 0.101$, $p = 0.008$). There was no relationship between PRO and any tested soil factor. Potential enzyme activity was also correlated with root tip colonization in low ($r = 0.611$, $p = 0.001$) and high N plots ($r = 0.445$, $p = 0.029$), but not in control plots ($r = 0.229$, $p = 0.281$).

The NMDS ordination showed significant differences in ECM community between N fertilization treatments based on enzyme activity (Figure 1b; $p = 0.001$ for PERMANOVA). This pattern was driven by PO activity, because high N plots had significantly higher levels of PO activity than control or low N plots (Figure 2).

Enzyme activity varied significantly between ECM species (Figure 3; Figures S1 and S2). Overall, *Helotiales* sp.1, and Thelephoraceae were the largest contributors to ECM community enzyme activity in most enzyme systems and N fertilizer treatments, and relative enzyme activity in these taxa was always significantly greater than the community mean (Figure 3a–c). Certain taxa were restricted to a specific enzyme system or N fertilization treatment (e.g., *Lactarius* for PO in N plots; Figure 3c), but for the most part, ECM taxa varied in their relative activity among enzyme systems and N fertilization treatments. For example, the activity of Atheliaceae has greater contribution to the community for AP in high N plots (Figure 3a), and for PRO in control plots (Figure 3b). There was also strong inter-specific variation in relative enzyme activity among the three species of Helotiales.

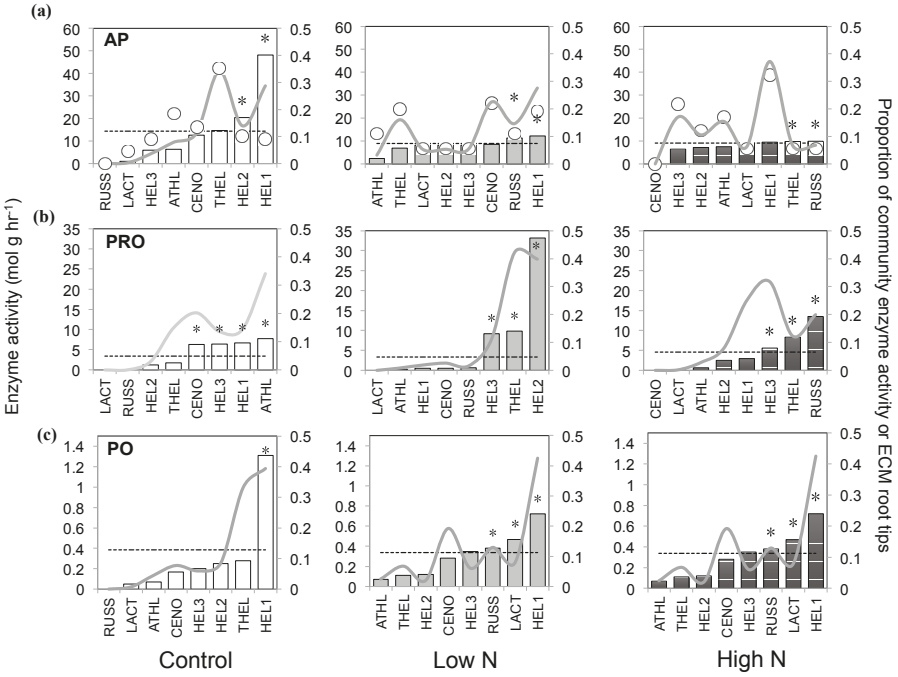

Figure 3. Levels of ectomycorrhizal (ECM) root tip relative abundance (open dot) and the relative contribution (grey curve) of individual ECM group to overall ECM community activity of (**a**) acid phosphatase (AP), (**b**) protease (PRO), and (**c**) phenol oxidase (PO) in response to N fertilization. Within each panel for enzyme and N fertilization treatment, broken lines within each panel indicate the mean community level of enzyme activity. Columns denoted with an asterisk (*) denote ECM species in which enzyme activity was significantly greater ($p < 0.05$) than the mean community value.

Fertilization altered the stoichiometry of enzyme activity by significantly increasing the depletion of AP (P-cycling) relative to PO and PRO (C-, N-cycling, respectively; Figure 2b). In N-fertilized plots, contact (Russulaceae) and medium-fringe (Atheliaceae) types showed a significant increase in the PRO:AP ratio, whereas short (*Cenococcum* and Helotiales) and medium-smooth (Thelephoraceae) types showed a decline (Figure 4a). Conversely, the PO:AP ratio increased in medium-smooth types and declined in medium-fringe types (Figure 4c). The PO:PRO ratio declined significantly (contact, medium-distance types) or did not differ significantly between treatments (short-distance; Figure 4b). Although the PO:AP ratio was higher in ECM species with hydrophilic mantles (groups of *Cenococcum*, Thelephoraceae, Russulaceae, and *Helotiales* spp.) compared to those with hydrophobic mantles (group of *Atheliaceae* spp.) (Figure 5), this difference was not statistically significant ($p = 0.223$). Similarly, PO:AP activity was greatest in high N plots, but again, this difference was not statistically significant (hydrophilic $p = 0.746$; hydrophobic $p = 0.391$).

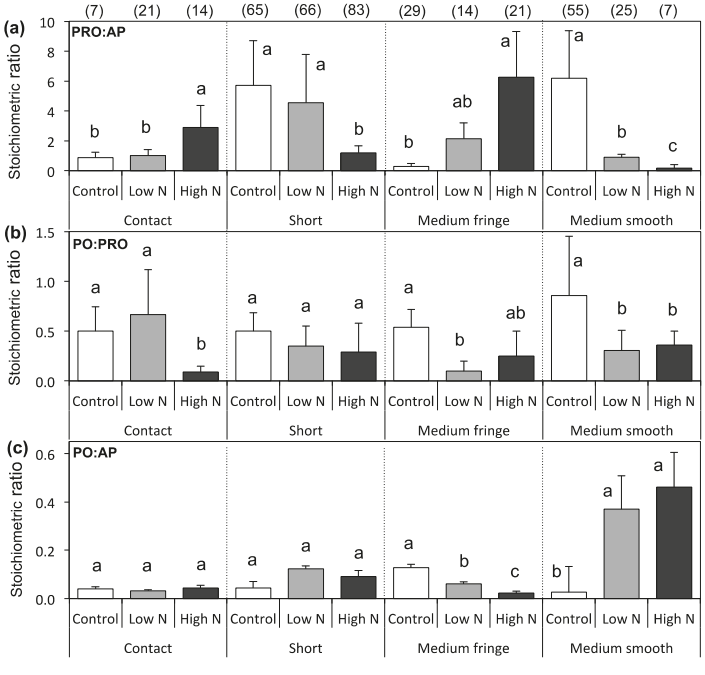

Figure 4. Mean (**a**) PRO:AP, (**b**) PO:PRO, and (**c**) PO:AP in ectomycorrhizal communities in control and N-fertilized plots based on hyphal exploration strategy. For each enzyme and hyphal strategy, columns with the same letter do not differ significantly at $p < 0.05$ based on Tukey's HSD test. Root tip number per exploration type is listed on the top panel.

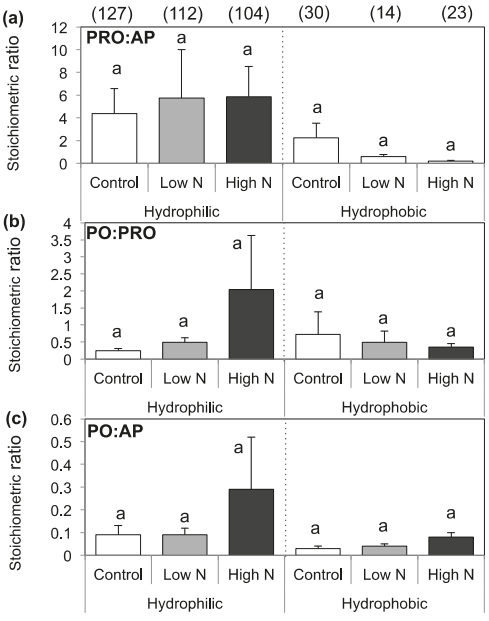

Figure 5. Mean (**a**) PRO:AP, (**b**) PO:PRO, and (**c**) PO:AP ratios based on mantle structure in control and N-fertilized plots. Mantle types with the same letter did not differ significantly at $p < 0.05$ based on Tukey's HSD test. Root tip number per hydrophobicity type is listed on the top panel.

4. Discussion

Despite the high levels of ambient N deposition, we found that three years of N fertilization produced shifts in ECM fungal community structure, and variously altered the abundances and potential enzyme activities of ECM fungal taxa as well as the stoichiometry of extracellular enzymes involved in P acquisition (AP) and lignin degradation (PO) (Question 1). Such shifts were related to some extent to changes in nutrient availability (P, K, OM) created by N fertilization (Question 2) and differences in enzymatic stoichiometry among ECM hyphal exploration types.

ECM fungal richness, diversity, and evenness were not significantly different across N fertilization treatments. These results reconcile with earlier studies in oak [30] and spruce forests [3,47–49], but seemingly contradict the majority of studies that show a rapid and substantial decline in ECM fungal diversity following N fertilization e.g., [6,9–11,30] and increasing soil N levels along natural gradients of productivity [50]. This result may reflect the short period (three years) over which the plots were fertilized [6,51] or (more likely) that high levels of ambient N deposition may have pre-empted any effects of the experimental N additions. Indicative of this condition, we found that even high N fertilization (300 kg N ha^{-1} $year^{1}$) did not significantly increase the levels of total and available N over those in non-fertilized plots, and the levels of available N across all plots (25–27 mg N kg^{-1} soil) exceeded those documented in an N-saturated pine forest in southern China [31]. Further, N additions are expected to alter the quantity and quality of resources (i.e., litter C:N) so as to influence soil C:N. However, we found that the soil C:N ratio did not differ significantly between N-fertilized and control (ambient N) plots. Taken together, these findings suggest the soil was N-saturated so that any new input of N (i.e., fertilizer) was likely leached from the system.

Even so, N fertilization was sufficient to alter ECM fungal community composition. The major genera found in our study (e.g., *Cenococcum*, *Thelephora*, *Tomentella*, *Sebacina*, *Inocybe*, *Russula*) are ubiquitous and dominant components in ECM communities. The most obvious indicators of

N-enrichment were the increased abundance of ECM taxa considered to be nitrophilic (*Russula*, *Tomentella*), the loss of N-sensitive fungi (*Cenococcum*), and the exclusion of protein-N-utilizing ECM species (*Suillus*, *Tricholoma*, *Cortinarius*, *Piloderma* [5,30,48,49]). These results also reflect the shift to an ECM fungal community comprising contact- (Russulaceae) and short-range exploration types (*Cenococcum*, Helotiales), and certain medium-distance types (Thelephoraceae, Atheliaceae). Our findings are in agreement with the well-documented patterns of ECM community change and species' abundances in response to N-deposition or fertilization noted elsewhere [8,11,30,49]. Unlike these former studies, soil K, P, and OM were the primary soil factors associated with changes in ECM fungal community structure, not N. This distinction could reflect the difference in major N input as NH_4-N versus NO_3-N in our systems. Nevertheless, our results show that imbalances of mineral nutrients will become increasingly important controls over ECM fungal community structure in ecosystems with chronic N deposition or saturation.

Forest soils can become increasingly deficient in K and P as a result of atmospheric N deposition or N fertilization, as occurred in our study plots [1]. However, ECM fungal communities respond differentially to deficiencies of K versus P. For instance, deficiencies of soil K reduce fine root growth and ECM colonization by impeding sucrose export from the leaves to the roots [52,53], but have no effect on ECM community composition [54]. Conversely, P-deficiency tends to increase C allocation belowground to stimulate root and ECM fungal growth and AP activity [55]. In our study, the decrease in ECM root colonization with N fertilization is consistent with K deficiency. In contrast, the decline in AP activity and changes in enzyme stoichiometry toward an increase in C-acquisition (PO) relative to P (AP) with N fertilization was a clear contradiction of previous studies [56]. The precise reason(s) for this response are beyond the scope of this study. However, it is possible that AP activity on ECM root tips may have been low relative to the activity in the foraging extraradical mycelium in other parts of the soil profile (i.e., functional compartmentalization [22,55]) or that there were changes in the physiological competence of ECM root tips owing to reductions in C-allocation from the host [52,53].

N fertilization also resulted in ECM communities with high PO activity. N-fertilized communities showed a net up-regulation in PO activity as individual ECM fungi showed an increased capacity to degrade SOM. This is in general agreement with the widespread capacity of ECM fungi to oxidize SOM [21,57,58] and the generally positive effect of soil N on litter degradation by ECM fungi [2,7]. Such increases in PO activity have been interpreted as a mechanism by which ECM acquire labile carbohydrates when host C allocation is low [22,58], or that ECM generally degrade polyphenolic-rich compounds as a consequence of mining for nutrients [20,22,59]. However, these mechanisms are not mutually exclusive, and may vary in their importance depending on the extent of N-enrichment and/or mineral nutrient deficits. Further studies are thus needed to determine the precise role(s) of each mechanism in this system. Even so, the positive relationship between PO and AP activity suggests that ECM fungi may have been similarly prospecting for P. Similarly, the significant correlations between PO and soil P, K, and Mg content are consistent with the concept that ECM use oxidative enzymes such as PO to mobilize mineral nutrients locked within organic matter-mineral complexes [13,17,20,24,57].

Without examining the relationship between ECM enzyme levels and chemical modifications of SOM, we cannot exclude the possibility that ECM fungi also acquired labile C from degraded substrates. In our analyses of ECM enzyme stoichiometry and potential energetic trade-offs, only ECM fungi with the medium-distance smooth exploration type (Thelephoraceae) showed an increase in SOM-degrading potential relative to P acquisition. This is not surprising, since the Thelephoraceae tend to dominate in organic-rich soil horizons [18,60] and show some of the strongest potential activities of degradative enzymes [4,6,61,62]. In addition, studies have shown that when host plants allocate less C to their ECM fungi, either due to dormancy or defoliation, the relative activity of C-degrading enzymes increases significantly [17,55,63].

ECM fungi may also experience trade-offs between the energetic demands associated with root tip colonization versus the metabolically expensive production of enzymes. Under ambient N conditions (control), ECM species with lower levels of root colonization showed disproportionately high levels of

potential enzyme activity—an outcome that is consistent with energetic trade-offs [46]. Communities in N-fertilized plots displayed a different pattern, whereby root tip abundance was positively associated with enzyme activity (Figure 3). Such results are more typical of competitive interactions; that is, where certain ECM taxa pre-emptively colonized roots and utilized resources [64,65] owing to competition for spatial co-existence (a limited availability of root tips) or overlap in resource utilization (limited C availability). Alternatively, the abiotic conditions created by N fertilization may have selected for more closely-related taxa than expected by chance (environmental filtering [62]; e.g., members of the Russulaceae and Thelephoraceae dominated N-fertilized plots). However, additional studies are needed to distinguish between these explanations.

There is now substantial evidence that labile-C compounds derived from roots and fungal tissues are the dominant inputs into stable SOC stores [66]. Up to 30% of the total C assimilated by plants may be transferred to their ECM partner, meaning that any change in the symbiosis will have profound effects on SOC storage. Our results address two sets of dynamics relating SOC to N availability. The first focuses on the dynamics associated with increasing potential enzyme activity for SOM degradation and the release of labile C compounds. Such elevated inputs of labile C may concomitantly stimulate microbial activity and SOC decomposition (the "priming effect" hypothesis). These changes would be expected to be associated with a more rapid turnover of the SOC pool and changes in litter chemistry. However, more rapid decomposition is not synonymous with reductions in total SOC stocks if coupled with similar increases in litter inputs and soil C stabilization. Conversely, soil microbes may preferentially metabolize these pools of labile C over the mining of complex polymers (the "preferential substrate utilization" hypothesis). This effect, combined with the observations that N fertilization can inhibit SOC decomposition [1,2], may lead to increases in SOC following N fertilization.

The second focuses on ECM community dynamics. Carbon transferred to ECM fungi can also contribute to SOC storage if fungal tissues decompose more slowly than non-mycorrhizal roots [67] or if the fungal residues persist in long-term SOC stores. As a corollary, the tissue quality (C:N, melanin content) of fungal necromass can influence degradation dynamics [68]. Thus, shifts in ECM community composition and root colonization—and especially the differences in the extent of root tip colonization between different ECM fungal species and their tissue chemistry—could feedback to influence the quantity and quality of fungal residues entering the long-term C pool [69].

In the near future, this region in China is predicted to experience increasing N deposition. Based on our results, we can hypothesize that increasing inputs of N are likely to exacerbate soil P and K deficiencies, and compromise the capacity of ECM fungal communities to acquire mineral nutrients. In addition, variations in ECM community composition and species' functional plasticity could undermine the contributions of fungal residues to long-term SOC stores. These findings add to the accumulating evidence that increasing inputs of N—either from atmospheric deposition or fertilization—will continue to impact forest health and productivity by altering soil mineral resources and ECM community structure and functioning. Such prospects point to the need for a better understanding of the role(s) of ECM functional traits, their interactions with host plant growth and nutrient status, and their link to the relative soil nutrient availabilities. This multi-faceted approach is urgently needed to improve forest health and productivity in this region, as well as patterns of C stabilization and loss.

Supplementary Materials: The following are available online at www.mdpi.com/1999-4907/9/3/99/s1; Supplementary S1: details of nutrient analyses. Figure S1: potential AP, PO, and PRO enzyme activity in ECM hyphal exploration types in response to N fertilization. Figure S2: potential AP, PO, and PRO enzyme activity in ECM hyphal hydrophobicity types in response to N fertilization.

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Rapid Shifts in Soil Nutrients and Decomposition Enzyme Activity in Early Succession Following Forest Fire

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Abstract: While past research has studied forest succession on decadal timescales, ecosystem responses to rapid shifts in nutrient dynamics within the first months to years of succession after fire (e.g., carbon (C) burn-off, a pulse in inorganic nitrogen (N), accumulation of organic matter, etc.) have been less well documented. This work reveals how rapid shifts in nutrient availability associated with fire disturbance may drive changes in soil enzyme activity on short timescales in forest secondary succession. In this study, we evaluate soil chemistry and decomposition extracellular enzyme activity (EEA) across time to determine whether rapid shifts in nutrient availability (1–29 months after fire) might control microbial enzyme activity. We found that, with advancing succession, soil nutrients correlate with C-targeting β-1,4-glucosidase (BG) EEA four months after the fire, and with N-targeting β-1,4-*N*-acetylglucosaminidase (NAG) EEA at 29 months after the fire, indicating shifting nutrient limitation and decomposition dynamics. We also observed increases in BG:NAG ratios over 29 months in these recently burned soils, suggesting relative increases in microbial activity around C-cycling and C-acquisition. These successional dynamics were unique from seasonal changes we observed in unburned, forested reference soils. Our work demonstrates how EEA may shift even within the first months to years of ecosystem succession alongside common patterns of post-fire nutrient availability. Thus, this work emphasizes that nutrient dynamics in the earliest stages of forest secondary succession are important for understanding rates of C and N cycling and ecosystem development.

Keywords: carbon; decomposition; disturbance; ecosystem process; extracellular enzymes; exoenzymes; forest fire; nitrogen; soil enzymes; succession

1. Introduction

Global change pressures have increased the prevalence of forest fires in western North America [1–3]. Therefore, a better understanding of the connection between resulting perturbations in environmental

factors and ecosystem processes, such as decomposition, will be vital to modeling ecosystem responses in the wake of such disturbance [4–7]. Microbial production of extracellular enzymes (EEA) involved in decomposition is regulated by quantity and quality of substrate that can change strongly after forest fires, although other factors such as moisture and pH are known to impact EEA as well [8–10]. In particular, fire disturbances dramatically alter soil pH, water holding capacity, and carbon (C) and nitrogen (N) pools [11,12], which may all continue to change through succession. These factors may thus affect microbial investment in both C- and N-targeting decomposition enzymes across short time scales of ecosystem recovery (e.g., months).

Past studies in post-fire forest soils have evaluated changes in edaphic properties, microbial communities, and related EEA over decadal time scales [13,14], while shorter-term successional dynamics are less well characterized. However, research has more recently shown the importance of changes in microbial function across short timescales of succession [15–17]. Work in similar forest ecosystems relating to mountain pine beetle kill has shown not only long-term effects of such disturbance, but also immediate, short-timeframe impacts on microbial function, such as respiration [17]. Altogether, this body of work has demonstrated that even when succession is considered over just months to years after a disturbance, shifts in nutrient pools—such as in ammonium (NH_4^+) and C availability—can have strong effects on microbial function [16–18]. Indeed, immediately after fires, burning of soil organic matter leads to alteration of soil C pools; fires can both burn-off C and alter its chemistry [12,19]. For N pools, a pulse of inorganic N occurs immediately after severe burns, which may either be rapidly exported from the ecosystem or persist into the first year following the fire [11,12]. Thus, even on a timescale of months to years after disturbance, these rapid and profound shifts in nutrient pools may influence microbial processes, such as the production of extracellular enzymes, which are central to nutrient cycling and ecosystem dynamics.

Here, we chose to examine early succession on a timescale of months after a high-severity forest fire to understand how soils and microbial enzyme production may change during this time period [20–23], with putative implications for the trajectory of ecosystem development [24,25]. Importantly, we focus on high-severity wildfires that are increasingly prevalent in montane forests of the U.S. Intermountain West [2] and can elicit particular responses in soil edaphic properties and microbial communities that are different from lower-severity burns; studies have shown that burn severity can differentially influence soil nutrient pools, C chemistry transformations, soil physical properties, and microbial community composition (for example [6,18,26]). While past work has evaluated shifts in EEA [14] and environmental controls on enzyme potential across secondary succession in general [27], our study examines how relationships between edaphic properties and decomposition enzyme activity may change within the first years of succession following a severe forest fire. We contrast these relationships between the fire-disturbed (burned) soils and undisturbed (reference) forest soils to highlight the effect of fires versus a control, as well as elucidate successional vs. seasonal patterns.

Specifically, we assessed edaphic properties, nutrient pools, and microbial enzyme activities relating to C and N cycling of soils across three time points in the initial stages of succession spanning 29 months after a major forest fire. We characterized the soil C and N resource environment and β-1,4-glucosidase (BG) and β-*N*-acetylglucosaminidase (NAG) decomposition enzymes that target these resources [9]. Across a global scale, BG and NAG both show strong positive relationships to soil organic matter, NAG shows strong negative relationship with pH, and BG is weakly related to mean annual precipitation [9]. As such, we assessed the activities of these enzymes alongside nutrient pools, pH, and moisture. Enzyme potential and soil nutrient pools are well suited for examining successional patterns as they may broadly persist over inter-seasonal time scales [28] and thus reflect successional patterns beyond seasonal variability.

We hypothesized that on month to year scales of forest secondary succession, changes in soil enzyme activity occur given rapid alterations to edaphic properties after fire disturbance. We further hypothesized that correlations between nutrient pools and EEA would vary between burned and reference soils given the different roles of nutrient limitation (such as stronger C limitations in burned

soils); and, that the strength of correlations between BG and NAG with nutrient pools would change over time to reflect common post-fire shifts in nutrient availability in burned soils. Accordingly, for burn soils we hypothesized that enzyme dynamics would reflect rapid shifts in nutrients that occur after fires: initially low C and high N pools would drive correlations between nutrient pools and BG, whereas with accumulating C and reductions in N over time, correlations between nutrient pools and NAG activity would become more prominent [29,30].

2. Methods

2.1. Site and Soil Properties

Samples were collected in the Fourmile Canyon, Boulder County, CO, USA. Samples were collected at 1 month (October 2010), 4 months (January 2011), and 29 months (June 2013) after a major, high-severity wildfire, which ignited on 6 September 2010. At all sample times, replicate samples were taken from both undisturbed (reference) forest soils and adjacent fire-disturbed (burned) soils. These sites were ~300 m apart and sampling areas fell within 650 m^2 landscapes at similar slope-aspect (northeastern facing mountainside slope) and elevation (~2100–2300 meters above sea level) within the extent of the Fourmile Fire (latitude: 40.036153, longitude: −105.400537) (Figure 1). Sample areas were free of tree mortality from bark beetles and fungi. Prior to the burn, this contiguous study area had similar soil conditions across the landscape given consistent topographical and vegetative features (Figure 1). Metamorphic and igneous parent material has resulted in coarse, poorly developed, sandy soils in Fourmile Canyon [31]. Soils are stony sandy loams of the Fern Cliff-Allens Park-Rock outcrop complex as per National Cooperative Soil Survey (NCSS) classification. Annual precipitation averages 475 mm and occurs primarily as snow in winter/spring [31]. Both reference and burned sites were dominated by *Pinus ponderosa* var. *scopulorum* and *Pseudotsuga menziesii* var. *glauca*; details of location vegetation and fire history as well as the Fourmile Canyon Fire dynamics have been previously described [31,32]. Photos and maps of the fire's extent and response to/control of the Fourmile Canyon Fire have been made available by the U.S. Department of Agriculture [31]. Trees in the burned site were severely burned (completely charred) and dead. Unlike the 1- and 4-month soils that were void of vegetation, 29-month soils were revegetated with understory herbaceous plants by seeding, dominated by sterile wheat.

Ten replicates for both burn and forested undisturbed soils were collected at each time point. Sampling locations within each treatment were 1 m from the base of a tree (burned or alive, respectively), and at least 3 m but no more than 25 m between individual trees used for sampling. At each sample location three 130.5 cm^3 soil cores of mineral soil at a depth of 5 cm were taken and bulked to constitute a single replicate. We avoided/removed belowground plant material. In reference site samples, the organic layer was removed prior to sampling; no organic layer was present in burned soils. One-month soils included an ash layer of <0.5 cm.

Within 2 h of sampling, soils were transported to the lab and then sieved through a 2 mm mesh, subsampled, and stored in a −70 °C freezer for molecular analysis or refrigerated at 4 °C for soil chemistry and enzyme assays. All samples were processed according to the methods enumerated in Ferrenberg et al. (2013), from which 1- (October 2010) and 4-month (January 2011) samples from the burned site, and 4-month samples from an undisturbed, unburned, forested site were also used. Soil moisture, pH, total dissolved nitrogen (TDN), extractable, non-purgeable, organic carbon (NPOC), and ammonium (NH_4^+) were evaluated.

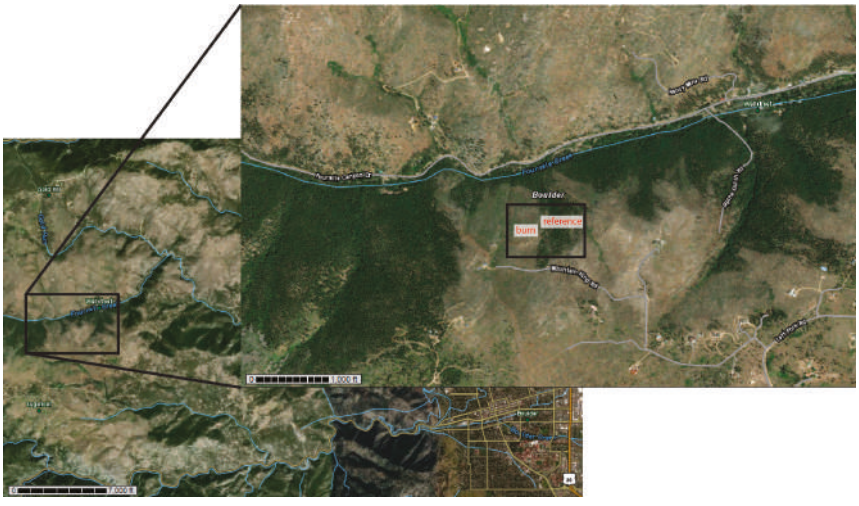

Figure 1. Maps of Boulder and Fourmile Canyon and surrounding region, with sample area demarcated in inset map. Maps are from the U.S. Department of Agriculture Natural Resources Conservation Service.

A subsample of each soil was dried at 100 °C for 48 h to determine gravimetric soil moisture; subsequent edaphic properties were calculated on a dry weight basis. Dried soils of all samples were ground and 50 mg were packed into tin capsules for %C and %N analysis using a Thermo Finnigan EA 1112 Series Flash Elemental Analyzer; (Thermo Fisher Scientific, Inc., Waltham, MA, USA) [33].

Immediately following collection, 8 g of soil were extracted for 1 h in 40 mL of 0.5 M K_2SO_4 and filtered with Whatman No. 1 paper (Whatman Incorporated, Florham Park, NJ, USA). Extract filtrate was frozen until analysis of NH_4^+, TDN, and NPOC. Filtrates were analyzed for NH_4^+ on a BioTek Synergy 2 Multidetection Microplate Reader (BioTek, Winooski, VT, USA) and TDN/NPOC, were measured on a Shimadzu TOC-V CSN Total Organic Carbon Analyzer (Shimadzu TOCvcpn, Kyoto, Japan). TDN, NPOC, and NH_4^+ analysis was completed for all 4 and 29 month soils. Soil pH was measured on soil slurries with a ratio of 2 mg dry soil: 4 mL water, which were shaken at 250 rpm for one hour and allowed to equilibrate for an hour before measuring.

2.2. Enzyme Analysis

Enzyme activities for β-1,4-glucosidase and β-1,4-*N*-acetylglucosaminidase were evaluated to assess microbial investment in C and N acquisition, the cycling of these nutrients, and connections with edaphic properties. BG and NAG enzymes are useful indicators of C and N cycling as they are produced across a wide variety of fungi and bacteria and importantly have been used widely in past research to assess microbial investment in C vs. N acquisition and the limiting nature of these nutrients in post-fire forest ecosystems [9,18,30]. While all ten replicates were used from 29-month samples, due to limited availability of samples, eight replicates of reference forested soils (4 months) and seven replicates of burned soils (1 and 4 months) were included for enzyme analysis. Enzyme activity was measured via fluorometric microplate methods [34,35]. The methods of Weintraub et al. [34,35] were used based on a 96-well assay plate method with 1 M sodium acetate buffer titrated to a pH of 7.0, and 4-methylumbelliferone standards. ~1 g of refrigerated soil was used from each sample [36]. Each sample (every experimental replicate) was run with 16 analytical replicates, quench corrections, standards, and negative controls for each enzyme assay. Fluorescence was measured using a microplate reader (Thermo Labsystems, Franklin, MA, USA) at 365 nm excitation and 460 nm emission to calculate nmol activity h^{-1} g $soil^{-1}$.

2.3. Statistical Analysis

The *pgirmess* package in the R statistical environment [37] was used to evaluate changes in edaphic properties within reference and burn soils across the various time points using Kruskal–Wallis contrasts. Enzyme activity was also analyzed as a BG: NAG ratio and tested for statistical differences across time within both burned and reference forest soils. Pearson product moment correlations were calculated between environmental factors of total C, total N, pH, C:N ratio, and percent moisture and BG/NAG activity both in burned and reference plot samples across all time points. Data were checked for normality and if nonconforming were transformed to achieve normality before correlation analysis.

2.4. Data Availability

All metadata have been made available at figshare [38].

3. Results

3.1. Extracellular Enzyme Activities

In burned soils, BG activity was significantly higher in 29-month soils than one- and four-month soils (Table 1), denoting a trend for increasing activity through time, becoming more comparable to reference soil activity levels. In contrast, NAG activity showed significant declines from 4- to 29-month soils. BG:NAG ratios exhibited a strong partitioning between the 1-/4-month and 29-month time points (Table 1). For instance, in burned samples, 29-month soils had significantly higher BG:NAG ratios than one- and four-month soils.

BG activity at all times was higher in reference soils than burned soils. While BG showed significantly higher activity in 29-month soils than four-month soils, no differences in NAG activity or BG:NAG ratios over time were observed in reference soils (Table 1).

3.2. Soil Properties

Burned soils showed patterns of change over time in ammonium (NH_4^+), total dissolved nitrogen (TDN), and percent moisture (Table 1). Significant decreases in NH_4^+ and TDN were observed between 4- and 29-month burned soils. Moisture declined from 1-month to 29-month time point; soil moisture at one month was significantly higher than at 29 months in burned soils. No significant changes were observed in total C and N pools as measured via %N, %C, or C:N ratio across any of the time points in burned soils.

Unburned reference soils showed declines in soil moisture over time with 4- and 29-month soils have significantly lower soil moisture than one-month soils. pH showed significant differences month to month (Table 1).

3.3. Soil Properties and Extracellular Enzyme Activity

In burned soils, EEA was uncorrelated to edaphic factors initially (i.e., at one month post-fire) but began to strongly relate to nutrient pools (%C and %N) at four months (r of >0.8) and onward (Table 2). These burned soils showed strong correlations between BG (but not NAG) and %C and %N. In contrast, the reference plots at this time point showed correlations of both NAG and BG with edaphic properties including C and N pools. By 29 months, both BG and NAG correlated with C and N pools, while no correlations were observed in reference plots (Table 2). Taken together, these analyses demonstrate that BG activity in burned soils correlated with soil nutrient pools during the 4–29-month post-fire interval, while NAG correlated with these same factors later in successional time only (e.g., at 29 months) (Table 2). In reference soils, both BG and NAG correlated with nutrient pools at four months and showed no correlation at 1- and 29-month time points.

Table 1. Mean (standard deviation) edaphic properties for reference/post-fire successional samples.

Category	pH	% Moisture	% N	% C	NH4 (mg/kg Soil)	NPOC (mg/g Soil)	TDN (mg/g Soil)	BG (nmol Activity/h/g Soil)	NAG (nmol Activity/h/g Soil)	BG:NAG Ratio
BURN										
1-month	7.21 (0.30) ^{AB}	9.93 (4.55) ^{BA}	0.14 (0.04)	2.43 (0.95)	N/A	N/A	N/A	53.94 (12.02) ^{C}	78.53 (31.46) ^{AB}	0.74 (0.23) ^{B}
4-months	8.08 (0.41) ^{A}	8.55 (2.36) ^{ABC}	0.11 (0.04)	2.33 (1.07)	48.94 (14.95) ^{A}	0.30 (0.16)	0.06 (0.02) ^{A}	76.38 (27.67) ^{BC}	107.54 (43.07) ^{A}	0.75 (0.18) ^{B}
29-months	7.00 (0.28) ^{B}	2.16 (0.80) ^{C}	0.12 (0.03)	2.48 (0.66)	2.30 (1.33) ^{B}	0.31 (0.30)	0.02 (0.02) ^{B}	127.00 (27.57) ^{A}	44.41 (14.61) ^{B}	3.01 (0.71) ^{A}
REFERENCE										
1-month	6.44 (0.67) ^{B}	19.74 (10.17) ^{A}	0.27 (0.08)	6.21 (2.01)	N/A	N/A	N/A	N/A	N/A	N/A
4-months	7.08 (0.46) ^{A}	6.35 (3.50) ^{C}	0.24 (0.17)	6.59 (5.08)	2.39 (1.40)	0.26 (0.18)	0.02 (0.01)	147.83 (54.97) ^{B}	136.29 (63.12)	1.17 (0.38)
29-months	6.63 (0.34) ^{AB}	8.40 (6.12) ^{BC}	0.24 (0.08)	5.20 (2.19)	1.24 (0.60)	0.09 (0.04)	0.01 (0.004)	254.41 (86.14) ^{A}	251.77 (116.38)	1.13 (0.38)

Letters denote significant differences across timepoints ($p < 0.05$) as per Kruskal-Wallis contrasts within Burn/Reference categories.

Table 2. Correlations between β-1,4-glucosidase (BG) and β-1,4-*N*-acetylglucosaminidase (NAG) enzyme activity and edaphic properties. Significant ($p < 0.05$) correlations (Pearson's r) shown for burned and reference soils across all time points.

		BURNED PLOTS		REFERENCE PLOTS	
Time	**Factors**	**BG**	**NAG**	**BG**	**NAG**
1-month post-fire October	pH	NS	NS	N/A	N/A
	moisture	NS	NS	N/A	N/A
	C	NS	NS	N/A	N/A
	N	NS	NS	N/A	N/A
	C:N	NS	NS	N/A	N/A
4-months post-fire January	pH	NS	NS	NS	NS
	moisture	NS	NS	0.9	0.76
	C	0.83	NS	0.81	0.79
	N	0.9	NS	0.78	0.72
	C:N	NS	NS	0.77	NS
29-months post-fire June	pH	NS	NS	NS	NS
	moisture	NS	NS	NS	NS
	C	0.69	NS	NS	NS
	N	NS	NS	NS	NS
	C:N	NS	0.69	NS	NS
33-months post-fire October	pH	NS	NS	NS	NS
	moisture	0.64	NS	NS	NS
	C	NS	NS	0.84	0.84
	N	NS	0.66	0.96	0.86
	C:N	NS	0.74	NS	NS

NS = not significant N/A = not available.

4. Discussion

Changes in edaphic properties and EEA of post-fire landscapes have been shown to occur across successional stages at decadal timescales [13,14,30,39,40]. Strikingly, we found that microbial EEA related to C and N acquisition varied significantly over a relatively short time span of 29 months of succession. Our results indicate that even within three years of succession [23] enzyme activity changes alongside rapid shifts in nutrient availability that are characteristic of post-fire succession. While microbes in early succession may be co-limited by both C and other macronutrients such as N [41–43], increasing BG:NAG ratios observed within the first 29 months of post-fire forest succession may reflect increasing C availability (e.g., revegetation) and a relative increase in microbial investment in C acquisition. Reference soils, however, showed no significant changes over time in BG:NAG ratios. Our work is consistent with past research in post high-severity forest fire soils that shows BG:NAG ratios of 2–3 at just over a year into succession, while lower disturbance environments displayed BG:NAG ratio around 1–1.5 [18]. In total, the observed shift demonstrates that within three years of succession EEA activity is responsive to the unique soil nutrient environment of burned soils and shows distinct dynamics from reference forest soils.

We more directly examined the relationship between edaphic properties and EEA within each stage of the burned landscape in contrast to corresponding reference soils. Within these stages of secondary succession, we observed a shift from correlations between only BG EEA and nutrient pools to correlations between NAG EEA and nutrient pools in the 29-month time point as well (Table 2). Although controls on microbial production of extracellular enzymes may vary, it is well known that the quantity and quality of available substrates can induce and structure the production/activity of both C and N acquiring enzymes [9,10]. The observed correlations indicate that even within the first years of secondary succession, nutrient limitation may control BG activity with eventual shifts toward more prominent connections between nutrient pools and NAG activity. This dynamic may

reflect a relative shift from C to N limitation (or relative changes in co-limitation) and is consistent with general patterns in nutrient dynamics across succession in post-fire landscapes [11,12,43–45]. Specifically, research has commonly observed that post-fire landscapes are characteristically low in C and experience a pulse of inorganic N in the form of ammonium and nitrate after severe wildfires, while slightly later successional soils may be more constrained by N with the buildup of soil C [11,12]. Accordingly, we witnessed evidence of a pulse of NH_4^+ and TDN in the four-month post-fire soils and a strong drawdown in these N pools at the 29-month time point (Table 2), consistent with a vast body of literature which notes a pulse of inorganic N immediately after a fire, but drawdowns in this pool on a timescale of months to years [11,12]. While C pools do not show significant increases over time in soils at the scale measured in this study, past work has shown that fires can strongly influence the composition of soil organic matter without significant impact on total stock [26]. For example, fires can alter C chemistry in forest soils, including the humification of C compounds which can influence substrate availability for microbial decomposition [18,19,46]. Changes in C pools over successional time with plant colonization (29-month soils) may also be in terms of composition and quality, not just quantity [27,47,48].

While we acknowledge that seasonality can influence variation in EEA [49], the observation that strong correlations between EEA and soil N and C pools correspond with common post-fire dynamics, such as a drawdown in inorganic N, likely reflects successional dynamics. Additionally, the fact that these observed patterns in EEA of successional soils are different from reference soils shows that such patterns are specific post-burn soil dynamics in the first months after a fire, illustrating EEA responses to geochemistry even within 4–29 months post-disturbance.

While pH and moisture have well-described successional dynamics, such as an immediate increase in pH after fire and decreases over time, or increases in water holding capacity with the buildup of soil organic matter over time [11,12], these factors may also vary on a seasonal basis. In the case of this study, we interpret moisture changes, for example, as largely a seasonal shift. Over 29 months, there is little change in soil organic matter and water holding capacity, and shifts in soil moisture occur in a similar manner in both successional and reference soils. This pattern of change is not unique to successional soils, but rather a seasonal dynamic true of reference forests as well. However, neither soil moisture nor pH correlated with enzyme activity in post-burn successional soils. While future work should seek to address how seasonality versus succession influences these ecosystems in the first months after fire, significant increases in BG:NAG ratios over time and correlations between C and N pools with BG/NAG EEA are different from patterns in reference soils and demonstrate dynamics that are unique to post-burn successional soils within the first three years following a severe wildfire.

Additionally, enzymes are well suited to studying inter-seasonal dynamics as they persist in the soil [28] and are assayed for enzyme potential (at controlled temperature, moisture, and pH) rather than in situ enzyme activity. Enzyme potential assays, such as those completed in the lab, may therefore reflect successional dynamics rather than seasonal ones where variable in situ temperature, pH, and moisture can strongly affect enzymatic activity.

Altogether, our work leads to a conceptual model of patterns in the coupling of nutrients and decomposition enzyme activity on short timescales after fires (Figure 2). Because of characteristic changes in nutrient pools over the first years in post-fire succession, and the role of C and N availability as a control on enzyme production, we propose that the initial limitation in C availability results in a connection between BG activity and the resource environment. Likewise, in subsequent stages where C pools begin to build and N is more limited in availability (though C and N may be co-limiting), NAG activity shows connections with the resource environment (Figure 2). Here, in particular with plant colonization and the accumulation of C, BG:NAG ratios increase, reflecting improved availability of C substrates (Figure 2). It is important to note that these dynamics are envisioned for short timescales within the first years of succession, as N limitation alone across longer timescales can yield declines in BG:NAG ratio [30] and more dramatic variation in other important controls such as pH and soil moisture may also become more influential.

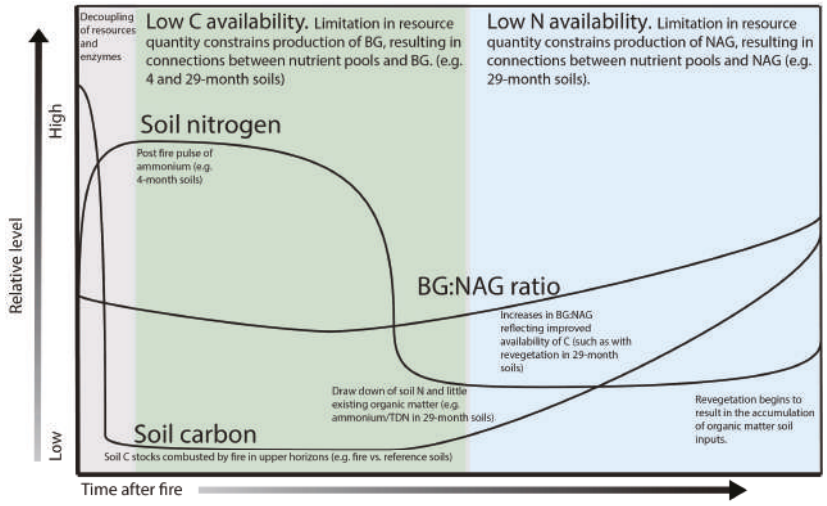

Figure 2. Soil resource and extracellular enzyme dynamics on a short timescale (<3 years) after a forest fire.

5. Conclusions

We found evidence for a connection between rapid shifts in nutrient pools and microbial decomposition enzyme activity in the first several years of secondary succession. We show that within 29 months of post-fire succession relative increases in BG:NAG ratios occur. These shifts are distinct from reference soils, and may represent rapid successional responses to changing nutrient dynamics. Our work demonstrates that soil nutrients first correlate with BG activity (C-targeting) and then correlate additionally with NAG activity (N-targeting) within 29 months of succession. This shift is likely driven by changes in substrate availability and quality as post-fire landscapes first show reductions in C pools, followed by reductions in NH_4^+/TDN pools over the timeframe examined in this study. Built on the empirical findings of this and other studies, our conceptual model suggests when and why we may expect to observe changes in nutrient–enzyme relationships across the initial stages of post-fire succession (Figure 2).

Despite the use of a single site in our research, such study systems and sampling schemes have traditionally been used in the study of ecosystem succession with great success in advancing the field empirically and theoretically [14,44,50,51]. Nonetheless, the research conducted herein represents samples from a single fire disturbance and thus we are limited in our ability to generalize such findings. We also note that scales of disturbance should be explicitly considered in future work and constrain the conclusions of this study, which were based on a high-severity fire. Past work has shown that high- vs. low-severity fires, for example, can modulate ecosystem responses in terms of soil chemistry and EEA [6,18].

We present our conceptual model as a hypothesis for further work (Figure 2). While this work describes shifts in EEA potential and the linkage between nutrients and soil enzyme activity within the first years of succession, future work should more closely examine the possible ecological mechanisms that underlie these patterns, such as how specific changes in microbial communities may be driving the observed differences in biogeochemical potential with EEA. Past work at this site in which bacterial communities were sequenced at each time point showed no correlation between bacterial community structure and EEA in the post-fire successional soils (data not shown); however, fungal communities are dominant drivers of EEA as well and further research may reveal to what extent microbial data can explain variation in soil EEA that is responsible for the cycling of C and N in these ecosystems [5].

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Conflicts of Interest: The authors declare no conflict of interest.

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forests

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Article

Effects of Nitrogen Deposition on Soil Dissolved Organic Carbon and Nitrogen in Moso Bamboo Plantations Strongly Depend on Management Practices

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Abstract: Soil dissolved organic carbon (DOC) and nitrogen (DON) play significant roles in forest carbon, nitrogen and nutrient cycling. The objective of the present study was to estimate the effect of management practices and nitrogen (N) deposition on soil DOC and DON in Moso bamboo (*Phyllostachys edulis* (Carrière) J. Houz) plantations. This experiment, conducted for over 36 months, investigated the effects of four N addition levels (30, 60 and 90 kg N ha^{-1} $year^{-1}$, and the N-free control) and two management practices (conventional management (CM) and intensive management (IM)) on DOC and DON. The results showed that DOC and DON concentrations were the highest in summer. Both intensive management and N deposition independently decreased DOC and DON in spring ($p < 0.05$) but not in winter. However, when combined with IM, N deposition increased DOC and DON in spring and winter ($p < 0.05$). Our results demonstrated that N deposition significantly increased the loss of soil DOC and DON in Moso plantations, and this reduction was strongly affected by IM practices and varied seasonally. Therefore, management practices and seasonal variation should be considered when using ecological models to estimate the effects of N deposition on soil DOC and DON in plantation ecosystems.

Keywords: soil organic carbon; dissolved organic matter; nitrogen addition; *Phyllostachys edulis*

1. Introduction

Dissolved organic matter (DOM) is a mixture of organic molecules of different sizes and structures that can pass through a 0.45 μm sieve and dissolve in water and acidic and alkaline solutions [1,2]. Although DOM accounts for a small proportion of the soil organic matter pool, it plays an important role in microbial growth and metabolism, which regulate soil nutrient loss and affect the decomposition and transformation of soil organic matter [3,4]. As the two important components of DOM, dissolved organic carbon (DOC) and nitrogen (DON) are the two common empirical indices that reflect the quantitative characteristics of DOM [5]. DOC affects the regulation of cation leaching, mineral weathering, soil microbial activity, and anion adsorption and desorption, as well as other soil chemical, physical and biological processes [6]. DOC is a very important and active factor associated with terrestrial and aquatic ecosystems in the geochemical carbon cycle [6,7]. DON plays a dual role in N cycling in terrestrial ecosystems: on the one hand, it can be directly absorbed by plants and therefore shorten the terrestrial nitrogen cycle; on the other hand, however, DON has high mobility and can cause pollution in aquatic ecosystems through surface runoff or leaching [8]. DOC and DON have been

recognized as the key components of forest C, N and nutrient cycling [9] and are therefore receiving a great deal of attention by researchers.

With the intensification of human activities, global atmospheric N deposition has increased rapidly, and several global N models predict that the subtropical regions in south central China will be among the areas most severely affected by atmospheric N deposition in the coming decades [10]. N is one of the essential elements for plant growth; thus, N deposition is thought to be beneficial to the plant. However, a few studies have shown that only a small proportion of atmospheric N deposited in forest ecosystems is utilized by plants, and that the major proportion of N is fixed in the soil [11]. Previous studies have shown the effects of N deposition on soil DOC and DON in forests. Frey et al. [12] found that more than half of the ecosystem C storage in a hardwood stand was attributable to an accumulation of soil organic matter, indicating that the soil has been more responsive to N addition than tree growth. Furthermore, they thought N enrichment resulted in a shift in organic matter chemistry and microbial community, thereby impacting DOM. Findlay et al. [13] reported that N deposition induces a great loss of soil DOC. Based on N saturation experiments, Gundersen et al. [14] showed that N input can increase the stability of soil humus and promote bacterial growth, thereby leading to a decrease in soil DON. Tu et al. [15] observed that N deposition significantly reduces soil microbial biomass carbon (MBC) but increases DOC in Sinocalmus bamboo (*Neosinocalamus affinis* (Rendle) Keng f.) plantations. However, the effects of N deposition on soil DOC and DON in Moso bamboo (*Phyllostachys edulis* (Carrière) J. Houz) plantations remain unknown.

Because of their rapid growth rate and high annual regrowth rate after harvesting, Moso bamboo forests are the most important source of non-wood forest products in China; they cover an area of 4.43 million ha and represent 73.7% of the country's bamboo forest area and 84.0% of the global distribution of Moso bamboo [16,17]. Our long-term investigation based on the Eddy covariance method showed that the Moso bamboo plantation ecosystem has a high C uptake capacity and might play an important role in mitigating climate warming [17]. In recent decades, intensive management (IM) has been implemented in more than half of these bamboo plantations to increase economic benefits. IM includes the removal of understory weeds, application of fertilizers, and soil tilling [18]. Typically, conventional management (CM) requires the regular harvest of bamboo stems and shoots, without any of the IM practices mentioned [19]. Thus, IM can affect soil organic carbon (SOC) [20], soil microbial biomass [21], and enzyme activities [22], which may affect the soil DOM. Moso bamboo plantations are mainly distributed in subtropical China, which is suffering severe N deposition of 30–37 kg N ha^{-1} $year^{-1}$ [19]. Our previous study [21] found that IM and N deposition significantly increased soil MBC but decreased bacterial diversity, and the combination of management practices and N deposition had greater effects on soil microbial biomass and diversity than either practice system or N deposition independently, which may impact soil DOC and DON. However, the mechanism of DOM response to these complicated factors and subsequent C and N cycles in Moso bamboo plantations remains unknown.

We conducted a more than three-year-long field experiment in Moso bamboo plantations to test the following three hypotheses: (1) IM practices decrease soil DOC and DON; (2) N deposition decreases soil DOC and DON; and (3) the combined effects of N deposition and management practices on soil DOC and DON are stronger than the effects of each of these factors independently.

2. Materials and Methods

2.1. Study Site

The study site was in Qingshan Town, Lin'an City (30°14′ N, 119°42′ E), Zhejiang Province, China. It has a subtropical monsoon climate, with mean annual precipitation and mean annual temperature of 1420 mm and 15.6 °C, respectively. The area receives an average of approximately 1847 h of sunshine and 230 frost-free days annually.

The CM Moso bamboo forests were originally established in the late 1970s from native evergreen broadleaf forests in sites of similar topography (southwest slope of approximately 6°) and soil type. The soils are named yellow-red soil and classified as Ferrisols derived from granite [19]. IM practices were conducted in half of the CM Moso bamboo forests since 2001. In September of each year, the IM Moso bamboo forests were fertilized and then plowed to a depth of 0.3 m. The application of the nitrate of S-based compound fertilizer (N-P_2O_5-K_2O: 15%-6%-20%, 450 kg ha^{-1}) is equivalent to the annual addition of 67.5 kg N, 11.8 kg P, and 74.7 kg K per hectare [19].

2.2. Experimental Design and N Treatment

Twelve CM plots and 12 IM plots, each with an area of 20 m × 20 m, were established. Detailed information on the experimental design can be found in Song et al. [19]. The local background atmospheric N deposition rate is 30–37 kg N ha^{-1} $year^{-1}$, with an average NH_4^+:NO_3^- of 1.28 [23]. Therefore, NH_4NO_3 was used as the N source for the low-N (30 kg ha^{-1} $year^{-1}$) treatment (N30), medium-N (60 kg ha^{-1} $year^{-1}$) treatment (N60), and high-N (90 kg ha^{-1} $year^{-1}$) treatment (N90). Three replicate plots for each treatment and the control (N-free) were randomized for each management practice. From January 2013, appropriate quantities of NH_4NO_3 were dissolved in 10 L water and sprayed evenly onto the forest floor of the corresponding plot every month. Each control plot received 10 L of N-free water every month to balance the effects of the water added.

2.3. Soil Sampling and Measurement

The experimental plots were sampled in the spring (30 April), summer (25 July), and winter (29 December) of 2016. The monthly mean air temperatures and precipitation quantities during the study period are shown in Table 1. Five soil cores at a depth of 0–20 cm were randomly collected from each plot and mixed. The samples were kept in an incubator, brought to the laboratory, and then sieved through a 2 mm mesh to remove roots, plant residues and stones. Next, we weighed two samples of 20 g fresh soil each and named them A and B. A was for determining DOC and total dissolved nitrogen (TDN) concentrations, and B was for determining soil moisture content. A was extracted with distilled water (soil:water ratio, 2:1), shaken for 0.5 h (170 rpm) at 25 °C, centrifuged for 20 min at 3500 rpm, and then filtered through a membrane (0.45 μm, Millipore, Xingya Corporation, Shanghai, China) into a plastic bottle [24]. DOC and TDN concentrations were determined using a total organic carbon analyzer (TOC-V_{CPH}, Shimadzu Corporation, Kyoto, Japan), and NH_4^+-N and NO_3^--N concentrations were determined using the SmartChem 200 Discrete Analyzer. DON was calculated as the difference between TDN and NH_4^+-N and NO_3^--N concentrations, according to Li et al. [24]. The ppm units of DOC, TDN, NH_4^+-N, and NO_3^--N were converted to mg/kg by the following formula:

$$M = \frac{P \times 50 \times 10}{20 \times (1 + S)} \quad (1)$$

where M is the combined value of DOC, TDN, NH_4^+-N and NO_3^--N in mg/kg; P is value of DOC, TDN, NH_4^+-N and NO_3^--N in ppm; 50 is the transformation factor from ppm to mg/kg; 10 is the dilution factor; 20 stands for 20 g of the soil sample; and S is the soil moisture content.

Table 1. Average monthly climatic data of the study site during the experimental periods in 2016.

Months	Total Monthly Precipitation (mm)	Average Monthly Air Temperature (°C)
April	352.5	11.49
July	244.8	19.34
December	67.2	3.46

2.4. Data and Statistical Analyses

One-way analysis of variance (ANOVA) and the least significant difference (LSD) method were used to determine the statistical significance of the differences in DOC and DON concentration among N addition treatments under the same management and season, between the two management practices under the same N addition treatment and season, and among three seasons under the same management and N addition treatment.

Two-way ANOVA was performed to evaluate the combined influence of N deposition and management practices in each season. All data were tested for homogeneity of variance and normality of distribution prior to conducting the ANOVA. The data satisfied the assumption of homogeneity of variance. These analyses were performed using SPSS 22.0 (SPSS Inc. Chicago, IL, USA) and SigmaPlot 12.5 for Windows.

3. Results

3.1. Soil DOC

In the CM plots, N deposition significantly reduced DOC concentration in April and July, but not in December (Figure 1). Moreover, in July, the DOC concentration under N60 treatment was significantly higher than that under N30 or N90. In the IM plots, the effect of N deposition on DOC concentration was similar to that in the CM plots in July (Figure 1). However, this effect was not significant in April and December. Nonetheless, a significant increase was observed under the N90 treatment.

The DOC concentrations in the CM plots were significantly higher than those in the IM plots in April and July, but not in December (Figure 1). Moreover, DOC concentration was significantly higher in July than in April and December under both management practices (Figure 1).

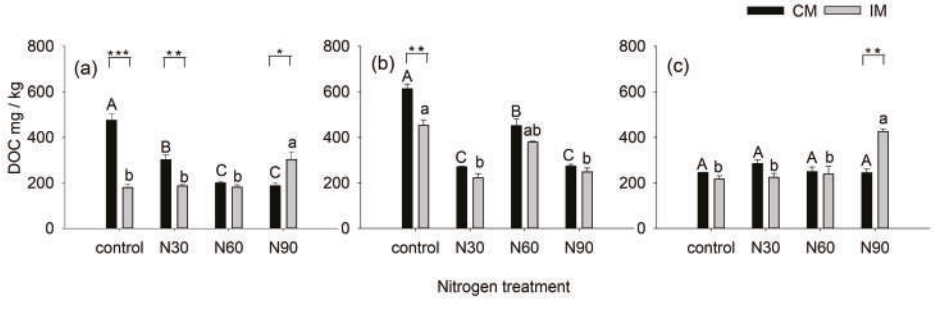

Figure 1. Dissolved organic carbon (DOC) in surface soil (0–20 cm) in different seasons ((**a**) April; (**b**) July; (**c**) December) under different management practices (CM: conventional management; IM: intensive management) and four nitrogen addition treatments (N30: 30 kg N ha^{-1} $year^{-1}$; N60: 60 kg N ha^{-1} $year^{-1}$; N90: 90 kg N ha^{-1} $year^{-1}$ and Control: N-free). Vertical bars indicate the standard error of three replicates. Different uppercase letters indicate significant differences among N addition rates under CM treatments ($p < 0.05$). Different lowercase letters indicate significant differences among N addition rates under IM treatments ($p < 0.05$). Asterisks indicate significant differences between CM and IM at the same N addition rate (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.2. Soil DON

In the CM plots, N deposition significantly decreased DON concentrations in April, but not in July and December, except for the N30 treatment in July ($p < 0.05$) (Figure 2). In the IM plots, only the N60 and N90 treatments significantly increased DON concentrations in both April and December, but not in July (Figure 2). The DON concentration was significantly higher in the CM plots than in the

IM plots in April, but not in July and December (Figure 2). Similar to DOC, DON concentration was significantly higher in July than in April and December, under both management practices (Figure 2).

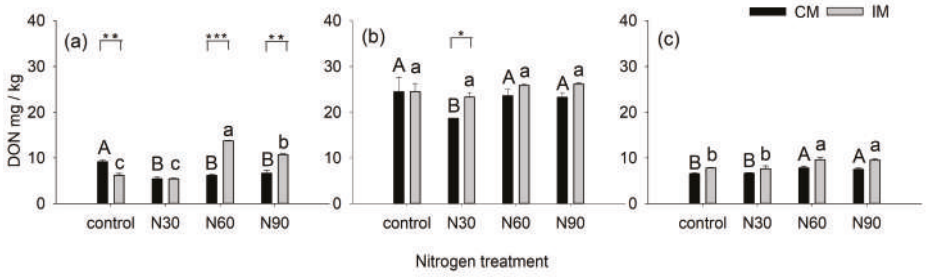

Figure 2. Dissolved organic nitrogen (DON) in surface soil (0–20 cm) in different seasons ((**a**) April; (**b**) July; (**c**) December) under different management practices (CM: conventional management; IM: intensive management) and four nitrogen addition treatments (N30: 30 kg N ha^{-1} $year^{-1}$; N60: 60 kg N ha^{-1} $year^{-1}$; N90: 90 kg N ha^{-1} $year^{-1}$ and Control: N-free). Vertical bars indicate the standard error of three replicates. Different uppercase letters indicate significant differences among N addition rates under CM treatments ($p < 0.05$). Different lowercase letters indicate significant differences among N addition rates under IM treatments ($p < 0.05$). Asterisks indicate significant differences between CM and IM at the same N addition rate (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.3. Combined Influence of N Deposition and Management on Soil DOC and DON

The two-way ANOVA showed that N deposition and management practices, independently and in combination, significantly affected DOC and DON in April ($p < 0.001$). In addition, the contribution of the interaction was greater than the independent effects of the two factors (Table 2). In July, N deposition and management practices, independently and in combination, significantly affected DOC ($p < 0.05$), whereas DON was significantly affected by the independent factors only ($p < 0.01$) (Table 2). Moreover, the contribution of separate factors was greater than of their interaction. In December, N deposition significantly affected the DOC only, whereas the management practices significantly affected DON only ($p < 0.05$), and their interaction significantly affected the DOC only (Table 2). The contribution of interaction was greater than that of the two factors separately on DOC only.

Table 2. Two-way ANOVA of the effects of N deposition and management practices on soil dissolved organic carbon (DOC) and nitrogen (DON) at 0–20 cm soil depths in Moso bamboo forests.

Months	Source of Variation/Factors	N Deposition			Management Practices			Interaction		
		F Value	*p* Value	Contribution (%) [a]	*F* Value	*p* Value	Contribution (%)	*F* Value	*p* Value	Contribution (%)
April	DOC	**18.98**	0.0000	22.00	**38.67**	0.0000	14.94	**49.07**	0.0000	56.88
	DON	**58.31**	0.0000	33.81	**74.61**	0.0000	14.42	**83.96**	0.0000	48.68
July	DOC	**66.26**	0.0000	82.92	**14.37**	0.0016	5.99	**3.53**	0.0390	4.42
	DON	**6.82**	0.0036	28.98	**32.99**	0.0000	46.74	0.38	0.7700	1.61
December	DOC	**6.23**	0.0052	26.46	0.36	0.5553	0.51	**11.87**	0.0002	50.38
	DON	2.30	0.1161	19.95	**7.54**	0.0143	21.80	1.39	0.2831	12.02

Significant contribution at $p < 0.05$ or $p < 0.01$ is shown in bold. [a] The contribution (%) is the percentage of overall variance explained by each factor.

4. Discussion

4.1. Effects of Management Practices on Soil DOC and DON

The present study showed that IM significantly decreased DOC and DON concentrations in spring (Figures 1 and 2), which partly supported our first hypothesis: IM practices decrease soil DOC and DON. Zhou et al. [25] showed that the total soil dissolved C at 0–20 cm soil depth in the IM Moso bamboo plantations was lower than that in the CM plots, which is consistent with the results of the present study. It is known that the concentrations of DOC and DON are mainly derived from ground litter, root exudates, soil humus, soil microbial biomass, and rainfall leaching [26]. Wu et al. [27] found that excessive consumption by microbial populations would decrease the DOC and DON in seasons with high temperature and high precipitation, which is consistent with our result. DOC and DON are important carriers of C and N loss in forest soil [28,29]. Furthermore, soil microbial consumption and leaching are the main output pathways of DOC and DON from forest ecosystems [30]. Yang et al. [31] proved that the growth of plant roots and soil microorganisms (represented by MBC) was enhanced by fertilization, increasing the amount of organic compounds (i.e., DOM) released by plant roots and soil microorganisms. Our previous study on this site showed that IM significantly increased soil MBC [21], indicating an increase in DOC consumption, which might greatly contribute to lower DOC concentrations in the IM plots than in the CM plots. Changed nutrient dynamics caused by management practices can also affect DOM concentrations between the native forests and plantations [27]. Long-term fertilization in the IM plots induced the loss of soil organic C and N and greatly decreased the chemical activity of the soil [20]. Soil acidification owing to long-term fertilization in the IM plots was more severe than that in the CM plots, which led to lower soil pH [19]. The decrease in soil pH might increase the adsorption capacity of Fe and/or Al oxides in soil [32], thereby reducing DOC and DON in the IM plots. Vance et al. [33] also reported a similar result. Generally, the DOM concentration is higher in forest topsoil than in cultivated soil, and plowing, weeding and fertilization in the IM plots alter the physical structure of soil, thus increasing the loss of DOC and DON through surface runoff and subsurface flow [34]. This, combined with high precipitation in spring (Figure 1), might lead to more export of DOC and DON from the IM plots and thereby decrease these concentrations more strongly in the IM than in the CM plots. The increased leaching effect on DOC in the rainy season was also observed by Neff and Asner [35]. Therefore, the high precipitation in spring (Table 1) might have washed a large amount of DOM and caused its loss by leaching, which might have contributed to the decline in DOC and DON between CM and IM in April and July.

In summer, DOC and DON concentrations were higher than those in spring and winter. However, IM largely reduced the concentration of DOC but not of DON in summer (Figures 1 and 2). Temperature can affect soil DOM concentration and turnover by controlling microbial biomass [36]. Jiang et al. [37] found that there was twice as much microbial biomass in summer than in spring in Moso bamboo plantations; thus, the high temperature in July (Table 1) might have contributed to the high DOC and DON. Furthermore, compared with spring, summer and winter had lower precipitation (Table 1), thereby inducing a smaller leaching loss of DOC and DON. At this point, the N input from fertilization might have contributed more to the slightly higher DON in the IM plots than that in the CM plots.

In winter, DOC and DON concentrations were low and did not show significant differences between IM and CM (Figures 1 and 2). A possible reason is that the low temperature in winter decreased the positive effects of temperature on DOC and DON. Moreover, the leaching loss of DON induced by plowing in the IM plots might have declined due to low precipitation (Table 1) and might have even been offset by N input from fertilization, which can contribute to slightly higher DON in the IM plots than that in the CM plots.

4.2. Effects of N Deposition on Soil DOC and DON

In the present study, N addition significantly decreased DOC and DON in the CM plots in spring (Figures 1 and 2), which partly supports our second hypothesis: N deposition decreases soil DOC and DON. N saturation experiments showed that exogenous N input can increase the leaching loss of DON [14] and induce a decrease in soil DON. Previous studies have reported that long-term N deposition reduces soil MBC [22,38]. A high degree of N deposition could affect the composition of the microbial community and inhibit the C of microbial degradation, thereby decelerating the decomposition of litter [39,40]. The effect mentioned above contributes to a decline in soil DOC and DON. Notably, N deposition can intensify soil acidification and decrease soil pH [21], which can potentially change the acidity of soil solutions [41–44]. This, in turn, may lead to the decline in DOC and DON in the CM plots.

In summer, N deposition significantly decreased the DOC concentration but did not significantly affect DON, except under the N30 treatment (Figures 1 and 2). In contrast to DOC, DON increased substantially from April to July, which might be attributed to the stronger adsorption of soil to DON than DOC at high temperatures [45]. Moreover, the high-temperature effect might be offset by the negative effect of N deposition on DON. DOC and DON concentrations in winter were not affected by N deposition (Figures 1 and 2). Probably, the low soil microbial activity due to low temperature and precipitation in winter alleviated the effect of N deposition.

4.3. Combined Influence of N Deposition and Management Practices on Soil DOC and DON

When combined with IM, N deposition significantly increased DOC and DON concentrations in spring and winter. This effect was opposite to that of the independent effect of N deposition in the CM plots. This result indicated that intensive management practices may change the direction of the effects of N deposition on DOC and DON (Figures 1 and 2), which did not support our third hypothesis: the combined effects of N deposition and management practices on soil DOC and DON are stronger than the effects of each of these factors independently. Our previous study at this site found that high N deposition decreased the decomposition of leaf litter and fine roots in IM plots [19,23], which could lead to more litter and fine root accumulation on the soil surface and subsurface. This accumulation may alleviate the leaching of DOC through surface runoff and subsurface flow, which partially explains the current finding that high N addition increased DOC in the IM plots. Nevertheless, in summer, the combined effects of N deposition and IM on DOC and DON were similar to the independent effect of N deposition in the CM plots (Figures 1 and 2), which indicated that the effect of interaction of N deposition and management practices on DOC and DON can vary with season.

The two-way ANOVA demonstrated that in spring, the combined influence of N deposition and management practices had greater effects on soil DOC and DON than the effects of each of these factors independently, which supported our third hypothesis (Table 2). Our previous study [21] at this site demonstrated that differences in microbial community structure were primarily due to a combination of N deposition and management practices (57.73%), with management practices alone accounting for 36.26% of the variation and N addition accounting for 21.47%, indicating that the combination of two factors has a stronger impact on DOM than each factor singly, which also supported our present result. In summer, the positive effects of high temperature partially offset the negative effects of N deposition and IM alone on DOC and DON, thus contributing to the low combination of these two factors (Table 2). Our previous study at this site elucidated that N deposition significantly decreased the diversity of soil microorganisms in both CM and IM plots [21], which indicates that the soil DOC and DON may be strongly correlated with microbial diversity in Moso bamboo plantations. Our results suggest, however, that N deposition has a higher contribution than management practices to soil DOC and DON (Table 2), except for DON in summer. Both DOC and DON concentrations showed the same tendency in the control treatment or the combined effects of N deposition and management practices: DOC and DON increased from spring to summer, then decreased in winter. It appears

to be the combination of high precipitation and temperature that increased soil adsorption to DOC, especially for DON [45].

5. Conclusions

The present study showed that DOC and DON concentrations were higher in summer than in spring and winter in Moso bamboo plantations. Both IM practices and N deposition independently decreased DOC and DON in spring, but not in winter. When combined with IM, N deposition increased DOC and DON in spring and winter. The effects of N deposition on soil DOC and DON strongly depended on management practices and season, suggesting that management practices and seasonal variation should be taken into account when applying ecological models to estimate the effects of N deposition on DOC and DON in terrestrial ecosystems, also indicating that anthropogenic management practices such as plowing and weeding would partially offset the negative effects of N deposition on DOM in the Moso bamboo plantation ecosystem. The results of the present study provide a new perspective for improving our understanding of the comprehensive effects of N deposition and management practices on C and N cycling in plantations. Our findings also offer a beneficial reference on how to manage plantations under the current background of increasing levels of atmospheric N deposition.

Supplementary Materials: The following are available online at www.mdpi.com/1999-4907/8/11/452/s1, Figure S1: Ammonium nitrogen (NH_4^+-N), Nitrate nitrogen (NO_3^--N) and Total dissolved nitrogen (TDN) in surface soil (0–20 cm) in different seasons ((a), (d), (g): April; (b), (e), (h): July; (c), (f), (i): December) under different management practices (CM: conventional management; IM: intensive management) and four nitrogen addition treatments (N30: 30 kg N ha^{-1} $year^{-1}$; N60: 60 kg N ha^{-1} $year^{-1}$; N90: 90 kg N ha^{-1} $year^{-1}$ and Control: N-free). Vertical bars indicate the standard error of three replicates. Different uppercase letters indicate significant differences among N addition rates under CM treatments ($p < 0.05$). Different lowercase letters indicate significant differences among N addition rates under IM treatments ($p < 0.05$). Asterisks indicate significant differences between CM and IM at the same N addition rate (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$), Table S1: The initial stand and soil characteristics of the study sites in the Moso bamboo forest (mean ± SD, $n = 4$).

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Conflicts of Interest: The authors declare no conflict of interest.

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