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Functional response of an Austrian forest soil to N addition

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Abstract

Elevated atmospheric reactive nitrogen (N_R) deposition is considered one of the key components of human induced global change, threatening biodiversity and possibly altering carbon sequestration, one of the forest’s key ecosystem services. Carbon sequestration is the net result of plant production and of soil organic matter (SOM) decomposition. Ignoring the impact of N deposition on plant growth, decomposition or any major physical, biological or anthropogenic process that alters the rate of conversion of soil organic matter to atmospheric CO2 (decomposition) will have profound implications for the global C budget and consequently climate change. Soil nitrogen cycling is predicted to change as a result of increased atmospheric N deposition and mineralization due to temperature increases. However, experimental results on the effects of increased N input on SOM decomposition in the field are inconsistent, reporting positive, negative and neutral responses of SOM to N input. We set out to test the impacts of elevated reactive nitrogen N_R addition, specifically on the soil processes in the field, independently of forest production effects. Using a suite of conventional, natural abundance and isotope pool dilution methods in situ, we traced C and N transformations of soil microbial and gaseous pools and monitored concomitant changes in gross mineralization and nitrification rates, as well as enzymatic activity. Over a number of growing seasons in a spruce dominated Austrian forest we found evidence to suggest N addition significantly reduces gross N mineralization rates and enzyme activity, in-line with an emerging consensus that N deposition reduces soil fungal abundance and activity, ultimately resulting in greater stocks of soil organic carbon. Simulated elevated nitrogen deposition decelerated SOM decomposition and consequently increased soil carbon storage, an N input effect on soil processes independent of the effect of N on tree growth and forest production.

1. Introduction

Forest ecosystems store vast amounts of carbon (C), typically 0.5 to 2 Mg C ha⁻¹ year⁻¹ in the form of standing biomass, and in temperate forest systems they store even greater or similar quantities of C in their soils [1]. Forests are the dominant component of the terrestrial carbon cycle [2, 3]. Forests are particularly sensitive to pollution, evident from their decline during the acid rain decades [4]. Although air pollution has declined, elevated atmospheric reactive nitrogen (N_R) deposition is considered to be a chronic threat, as it is one of the proximate drivers of global climate change, threatening biodiversity and possibly a key forest ecosystem service, C sequestration, since anthropogenic N_R has been linked to forest C uptake [5, 6]. Soils contain the largest terrestrial organic C pool (∼2400 Pg C when calculated to a soil depth of 2 m), therefore storing twice as much C than is found in all the Earth’s plants and atmosphere combined. To put that in perspective, soils store 240 times the global annual fossil fuel emission (∼10 Pg) [7]. Soil carbon is dominantly in the form of soil organic matter (SOM) [8]; a number of meta-analyses have suggested that elevated N addition increases the amount of soil organic C (SOC) [9]. This could be a consequence of higher forest litter inputs due to a positive N fertilization effect and/or increases in the rate of soil organic matter turnover. To evaluate how the relative contribution of these two factors changes with elevated N deposition, it is important to gain a better understanding of the fate of the carbon fixed by fertilization by simulating elevated N deposition and associated changes in SOM decomposition rates.

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effect on tree growth and/or inhibition of SOM decomposition, although few studies have teased out these opposing influences.

In Europe, although $N_R$ compounds in precipitation have decreased since the year 1990, with a 50% drop in NO$_x$ and 30% decline in NH$_3$ between 2009 and 2015 [10–12], future reductions are not expected to remove the risk of eutrophication [10, 13]. In a world where commercial forestry is becoming more important, the impacts of increased N (from deposition or from fertilizer usage) have to be studied experimentally rather than using pollution gradient based studies in order to gain a mechanistic understanding.

The details of microbe-dependent biogeochemical feedback mechanisms on N and C dynamics in European forest ecosystems are limited; they are often based on nitrogen pollution deposition gradients where correlation rather than causality is established, or are based on laboratory studies. Although much is known about the microbial diversity of these systems [14], determining the impact of community structural changes on ecosystem processes such as carbon or nitrogen mineralization has been lacking. Cheng et al [15] concluded that only a handful of studies have directly quantified the effect of N deposition on gross N transformation rates in temperate forests in the field. They noted that the responses of a number of specific N transformation processes to N deposition are poorly studied, for example processes such as autotrophic nitrification (conversion of ammonium to nitrate), heterotrophic nitrification–production of nitrate from organic and inorganic sources (carried out by organisms requiring an external carbon sources for nutrition), dissimilatory nitrate reduction to ammonium (DNRA), N mineralization–conversion of organic nitrogen to inorganic nitrogen and N immobilization (microbial N uptake), in situ, are scarce. They suggested this lack of knowledge is limiting our understanding of the response of soil N cycling to N deposition. Moreover, labile C and N inputs can accelerate organic matter decomposition; this is termed the ‘positive priming effect’ [16]. Labile carbon inputs are dependent on primary productivity, however labile N inputs can be in the form of inorganic–fertilization, N fixation or N deposition/pollution. Due to the foreseen rise in soil temperature associated with global warming, labile soil inorganic-N concentrations in forests are predicted to increase as the result of increased SOM mineralization [2, 17]. At the global scale, N deposition rates are also predicted to double by 2050 [18], which will also have significant consequences for above-ground plant diversity, especially in low N ecosystems such as forests [19, 20]. This could lead to changes in system productivity and/or quality of the litter inputs. Forest growth is generally constrained by available soil N and in N limited soils there is approximately a ninety-fold tree-biomass response to increased soil nitrogen (gram on gram) [21]. $N_R$ would be expected to boost productivity, resulting in increased carbon inputs into the system [22]. However, experimental results on the effects of increased N inputs on SOM decomposition are inconsistent, reporting positive [23, 24], negative [25], and neutral outcomes [26]. Soil organisms and microbial biomass are the decomposition workforce and are generally assumed to be limited by the availability of carbon and nitrogen [27]. This proximate N mediated mechanism of decelerated or accelerated SOM breakdown by the soil microbial biomass could enhance soil carbon storage or push climate change towards a tipping point where increased inorganic N availability leads to greater decomposition leading to a dangerous feedback loop.

The true impacts of N deposition can only be elucidated by conducting medium to long-term studies, where the same response variables are measured over comparable timescales in the field, as the properties of complex systems are often emergent and cannot be readily replicated in the laboratory or glasshouse, as such systems often fail to replicate forest conditions adequately, or capture nuances of interactive or seasonal effects. The recently appreciated role that networked mutualistic mycorrhizal fungi play in forest nutrient cycling, highlight this. Ex-situ studies of sieved soils, where mycorrhizal networks are severed, may not adequately reflect the on-site soil nutrient cycling processes. To capture the inherent complexity in these complex forest ecosystems it is necessary to use methods that allow us to trace soil processes in situ. A suite of methods have been developed such as isotope pool dilution to measure soil N gross mineralization, conversion of organic N to inorganic N (or more precisely ammonium), and gross nitrification conversion of ammonium to nitrate, as well as in-growth bags to study mycorrhizal network growth. These methods can provide critical information in these ‘undisturbed’ systems.

Despite a growing recognition of the importance of organic nitrogen cycling in forests and increasing doubt over the validity of the mineralization immobilization turnover (MIT) model to fully describe N turnover in forest ecosystems, MIT still plays an important role in SOM decomposition and could be affected by elevated N deposition [28]. The central idea of the MIT model is that all soil organic nitrogen is broken down into inorganic nitrogen, specifically ammonium, then possibly nitrate, prior to plant uptake. This implies that all organic nitrogen mineralized passes through the ‘eye’ of the ammonium pool prior to plant N uptake. If we label the ammonium pool with an isotopic tracer, it is then possible to follow the change in pool size and the dilution of the tracer as the unlabeled ammonium products of the organic matter breakdown enter the pool. Similarly, if we label the nitrate pool with a tracer we can measure the dilution due to nitrification of unlabeled ammonium or organic N. The relative sizes and turnover rates of the nitrate and ammonium pools measured in forest soils suggest that the isotope pool dilution (IPD) methods described herein still offer a reasonable viewing window on
the soil N turnover processes. Isotope pool dilution (IPD) methods allow us to measure gross ammonification and nitrification processes irrespective of other influences on the target pool, such as plant N or microbial uptake, leaching, gaseous losses etc with high sensitivity, assuming that the systems are in steady state and all reactions are first order reactions [29]. A number of studies have shown that mycorrhizal fungi can take up and transport low molecular weight organic N, peptides and amino acids before they are mineralized to ammonium, thus circumventing the MIT pathway and possibly leading to underestimation of absolute N mineralization rates [22]. Despite this, in situ IPD experiments offer unique insights into underlying process of SOM-mineralization and nitrification in the field and allow us to compare the influence of externalities such as NR deposition or N addition. Given the potential dual role of N in forest ecosystems of 1) increasing carbon sequestration by stimulating plant production and 2) possibly altering SOM decomposition-carbon mineralization, it is imperative that we have a clear understanding of how these processes are driven.

Based on Cheng’s review [15] it appears that there are no in situ gross rate measurements on temperate forest soils where reactive nitrogen has been experimentally added in the form of nitrate. Given the low nitrification rates in forest soils and high potential reactive nitrate inputs from NOx pollution, which is often co-deposited with ammonium, this is a significant research gap which we identified; to make predictions on how pollution scenarios or fertilization will affect the soil carbon storage response requires this knowledge.

Global carbon budgets rely on underlying process-based models linking shifts in carbon stocks with global drivers of resources. Herein we aim to provide clarity on soil processes associated with SOM decomposition that could inform global models.

In this study we set out to examine the medium-term impact of N addition on gross ammonification, nitrification and heterotrophic nitrification in an Austrian forest soil subject to experimental reactive nitrogen inputs in the form of ammonium nitrate for more than five years. Moreover, we simultaneously studied the respiration responses and soil microbial community structure in an attempt to link them to these ecosystem functions. Small treatment plots within a large experimental forest area were repeatedly fertilized in order to focus specifically on the soil processes in situ. We investigated the impact on N on soil processes independent from the influence of experimental N-addition on tree productivity and subsequent soil inputs as a result of altered litter quality and quantity. Explicitly, we set out to answer these questions: Does inorganic N availability control the rate of organic matter breakdown? Is forest carbon decomposition primed by N addition? Is the change in SOM breakdown rate related to a shift of the community structure or community activity? Does increased N availability influence the gross ammonification and nitrification rates in Austrian soil/forest ecosystems?

2 Materials and methods

The experimental site Zöbelboden is a core site of LTER (Long-Term Ecological Research) Europe. Zöbelboden is located within the Northern limestone Alps (latitude: 47° 50′ 30″, longitude 14° 26′ 30″; www. umweltbundesamt.at/im). It is part of the Upper Austrian ‘Kalkalpen National Park’. The bedrock is mainly carbonaceous, Norian dolomite and limestone. The experimental site is on a flat plateau (altitude 850–956 m). Average annual temperature is 7.2 °C. Minimum monthly temperature is −1 °C (January), the maximum is 15.5 °C (August). Annual precipitation is 1500 to 1800 mm and monthly precipitation is between 75 mm to 182 mm in February and July, respectively. Snow falls between October and May with an average snow cover duration of about 4 months. The forest was clear-cut in 1910 and planted to pure Norwegian spruce in February and July, respectively. Snow falls between October and May with an average snow cover duration of about 4 months. The forest was clear-cut in 1910 and planted to pure Norwegian spruce in February and July, respectively. Snow falls between October and May with an average snow cover duration of about 4 months. Snow falls between October and May with an average snow cover duration of about 4 months. Snow falls between October and May with an average snow cover duration of about 4 months.

The fertilization treatments were carried out in a randomized block design, blocks were repeated 5 times. Blocks were randomly distributed among all trees of the plateau within an area of about 20 × 20 m, but with at least 2 m between blocks. The control and treatment plots were 1 by 1 m and treeless, with an average distance from the nearest tree of around 1.5 m and well marked. Understory vegetation was less than 5% cover on the plots. +N treatment plots were less than 2% of total area; we assume this had minimal impact on overall tree productivity, litter quantity or quality over the experimental period.

+N fertilized plots were watered (carefully hand sprayed, avoiding any drift) with tap water containing ammonium nitrate, giving an equivalent to 80 to 100 kg N ha−1 year−1 (or 8 to 10 g N m−2 year−1). Unfertilized...
control plots were watered with tap-water only in the same manner. Each plot received 1 l of the solution, every 3rd week. Fertilization was not conducted during windy or heavy rain events. A full complement of climatic data were noted on the day before and day after fertilization (precipitation, windspeed, min max temperature etc.). The treatment applications were started in June 2010 and continued throughout the experiment. Rate measurements were conducted once per season over the snow free period between Summer 2016 and Autumn 2017, having received at least six years of elevated nitrogen inputs.

Gross rates of N transformation processes were determined using the $^{15}$N isotope pool dilution (IPD) technique [32–34]. In the IPD technique, the ammonium pool is labelled with an isotopic tracer, the pool size and the isotopic dilution of the pool measured. The isotopic dilution is due to the unlabeled ammonium products from the breakdown of unlabeled organic matter entering the pool. Similarly, if we label the nitrate pool in a different sample with a tracer it is possible to measure gross nitrification. $^{15}$N additions and sampling times were calculated in a series of lab pre-experiments to calculate enrichments and concentrations needed for detection and to ensure the methods fulfilled the assumptions of steady state [34]. $^{15}$N tracer was added at a rate which equated to 5 μg N g soil$^{-1}$. Both labelled potassium nitrate (3.5 atom% $^{15}$N) and ammonium chloride (10 atom% $^{15}$N) were added separately at different ends of each of the plots to determine gross ammonification and gross nitrification separately, and 15 cm within the plot boundaries to avoid edge effects. Label was applied in situ in the field on each replicate plot using a bespoke 7.2 ml injection device, diameter 8 cm. Soil injection volume was approximately 100 ml, the center of the injection site was marked with a small plastic marker. Only the A layer organo-mineral layer was injected and sampled, after having rst scraped back the litter dominated O horizon. Soil sampling was conducted at 4 and 24 h after injection, by taking three 0.7 by 4 cm long cylindrical cores randomly from across the small injection site. Soils from the cores were combined and immediately sieved to 3 mm and subsampled to give a 2 g fresh homogeneous sample, which was weighed and mixed with 15 ml of cold 0.5 M K$_2$SO$_4$ shaken for 1 h then filtered. We also took sub-samples to determine soil dry weight (d.w.) at 105°C. Other bulk soil sub-samples were dried for total C and N and isotope analysis at 50°C. K$_2$SO$_4$ extracts were immediately stored at 4°C and kept for further N analyses. Ammonium and nitrate were determined calorimetrically using the VCL$_{Griess}$ and modified indophenol method in micro titer plates as described Hood-Nowotny et al [35]. Isotope ratios in the extracts were determined using a modification of the micro-diffusion technique [36]. All micro-diffusion discs and soil samples were transferred into tin cups and measured using an Elemental Analyser Isotope Ratio Mass spectrometer (EA-IRMS), connected via a ConFlo III interface (Thermo Fisher) to a Thermo Delta V (Bremen, De). A full series of isotopic and N internal and external International standards were included in each run. DNRA was measured according to Silver [37], gross ammonification and nitrification were measured using a matrix calculation using the standard IPD equations for gross pool inputs (GP) and gross consumption rates (GC) (see SI). All negative rate values were re-assigned to zero (as it is impossible to have negative rates). Missing values were reported as such and all values were reported as μg N g dry soil$^{-1}$. To evaluate the rate data both a linear model and a mixed linear effect analysis were performed using R Core Team 2012 and lme4 [38, 39]. We analyzed the relationship between both gross ammonification and gross nitrification independently, as fixed effects we entered treatment, season and year. Random effects were block. For the mixed models P values were obtained using likelihood ratio tests of the full model with and without treatment. All other statistical analyses were performed using one, two or three way ANOVA’s using Sigma Plot 14.0; data were transformed to conform to normality (Shapiro Wilk test) or run using Dunn’s test on rank based ANOVA’s.

For carbon and nitrogen inventories soil sampling was conducted on all plots; ten replicate soil cores down to a depth of 30 cm, from the top of the O layer, were taken per plot. We chose to take samples based on the layer characteristics, so O layer the dominantly organic litter layer, A the layer mineral and organic fraction, B layer subsoil. We sampled down to the bed rock which was generally at 30 cm depth using a 1.5 cm diameter auger. On each plot we measured and noted the horizon depth carefully. Sampling was done in a W formation across the plot, resulting in less than 0.5% of the plot area being disturbed. Average bulk density (BD) of each horizon was also measured (see SI) and reported on a g dry soil$^{-1}$ per cm$^2$ basis, to calculate carbon stock, care was taken to establish layer specific BD values [40]. Mycorrhizal carbon isotope values were obtained from 12 cm by 5 cm bags containing 50 g of washed sand with a mesh of 50 ± 10 micron (allowing entry of mycorrhiza but not roots) placed in the A horizon over the 2017 soil growing season, two replicates per plot were analyzed for C and N concentrations, and their isotopes, in the same way as the soil. Blank sands (initial values) were also run.

Soil respiration amounts and the respective isotopic ratios were measured on A horizon field moist samples taken in Summer and Autumn in 2016 and 2017. Soils were sieved through a 3 mm sieve on site and returned to the laboratory, on arrival samples were immediately stored at 4°C.

Respiration measurements were made on one-gram fresh soil samples. Concentrations and δ$^{13}$C of CO$_2$ was measured on a gas chromatograph linked to an isotope ratio mass spectrometer and fluxes reported on a dry weight basis. δ$^{13}$C values were reported on the internationally recognised scale of VPDB (Vienna Pee Dee
Belemnite) and uncertainty of the δ^{13}C measurement was 0.2‰ (see SI). Respiration rates were calculated from the slopes of the concentrations and δ^{13}C of CO₂ isotope values based on Keeling plots where r^2 > 0.98.

**Soil respiration and soil moisture** was also measured in the field intermittently using a handheld infrared gas analyzer device (PP Systems-EGM-4, MA, USA connected to 13 cm diameter round static plastic chambers buried permanently in the soil. The chamber closure time was 100 s. Soil moisture was measured using a Field Scout TDR 100 (Spectrum, USA)

**Permanganate oxidizable carbon (Pox-C)** which is widely considered an ‘easily digestible available’ pool which is sensitive to soil management, was measured based on the method of Weil et al [41] (see SI for details). Sucrose and cellulose standards were included for cross comparison.

**To measure potential extracellular enzyme activity** either 4-Methylumbelliferone (MUF) or 7-amino-4-methyl coumarin (AMC) conjugated fluorescent substrates were used [42]. These were added to the soil suspension in different concentrations for different enzyme-conjugate complexes respectively: Exoglucanase/4-Methylumbelliferyl β-D-celllobioside, β-Glucosidase/MUF-β-D-glucopyranoside, exochitinase/MUF-N-acetyl-β-D-glucosaminide, phosphatase/4-Methylumbelliferyl phosphate, protease/L-Leucine-7-amido-4-methylcoumarin hydrochloride (see SI). Activity was calculated and reported as activity nMol g dry soil⁻¹ h⁻¹.
Samples for PLFA analysis, which provides insight into microbial community structure, were prepared using the standard procedure [43–45] (see SI). We used the PLFA functional assignment according to Djukic et al [45]. Bacterial were assumed to be the sum of \( \text{i14:0, i15:0, a15:0, i16:0, i17:0, a17:0, cy17:0, cy19:0, 16:1} \) \( \omega_7c \), 14:0, 15:0, 17:0 groups. Gram positive bacteria (Branched PLFAs) the sum of \( \text{i14:0, i15:0, a15:0, i16:0, a17:0} \). Gram Negative bacteria (cyclopropyl, monounsaturated and straight chain PLFAs), the sum of \( \text{cy17:0, cy19:0, 16:1} \) \( \omega_7c \), 14:0, 15:0, 17:0 groups. Fungi \( \text{18:2} \) \( \omega_6,9 \) and arbuscular mycorrhiza fungi as \( \text{16:1} \) \( \omega_5c \) [45].
3. Results

Total inorganic nitrogen concentrations in the +N treatment were significantly higher in the A horizon (figure 1, Two-way ANOVA, $F_{(1,11)} = 7.316, p = 0.027_{\text{treatment}}$). Nitrate concentration in the control soils were minimal, less than 2 $\mu$g g$^{-1}$ dry soil. Inorganic N was dominantly in the ammonium form in the +N treatments.

There were significantly higher bulk soil carbon and nitrogen stocks, calculated to 30 cm depth, in the +N treatment. This was mainly attributable to differences in the O horizon, where both carbon and nitrogen concentrations were significantly higher (table 1). Notably there were no significant differences in the A and B horizon stocks. Despite higher C concentrations in the O horizon, bulk density-based calculations suggested that the A and B horizons store greater quantities of organic matter than the O horizon.

From the laboratory soil respiration measurements, no significant annual differences between 2016 and 2017 were observed in the A horizon soil respiration rates ($\mu$g CO$_2$ g$^{-1}$ h$^{-1}$), so averages for the two years are given in table 2. Three-way ANOVA revealed no annual or treatment differences in respiration rates, only significant seasonal differences ($F_{(1,32)} = 3.8 p < 0.05_{\text{season}},$ figure 2). Although highly variable and not significant, they point at an explanation for the difference in total carbon stocks; results suggest that on average a third more carbon was respired from the control compared to the +N treatment, lack of significance may be a result of type II errors (high variation, insufficient replication).

Three-way ANOVA revealed season-treatment interactions for the $\delta^{13}$C of the respired CO$_2$, so seasonal effects were analyzed separately. The $\delta^{13}$C of the respired CO$_2$ in the +N treatments was significantly higher in the Autumn ($F_{(1,17)} = 6.5 p = 0.023,$ figure 3), but not in the summer months.

Moreover the $\delta^{13}$C of the respired CO$_2$ in the controls was significantly higher ($-26.7_{\%}o$) in Summer months compared to Autumn ($-27.5_{\%}o$) (Three-way ANOVA $F_{(1,32)},$ post hoc Holm-Sidak $p = 0.035,$ figure 3). Recovered bulk-root $\delta^{13}$C was on average $-27.9_{\%}o \pm 0.9$, SOM $-27.5_{\%}o \pm 0.5$ and soluble organic

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Table 3. Permanganate oxidizable carbon calculated from soil profile measurements ($n = 25$), B horizon normalized to 30 cm depth, using average bulk density measurements per horizon ($n = 5$) and mean values for the different treatments ($n = 5$). Figures in italics are standard deviation of the mean.

|          | mg Pox C per cm$^2$ to a depth of 30 cm | A  | B  | To depth 30 cm Kg Pox C ha$^{-1}$ |
|----------|----------------------------------------|----|----|----------------------------------|
| +N       |                                        | 1.8| 6.7| 5.6                              |
|          |                                        | 1.2| 2.2| 2.3                              |
| Significance |                                      | NS | NS | NS                              |
| Control  |                                        | 1.5| 8.3| 6.4                              |
|          |                                        | 1.7| 3.8| 3.5                              |

Figure 4. Extracellular enzyme activity ± standard error. * indicates significant difference as determined by two-way ANOVA (plot & treatment) $^*P < 0.01,$ $^{**}P < 0.001$ ($n = 5$).
carbon and carbon measured in fine sand bags $-25.5\%$. $\delta^{13}C$ of bulk soil increased significantly down the profile ($F_{\text{soil horizon}} = 10.5, p < 0.001$), average $\delta^{13}C$ values were SOM-O $-27.7\%$, SOM-A $-27.5\%$, and B $-26.7\%$, but there were no significant treatment effects. Pox-C stock concentrations were significantly higher in the control compared to the $+N$ treatment overall, but only when calculated up to a field scale, based on depths and bulk density of the soil horizons (table 2).

Field measurement of soil respiration indicated that respiration was on average 15% lower in the $+N$ treatment but was not significantly different from the control, although significant seasonal differences were observed based on data measured monthly over 12 months but measured only in the growing season (data not
Table 4. Mean phospholipid fatty acid (FA) concentrations in A horizon, \( \mu g \) per g dry soil \(^{-1} \) (\( n = 5 \)). * indicates significant difference as determined by Students T test.

| FA   | FG   | Control | +N | T-test value | +N treatment %-of control |
|------|------|---------|----|--------------|--------------------------|
| i14:0| Gram+| 0.7     | 1.0| 0.80         | 138                      |
| 14:0 | Gram−| 2.0     | 2.0| 0.69         | 99                       |
| i15:0| Gram+| 17.1    | 16.1| 0.72       | 94                       |
| a15:0| Gram+| 7.6     | 8.1 | 0.46        | 106                      |
| 15:0 | Gram−| 1.7     | 1.6 | 0.84        | 97                       |
| i16:0| Gram+| 10.7    | 9.9 | 0.78        | 93                       |
| 16:1w7c| Gram−| 20.3    | 22.4| 0.54       | 111                      |
| 16:1w6c| Gram+| 2.6     | 3.1 | 0.05*       | 123                      |
| 16:1w5c| AM fungi| 12.4   | 12.1| 0.47        | 98                       |
| i16:0| Gram+| 37.2    | 38.2| 0.82        | 103                      |
| 17:1w8| Gram+| 3.6     | 4.0 | 0.58        | 109                      |
| 10Me16:0| Gram+| 9.9     | 11.0| 0.18        | 111                      |
| i17:0| Gram+| 3.6     | 3.7 | 0.55        | 104                      |
| a17:0| Gram+| 5.1     | 5.0 | 0.66        | 99                       |
| 17:1w8| Gram+| 1.2     | 1.2 | 0.88        | 100                      |
| cy17:0| Gram−| 6.1     | 7.9 | 0.48        | 129                      |
| 17:0 | Gram−| 1.7     | 1.8 | 0.11        | 109                      |
| 10Me17:0| Gram+| 2.8     | 2.6 | 0.74        | 94                       |
| 18:2w6:9| Fungi| 19.2    | 20.1| 0.69        | 105                      |
| 18:1w9c| Fungi| 43.7    | 43.3| 0.41        | 99                       |
| 18:1w7c| Gram+| 48.0    | 52.5| 0.81        | 109                      |
| 18:1w5c| Fungi| 5.0     | 4.9 | 0.33        | 97                       |
| 18:0 | Gram+| 8.2     | 8.6 | 0.47        | 105                      |
| 10Me18:0| Gram+| 6.8     | 6.4 | 0.53        | 94                       |
| cy19:0a| Gram−| 0.7     | 0.8 | 0.58        | 111                      |
| Total PLFA |     | 314.5   | 321.4|      | 102                      |

Table 5. Gross process data (+/−standard error of the mean) treatment differences as determined by ANOVA on ranks Kruskal-Wallis * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \) indicate significant differences between the means.

| Flux data \( \mu g \) N g dry soil \(^{-1} \) day \(^{-1} \) | Gross ammonification | Gross nitrification |
|-------------|----------------------|----------------------|
|             | Control | +N    | Control | +N    |
|             | H       |        | H       |        |
| SU16        | 66.57   | 15.55  | **9.5   | 31.48  | 10.33  |
| AT16        | 51.34   | 8.12   | ***17.8 | 2.46   | 0.94   |
| SPR17       | 19.50   | 4.45   | 37.39   | 9.84   | 10.52  | 3.99   |
| SU17        | 56.71   | 15.42  | 41.28   | 12.38  | 2.48   | 0.82   |
| AT17        | 87.93   | 20.87  | 72.92   | 23.51  | 3.65   | 1.43   |
|             | 3.8     | 3.38   | 0.63    |        |

shown). When ambient temperature field values were normalized (by simple arithmetic scaling on a Kelvin basis) to 20 °C, the approximate temperature at which the laboratory samples were taken, we observed significantly higher apparent respiration rates in the Autumn which matched well with the laboratory data (table 3).

Extracellular enzyme activities in the A horizon were suppressed in the +N treatment, specifically β-glucosidase \( (F_{\text{treatment}} = 38.89, p < 0.01) \), exochitinase \( (F_{\text{treatment}} = 45, p < 0.001) \) and protease \( (F_{\text{treatment}} = 21.9, p < 0.001) \) showed half the activity of the control treatment (figure 4).

Total PLFA concentrations, a measure of total microbial biomass, were not significantly different between treatments. PLFA patterns and concentrations in the A horizon were generally not significantly different between the treatments as determined by T-test (table 4). Only the 16:1ω6c concentration, representative of a gram negative bacterial strains, was significantly higher in the +N treatment. Bacterial to fungal ratios were not significantly different between treatments and bacterial PLFAs were more dominant than fungal PLFAs in the A horizon.

Gross ammonification rates in the control plots were significantly higher than in the +N treatment, with ammonification rates in the +N treatment lower in four of the five sampling dates (figure 5, table 5, \( (F_{1,221} = 3.88 \ p < 0.05) \), ANOVA of linear mixed model including the factors season, plot, treatment and year
as variables); however, seasonal effects were stronger ($p < 0.005$). The mixed effect model analysis, with block as a random factor suggested that there was a smaller effect of the +N treatment on gross ammonification rates ($\chi^2(1) = 3.4, p = 0.066$), showing a trend across all the sampling dates. The linear model analysis suggested there were strong seasonal effects. Gross ammonification rates in the Autumn were double that of Spring. Mean values in Autumn in the control and +N treatment plots were 70 and 50 μg N g dry soil$^{-1}$ day$^{-1}$, compared to 19 versus 37 μg N g dry soil$^{-1}$ day$^{-1}$ in Spring, and 61 and 29 μg N g dry soil$^{-1}$ day$^{-1}$ in Summer in the control and +N treatments respectively ($F(2,221) = 5.31, p = 0.005_{\text{season}}$). Influences of season were greater than those of treatment, year and block reflecting the dramatic differences in seasonal weather patterns in the region.

Gross nitrification rates in the controls were lower than the rates in the +N treatment, less than 11 μg N g dry soil$^{-1}$ day$^{-1}$, both the linear model ANOVA ($F(1,210) = 4.25, p < 0.05$) and mixed effect model analysis (with block as a random effect, figure 6), suggesting that there was a significant effect of treatment ($\chi^2(1) = 4.2, p = 0.04$). In summer these differences were clearly driven by ammonium addition (the availability of ammonium for nitrification); gross nitrification rates were 14 versus 2 μg N g dry soil$^{-1}$ day$^{-1}$ in the +N treatment and control respectively. In Autumn this effect persisted, but differences between treatments were not significant. In spring, rates were higher in the control, but not significantly, 10 versus 3 μg N g dry soil$^{-1}$ day$^{-1}$ in the control and +N treatment. Again season had significant effects on rates. It appeared that nitrification was dominated by heterotrophic nitrification as direct rates of nitrification measured were less than 0.3 μg N g dry soil$^{-1}$ day$^{-1}$ in both control and +N treatment, even though we added sufficient ammonium to trace it. DNRA rates were similar in both control and the +N treatment, consistent over seasons and low, but detectable, around 3 μg N g dry soil$^{-1}$ day$^{-1}$. However, these rates were only measured where we added a nitrate label and were possibly overestimates of background rates, as we observed low nitrate concentrations throughout the whole experiment in both control and +N plots respectively (>2 μg and 15 μg N g dry soil$^{-1}$). There were significant differences in the A-horizon sand bag carbon concentrations with mean values of 2159 compared 852 mg C kg

![Figure 7. Mean carbon and nitrogen concentrations in fine mesh, fine sand bags buried in the A horizon in Autumn 2016, removed and measured Autumn 2017 (n = 10), error bars are standard error of the means.](image-url)
in response to N pollution evidenced by the inorganic N data, had little impact microbial community assemblage in the A-horizon. This is as a result of repeated measures, suggesting that the imposition of the abundance of one gram negative bacterial indicator species, but this may have been the result of a Type II error, similar in both treatment and control in the A horizon, suggesting retention of community structure over the only investigated the A horizon. The PLFA total and individual average values in our study were remarkably their data were skewed by the higher microbial abundance and diversity in the uppermost soil layers, whereas we structure, presence or abundance of the soil microbial biomass is a more nuanced controller of biogeochemical measure of changes in microbial activity. Taken together the results suggest that microbial activity rather than

4. Discussion

The significantly higher carbon and nitrogen total soils stocks in the +N treatment were attributable to differences in the O horizons, as there were no significant differences in the C & N stocks in the other soil horizons. This phenomenon was probably due to the inhibition of fungal activity in the O horizon, as a result of the high inorganic nitrogen load, given the sensitivity of fungal community structure to inputs of N [46]. Microbial data on the O horizon was not reported in this study. Overall C stocks in this study ~110 Mg C ha\(^{-1}\) were higher than those reported for typical cambisols (50 Mg C for top 30 cm) [7]. Similar significant increases in soil organic carbon and nitrogen stocks in the O horizon have been previously observed in Swiss and Danish soils subject to long-term N deposition treatments [47], but to a lesser extent, ~25% as opposed to ~40% increases that we observed, although we used higher N rates than their study. Moreover, we saw no evidence of vertical redistribution of organic carbon pools as had been previously observed [47]. In many of the previous studies, teasing out the increase as a result of forest production from decomposition was not possible, as the large +N treatment plots included the trees, therefore soil effects observed are often a combination of effects from the above ground tree litter input and the soil response. This experiment attempted to overcome this issue using a simple approach; soil was treated only in plots small enough to not significantly influence tree inputs (less than 2% of total experimental area) but large enough to conduct experiments and overcome edge effects. These data clearly show increases in carbon stocks are in part due to the inhibition of soil decomposition rates as a result of high inorganic N inputs and not only increased forest production. Furthermore, profile Pox-C concentrations were around 15% greater in the controls and significantly higher than in the +N treatment, suggesting that more labile carbon was present, presumably a reason for the higher enzyme activity observed in the controls. +N treatment reduced nearly all soil extracellular enzyme activities. Although the activity of the cellulose degrading enzyme exoglucanase was reduced to half its activity, the effect was not significant, despite the fact that the activities of other enzymes involved in complex polymer substrate breakdown (such as β-glucosidase and exochitinase) were significantly reduced. The protease enzymes involved in protein depolymerization were almost completely inhibited in the +N treatment. Notably, the enzymes involved in phosphate metabolism were not affected. The inhibition of enzyme activity involved in forest soil organic matter decomposition by inorganic N availability has been previously documented [48–50] and has been attributed to a feedback mechanism of N availability controlling microbial enzyme production and N mining. Here we also clearly show that increased inorganic N concentrations reduced enzyme production which could explain the significant reduction in gross N mineralization rates in the N treated soils. It should be stressed that we added at least five times the estimated annual N\(_R\) input due to deposition into these systems.

Interestingly in the A horizon we saw no significant differences in the viable microbial biomass pool size, derived from total PLFA data, or big differences in microbial community structure evident from relative PLFA abundances. Control and +N values were very similar, although there was one significant difference in the abundance of one gram negative bacterial indicator species, but this may have been the result of a Type II error, as a result of repeated measures, suggesting that the imposition of the +N treatment, although clearly there, as evidenced by the inorganic N data, had little impact microbial community assemblage in the A-horizon. This is in contrast to global genetic studies which have revealed a global decline in both fungal diversity and abundance in response to N pollution [14] in soils cores of 25 cm depth. One explanation for the discrepancy could be that their data were skewed by the higher microbial abundance and diversity in the uppermost soil layers, whereas we only investigated the A horizon. The PLFA total and individual average values in our study were remarkably similar in both treatment and control in the A horizon, suggesting retention of community structure over the midterm, despite the large N inputs we applied and differences in inorganic N concentrations we measured. However, there were significantly higher carbon contents in fine mesh sand bags of the A horizon suggesting higher mycorrhizal activity in the control soils, suggesting that sand bags could possibly provide a more sensitive measure of changes in microbial activity. Taken together the results suggest that microbial activity rather than structure, presence or abundance of the soil microbial biomass is a more nuanced controller of biogeochemical processes in these forest systems. This is in contrast to findings from Ribbons et al [51] who found that isotopic pool dilution based gross process rates measured in the laboratory were related to microbial C:N ratios. However, on closer analysis they had made immediate extractions following injection of the isotope label, which may have unwittingly biased their data. We suggest that it is unlikely that their soils would have reached steady state conditions immediately, leading to non-compliance with the assumptions of IPD, and consequently an overestimation of gross process rates measured; [52, 53] as a result of pool substitution, a phenomenon outside the scope of this study, but particularly problematic in forest soils. In this study, tests were made prior to main
study to ensure all assumptions of the IPD were fulfilled and is why the four hour and twenty-four-hour sampling times were selected.

The lack of significant treatment differences in soil respiration measured in the laboratory was probably due to insufficient replication and high forest heterogeneity. A trend, however, was observed; on average a third more carbon was respired from the control compared to the +N treatment. Soils are often more heterogeneous than plant related traits, underlying the need for sufficient replication [54]. Although we did observe significant seasonal differences in soil respiration and mineralization. In Autumn, rather intuitively, significantly more carbon was respired and mineralization rates were higher, possibly a consequence of higher fungal decomposition activity, also evident from common observations of fungal fruiting behavior in forests. However, care must be taken when interpreting these results as all the soil respiration tests were run at laboratory temperature of 20 °C; temperatures to date rarely seen in the field in Autumn in Austria. Isotope data suggest that more recalcitrant SOM carbon was respired in the control in Autumn, as mean respired soil values were of −27.54% ±0.47 and the value of measured assumed to be mycorrhizal derived from ‘tree’ carbon was −25.5% the value we measured in the sand bags. The higher respiration rates in conjunction with more negative values in the control suggest that more than 50% of the C in the control was derived from the breakdown of SOM, calculated using simple mass balance. In the bulk soil we measured a significant, although not particularly predictive, negative relationship between % soil carbon and δ13C (p < 0.001 r² = 0.22, n = 48). Soils with high SOC had more negative δ13C values and those with low % soil carbon less negative δ13C values. The fact that the control respiration had lower δ13C values in Autumn suggests that, in the control plots at least, the soil microbial biomass was catabolizing more of this recalcitrant soil organic carbon, and in doing so was releasing nitrogen, in line with previous findings [35]. In summer the direct or mycorrhizal ‘tree’ input signal appears to swamp the SOM breakdown isotope signal. This suggests a divergent microbial strategy of Summer mutualism and Autumnal SOM breakdown in these forest soils. These results confer well with the mineralization data which showed that in Autumn gross ammonification was higher in the controls, as too were Pox-C concentrations and enzyme activities. Also, despite the fact we saw no significant differences in the A horizon bulk % SOM carbon between treatments, we did observe overall higher carbon stocks in the +N treatment concurrent with these observations. Moreover, we observed no correlation between gross N mineralization and SOM content. The in-growth bag data confirmed that the +N treatment had a significant effect on what was assumed to be the fungal-mycorrhizal community. These isotope and in-growth bag methods are useful indicators of changes in forest biogeochemistry, possibly overcoming some of the problems with the forest heterogeneity we experienced.

Gross ammonification rates were significantly decelerated as a result of the +N treatment or negatively primed [16]. Although we saw no significant shifts in microbial communities as determined by PLFA analysis in the A horizon we did observe significant functional changes in enzyme activity, suggesting that microbial activity rather than abundance is a more important predictor of process rates in these systems. No positive priming effects of the N addition were observed in this system, although they have been observed in forests [56]. We suggest this lack of positive priming was the result of the very high C:N ratios measured in this forest, in the order of ~21:1 in the O horizon and ~20:1 in the A horizon. In these low fertility coniferous forests with high carbon contents, tipping the SOM to a C:N ratio of 12:1 ratio, where mineralization is known to exceed immobilization, would require huge quantities of N, in the order of 9 t ha⁻¹ for this system as a whole to respond. We show in line with Janssens et al [25] that adding inorganic N to soil high in recalcitrant SOM leads to a decrease in decomposition and effective increase in soil carbon storage. However, we have managed to explicitly demonstrate that SOM sequestration under +N treatments is in part due to decelerated SOM decomposition.

In light of recent evidence of climatic controls on forest litter decomposition, it is increasingly clear that these ectomycorrhizal dominated forests are particularly adapted to the alpine conditions of Zöbelboden and that these pine dominated forests have evolved an evolutionary stable strategy [57, 58]. Clearly the tight native nitrogen cycle in these forest soils confer an ecological benefit to the dominant tree species and their ectomycorrhizal fungi, leading to the formation of these characteristic forest soils. We posit that inorganic nitrogen addition in the form nitrate is so alien to these systems that they are not adapted to nitrate uptake and that the added nitrate simply leaches out of the system with known acidification effects [39]. The nutrient tight nitrogen cycle of the system is clearly an adaptation to both temperature constraints and high precipitation.

Below-ground storage of immobile nitrogen in the form of organic or ammonium N, which is not subject to leaching losses, becomes an effective extended-phenotype strategy for nutrient uptake and growth, particularly in times of snow melt where nitrate would be vulnerable to leaching.

We found significant negative impacts of N enrichment on community function, evident from reduced rates of SOM decomposition based on respiration measurements and detailed N cycling data. Lack of response on the microbial community size or structure might have been due to the fact that these Austrian sites are considered N saturated and that our controls are already so altered by legacy N pollution that we see little effect of the nitrogen treatment. We conclude that these coniferous forest soils, which have an extremely high C:N ratio, are not
sensitive to changes in the stoichiometry per se, but to deactivation of the fungal, probably ectomycorrhizal enzyme production.

We measured the impact of simulated atmospheric nitrogen deposition on forest soil carbon sequestration and nitrogen and carbon turnover, in doing so we examined the consequences for ecosystem function using an experimental approach. The work highlighted that it is possible to directly measure changes in organic matter turnover in forest soils in realistic time frames in the field using the novel isotope techniques described herein. These sensitive measurements are essential to underpin the assumption made in the models and can only be executed where the true complexity of the ecosystem is captured in the experimental design. It should be stressed that to date most of the studies of this isotopic and molecular nature have been conducted in short term experiments in the laboratory and then extrapolated back to the field. As has been so eloquently stressed a more ‘mycocentric’ view of plant–mycorrhizal relationships is required [60] to improve our ability to predict impacts of environmental disturbances. Such nuanced field experiments are surely necessary, albeit bearing in mind the need for greater replication.

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Data availability statement

The data that support the findings of this study are available upon reasonable request from the authors.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

RH, ID, MG and TD wrote original proposal and designed experiments. RH was PI. RH, ID, MG, KS, EZ, SL conducted the experiments and analysed data. RH, EZ, AW and SL analysed samples. RH wrote manuscript with editorial contributions from all authors.

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