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Anthropogenic nitrogen enrichment enhances soil carbon accumulation by impacting saprotrophs rather than ectomycorrhizal fungal activity

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Abstract

There is evidence that anthropogenic nitrogen (N) deposition enhances carbon (C) sequestration in boreal forest soils. However, it is unclear how free-living saprotrophs (bacteria and fungi, SAP) and ectomycorrhizal (EM) fungi responses to N addition impact soil C dynamics. Our aim was to investigate how SAP and EM communities are impacted by N enrichment and to estimate whether these changes influence decay of litter and humus. We conducted a long-term experiment in northern Sweden, maintained since 2004, consisting of ambient, low N additions (0, 3, 6, and 12 kg N ha^{-1} $year^{-1}$) simulating current N deposition rates in the boreal region, as well as a high N addition (50 kg N ha^{-1} $year^{-1}$). Our data showed that long-term N enrichment impeded mass loss of litter, but not of humus, and only in response to the highest N addition treatment. Furthermore, our data showed that EM fungi reduced the mass of N and P in both substrates during the incubation period compared to when only SAP organisms were present. Low N additions had no effect on microbial community structure, while the high N addition decreased fungal and bacterial biomasses and altered EM fungi and SAP community composition. Actinomycetes were the only bacterial SAP to show increased biomass in response to the highest N addition. These results provide a mechanistic understanding of how anthropogenic N enrichment can influence soil C accumulation rates and suggest that current N deposition rates in the boreal region (≤12 kg N ha^{-1} $year^{-1}$) are likely to have a minor impact on the soil microbial community and the decomposition of humus and litter.

KEYWORDS

carbon sequestration, ecological stoichiometry, Gadgil effect, high-throughput sequencing, ingrowth mesh bags, ITS amplicons, litter decomposition, root exclosure, soil organic matter

1 | INTRODUCTION

In northern ecosystems, plant growth is usually limited by the availability of soil nitrogen (N; Tamm, 1991). Soil microbes provide the main source of N taken up by plants as a result of their decomposition of plant litter and humus, and thereby play a key role in regulating both N and carbon (C) cycling. During the past century, the increased use of fertilizers and combustion of fossil fuels have affected the global N cycle (Galloway et al., 2008; Vitousek et al., 1997), resulting in higher N inputs to terrestrial ecosystems. There is increasing

evidence that anthropogenic N deposition enhances C accumulation in both vegetation and soils in N-limited ecosystems, such as boreal and temperate forests. However, the mechanisms by which C accumulates belowground in response to N deposition remain poorly understood (Frey et al., 2014; Maaroufi et al., 2015; Zak, Freedman, Upchurch, Steffens, & Kögel-Knabner, 2017).

In boreal forests, two main fungal guilds are involved in soil C dynamics, ectomycorrhizal (EM) and saprotrophic fungi (Fernandez & Kennedy, 2015; Read, 1991). The vast majority of trees in these ecosystems form symbioses with EM fungi where the host plant provides photosynthates that serve as a primary C source to EM fungi (Talbot, Allison, & Treseder, 2008), while EM fungi explore the soil through their hyphal network and provide water and nutrients, such as N, to their host plant (Smith & Read, 2008). EM fungi can affect soil C dynamics in several ways. On the one hand, certain EM fungal species can negatively affect soil C pools by stimulating C losses through the production of enzymes that degrade soil organic matter and potentially further enhance C availability to saprotrophic fungi and other soil organisms (Högberg & Read, 2006). On the other hand, EM fungi have been shown to contribute to soil C accumulation through the production of recalcitrant fungal tissues (i.e., fungal necromass; Clemmensen et al., 2013, 2015). Other studies have also suggested that plants and their EM fungi may enhance soil C accumulation through direct competition with saprotrophs (SAP) for nutrients, referred to as the 'Gadgil effect' (Averill & Hawkes, 2016; Gadgil & Gadgil, 1975; Orwin, Kirschbaum, St John, & Dickie, 2011), thus reducing saprotrophic activity and the decomposition of soil organic matter (Averill, Turner, & Finzi, 2014; Lindahl, Boer, & Finlay, 2010; Sterkenburg, Clemmensen, Ekblad, Finlay, & Lindahl, 2018). Several studies have shown that N addition can negatively impact the abundance and diversity of EM fungi as a result of reduced belowground C allocation by trees (Högberg, Bååth, Nordgren, Arnebrant, & Högberg, 2003; Högberg et al., 2010; Treseder, 2004). However, how N-induced changes in EM fungal communities impact other components of the microbial community (e.g., free-living SAP) and soil C dynamics remains poorly understood.

It is also uncertain how EM fungi and other soil microbes are impacted by the rate of N addition. Several studies have shown a reduction in microbial biomass in response to high doses of N fertilizers (50–150 kg N ha^{-1} $year^{-1}$), particularly for EM fungi (Frey, Knorr, Parrent, & Simpson, 2004; Hasselquist & Högberg, 2014; Nilsson & Wallander, 2003), as well as concomitant decreases in fungal and bacterial biomasses (Blaško, Högberg, Bach, & Högberg, 2013; Demoling, Nilsson, & Bååth, 2008; Treseder, 2008). Some additional studies applying lower N addition rates (<50 kg N ha^{-1} $year^{-1}$) have also shown declines in EM root tip abundance, as well as shifts in fungal community composition along N deposition gradients (27–43 kg N ha^{-1} $year^{-1}$; Kjøller et al., 2012). However, other studies applying low annual rates of N to highly N poor forests have found either no effect on fungal or bacterial biomass (12.5 kg N ha^{-1} $year^{-1}$; Maaroufi et al., 2015), or an increase in EM respiration and sporocarp production (20 kg N ha^{-1} $year^{-1}$; Hasselquist & Högberg, 2014; Hasselquist, Metcalfe, & Högberg, 2012). Thus, these contrasting results suggest that it remains poorly understood whether key soil microbial community functional groups respond differently to low versus high long-term N addition rates.

Furthermore, the effect of N additions on microbial mediated organic matter decomposition may depend on the type of substrate that is decomposed. Studies have shown that N addition may accelerate decomposition rates of fresh litter, while deeper in the organic soil horizon, N addition can decrease decomposition rates of humus (Berg & Matzner, 1997; Magill & Aber, 1998; Melillo, Aber, Muratore, & Jun, 1982). In addition to differences in substrate quality, these findings may be the result of differences in fungal guilds having contrasting enzymatic capacities at different soil depths. Saprotrophic and EM fungi are often spatially separated in fresh litter and deeper humus layers, respectively (Lindahl et al., 2007; O'Brien, Parrent, Jackson, Moncalvo, & Vilgalys, 2005). However, studies have also demonstrated that some EM fungi may have similar oxidative and hydrolytic enzymatic capacities to saprotrophic fungi (Bödeker, Nygren, Taylor, Olson, & Lindahl, 2009; Firoz, 2014; Phillips, Ward, & Jones, 2014), and that gene expression for their oxidative degradation may be regulated by belowground tree C allocation (Rineau et al., 2013; Voříšková, Brabcová, Cajthaml, & Baldrian, 2014). Furthermore, a recent study conducted in a boreal forest suggested that saprotrophic and EM fungi may have overlapping niches with regard to their colonization of different substrate qualities, suggesting that EM fungi may colonize fresh litter as well as humus (Bödeker, Lindahl, Olson, & Clemmensen, 2016). While it is clear that both EM fungi and saprotrophic microbiota (i.e., fungi and other microbes) play an important role in decomposition, less is known about how N enrichment influences the relative contribution of these functional groups to the decomposition of new (fresh litter) versus old substrates (humus).

We conducted a long-term experiment in the middle boreal zone of northern Sweden to understand how low versus high chronic N addition impacts soil microbial communities, and to determine whether these changes influence litter and humus decomposition rates and, consequently, soil C stocks. The study was established in a Scots pine forest by applying N addition treatments annually since 2004 to simulate a range of N deposition rates that occur in boreal forests (Dentener et al., 2006) as well as higher rates used in numerous long-term N fertilization studies (Hyvönen et al., 2008). We also set up root exclosure plots to separate the contribution of root-associated biota such as EM fungi from the rest of the soil microbial community (Hanson, Edwards, Garten, & Andrews, 2000; Wallander, Nilsson, Hagerberg, & Bååth, 2001). We tested the following hypotheses: (a) that N addition will impact decomposition of both litter and humus, with low N addition levels promoting microbial activity, and high levels of N addition reducing it; (b) that changes in decomposition in response to N would differ depending on whether root-associated microbes are included or excluded, due to competitive interactions between EM fungi and SAP; (c) that the decomposition response to N would correspond to changes in both the biomass and composition of the microbial community, with SAP contributing more to the decomposition response of fresh litter, and EM fungi contributing

more to the decomposition response of humus. Testing these hypotheses together will provide a more mechanistic understanding of why soil C accumulates in response to anthropogenic N inputs, which occurs at our study site (Figure S1), and has been shown at several other N addition experiments in the boreal region (Hyvönen et al., 2008; Maaroufi et al., 2015).

2 | MATERIAL AND METHODS

2.1 | Study site

The study was performed at Åheden Experimental Forest (64°14′N, 19°46′E) in the middle boreal zone of northern Sweden. The forest site consists of a naturally regenerated ~140 year old Scots pine forest (*Pinus sylvestris* L.). The plant community at the site is classified as xeric-to-dry dwarf-shrub type (Arnborg, 1990). The understory layer is dominated by ericaceous shrub species, *Vaccinium vitis-idaea* L. and *Calluna vulgaris* (L.) Hull. The bottom layer is composed mainly by the bryophytes *Pleurozium schreberi* (Brid) and to a lesser extent by *Dicranum* sp. and, by the lichens *Cladonia rangiferina* (L.) Weber and *C. arbuscula* (Wallr.) Flot. Soils at the site are typic haplocryods developed from fine sandy and silty glacial outwash sediments (FAO, Cambic Podzol). The background N deposition rate in the area is approximately 2 kg N ha^{-1} $year^{-1}$ (Pihl-Karlsson, Karlsson, Akselsson, Kronnäs, & Hellsten, 2013). Mean annual precipitation and temperature at the site are approximately 583 mm and +1.0°C, respectively.

2.2 | Experimental design

In 2004, a long-term N addition experiment was set up at the site, consisting of five N addition levels (0, 3, 6, 12, 50 kg N ha^{-1} $year^{-1}$) being applied to 0.1 ha plots in a fully randomized design (n = 6 per treatment). The low N addition treatments (3, 6, and 12 kg N ha^{-1} $year^{-1}$) were chosen to simulate the range of N deposition rates that occur in the entire boreal region (Gundale, Deluca, & Nordin, 2011), with 12 kg N ha^{-1} $year^{-1}$ representing the upper rates of N deposition, which are maximal in the southern boreal zone of northern Europe. For a map of N deposition rates in the boreal region, please see Gundale et al. (2011). In contrast, the highest N addition treatment (50 kg N ha^{-1} $year^{-1}$) serves as a useful comparison with many previous long-term forest fertilization experiments. The N addition treatments consist of manually spreading solid granules of ammonium nitrate (NH_4NO_3) throughout individual plots once each year directly after snow melt (i.e., May).

In order to separate the contribution of EM fungi from the rest of the microbial community, we established two 0.25 m^2 root-EM fungi exclosure plots (Wallander et al., 2001) within each 0.1 ha main plot during July 2014 (i.e., 10 years after the start of N addition). Root-EM fungi exclosures (hereafter referred to as trenched plots) consisted of square steel collars (50 × 50 × 50 cm) that were permanently inserted into the soil in order to avoid any ingrowth of tree roots and EM fungi. Tree seedlings were also removed within each collar in order to prevent EM fungi networks to establish. After the subplots were established, they were left to equilibrate for 9 weeks in order to allow EM fungi to die, and fine roots to decompose. Paired non-exclosure subplots (0.5 × 0.5 m) were established adjacent to each exclosure subplot in order to assess the contribution of the total soil microbial community to soil C dynamics (hereafter referred to as untrenched plots).

2.3 | Soil C accumulation

In autumn 2017, the organic horizon was sampled at 10 predefined locations in each main plot by collecting the entire organic horizon (i.e., from the top of the forest floor down to the mineral soil surface) with a 10 cm diameter PVC tube fitted with a serrated blade. The 10 cores were then pooled to form one composite sample per plot and sieved through a 1 cm sieve and then a 2 mm sieve to remove intact roots. The composite sample was then thoroughly homogenized. A subsample of this composite was dried at 60°C and ground to a fine powder using a roller mill, upon which the organic matter content was measured by loss on ignition (550°C, 6 hr) on analytical triplicates, each of approximately 10 g soil. The soil organic matter stocks were upscaled to an area basis using the composite core mass within each plot, the composite core surface area, and the average % organic matter of the composite sample. Carbon stocks were finally calculated as 50% of the organic matter mass (Pribyl, 2010). In addition, pH was measured from these same samples using 1 g dried soil in 10 ml of calcium chloride ($CaCl_2$ 0.01 mol/L).

2.4 | Humus and litter decomposition

In summer 2014, organic horizon soil (i.e., humus) and freshly senesced *P. sylvestris* needle litter were collected from unfertilized plots in the vicinity of the experimental site. Humus and plant litter were chosen because they provided an old versus new C substrate for microbial decomposition, respectively. Humus samples were passed through a 4 mm sieve to remove roots, stones, and coarse materials, then homogenized and oven-dried at 60°C for 48 hr. The collected litter was air-dried and thoroughly homogenized. Subsamples of litter were dried at 60°C for 48 hr to determine their dry weight and thus correct the initial litter bag biomass for moisture content. Subsamples of both humus and litter were also taken and ground for chemical characterization (i.e., %C, %N, %P); the initial chemical parameters of humus and litter are reported in Table S1. The remaining humus and litter were used to make decomposition bags, following the same design as the ingrowth mesh bags (see below). The mesh bags were filled with 3 g dry weight of humus or *P. sylvestris* needle litter. Two bags of each type (i.e., humus and litter) were placed at the interface between the mineral and organic soil horizons, both inside and outside each trenching subplot. Because there were two subreplicate trenched and untrenched plots per main plot, this yielded a total of 240 decomposition bags in total (i.e., 30 plots × 2 substrate types × 2 trenching treatments × 2 subreplicates of each trenching treatment). The bags were collected 12 months later.

After collection, the mesh bags were oven-dried at 60°C for 48 hr to prevent further decomposition, and then weighed to estimate total mass loss. The content of the two subreplicate mesh bags of each substrate type removed from each plots were composited, ground, and analyzed as the initial soil and litter samples. Total C and N were determined by dry combustion (Flash EA 2000; Thermo Fisher Scientific, Germany), while total P was measured by Kjeldahl acid digestion (Auto Analyzer III Spectrophotometer; Omniprocess, Germany). Humus and litter mass changes were expressed as relative to the initial mass (% mass change). We further calculated the mass change in each specific element (i.e., C, N, and P), using concentrations (mg/g) of each element before and after the incubation, which allowed calculation of the final mass of each element. Substrate mass losses and final substrate C, N, and P concentrations are reported in Table S2.

2.5 | Phospholipid fatty acid analysis

Soil microbial communities within and outside the trenching plots for all N addition treatments were characterized using the microbial phospholipid fatty acid method (PLFA). In September 2015 (11 years after the start of N addition and 1 year after the establishment of trenching plots), soil samples from the two untrenched and trenched subplots were collected, and were pooled into a single composite sample per subplot type within each plot. Soil samples were immediately frozen and freeze-dried, and a subsample of 1 g was used to extract PLFAs using a modified method of Bligh and Dyer (Bligh & Dyer, 1959; Mcintosh, Macdonald, & Gundale, 2012; White, Nickels, King, & Bobbie, 1979). A total of 28 PLFA markers were detected with some subsets of microbial PLFAs representing different functional groups. Total bacteria were represented by i-15:0, a-15:0, 15:0, i-16:0, 16:1ω9, 16:1ω7, 16:0, i-17:0, cy-17:0, a-17:0, 18:1ω7, and cy-19:0 bacterial PLFAs (Frostegård & Bååth, 1996). Gram-positive bacteria were represented by branched chain fatty acids i-15:0, a-15:0, i-16:0, i-17:0, and a-17:0, while cy-17:0, cy-19:0, and 18:1ω7 were used as a measure of gram-negative bacteria. The branched chain fatty acids 10me16:0, 10me17:0, and 10me18:0 were used to estimate actinomycete contribution (Kroppenstedt, 1985; Wardle, Gundale, Jäderlund, & Nilsson, 2013). PLFAs 18:2ω6 was used to estimate the contribution of fungi (Frostegård, Tunlid, & Bååth, 2011).

2.6 | Fungal community analysis

The fungal community composition was estimated using nylon ingrowth mesh bags (Wallander et al., 2001). Triangular ingrowth bags (9 × 9 × 12.5 cm sides) were constructed using a 50 μm mesh and were filled with 30 g acid-washed and burned quartz sand (0.5–1 mm). This mesh size was chosen to allow fungal hyphae to grow in or through the bags while excluding root ingrowth, while the substrate was chosen because it allows easy extraction of fungal tissues. In September 2014 (3 months after the establishment of trenching subplots), ingrowth bags were gently placed (to minimize soil disturbance) at the interface between the mineral soil and the organic horizon (Lindahl et al., 2007). The ingrowth bags were buried within the trenched plots and within the paired untrenched plot directly adjacent to each trenched plot (i.e., 120 ingrowth bags in total). The buried mesh bags were collected after 12 months (11 years after the start of N addition), and were stored at −20°C until chemical analysis was performed.

The ingrowth bags were freeze-dried and the contents from bags derived from the two subplots within each main plot were pooled, yielding a single sample from inside and a single sample from outside trenching plots. These samples were then homogenized using a ball mill with the ball removed (MM 301; Retsch, Germany). DNA was extracted from a 5 g subsample of homogenized sand by adding 10 ml CTAB-SDS buffer (2% cetrimonium bromide, 2% sodium dodecyl sulfate, 2 mmol/L EDTA, 150 mmol/L Tris-HCl, pH 8), vortexing, and then incubating at 65°C for 1.5 hr, followed by chloroform addition, vortexing, supernatant removal, and isopropanol/ethanol precipitation. The DNA pellet was resuspended in 50 μl of MilliQ-water, and then purified using a Nucleospin gDNA clean up kit (Machery-Nagel, Düren, Germany). DNA from the ITS2 region of the Internal transcribed Spacer (ITS) was amplified with the fungal-specific primers ITS7g (Ihrmark et al., 2012) and ITS4 (Gardes & Bruns, 1993), which included adapter sequences for Illumina sequencing, and subjected to a second eight-cycle amplification to attach Nextera (Illumina Inc., San Diego, CA) sample barcodes. Equimolar amounts of DNA from each sample were pooled and submitted for Illumina sequencing with paired-end (325 bp forward; 275 bp reverse) sequencing on a MiSeq sequenator using the MiSeq Reagent Kit v3 chemistry (Illumina Inc.) at the next-generation sequencing facility at Lund University, Lund, Sweden.

2.7 | Bioinformatic processing of fungal community data

The mesh bag sequences obtained from Illumina sequencing were trimmed and filtered using Mothur v1.34 (Schloss et al., 2009), clustered using the Gaussian mixture model clustering algorithm CROP (Hao, Jiang, & Chen, 2011) at 97% sequence similarity, and a set of operational taxonomic units (OTUs) thus obtained. All OTUs representing less than 10 total reads or occurring in only one sample were excluded. All nonfungal and chimeric sequences were removed and sequences were trimmed to include only the ITS2 region using ITSx extractor v1.5.0 (Bengtsson-Palme et al., 2013). The taxonomic identity was then assigned to the set of clustered sequences by searching the Full 'UNITE + INSD' (Kõljalg et al., 2014) dataset (476,000 seqs, release date 1 August 2015) and using the Basic Local Alignment Tool (BLASTN program 2.2.25, blast.ncbi.nlm.nih.gov). Sequences that presented a 96% similarity between the query sequence and top hit, with at least 80% coverage of the query sequence length, were assigned to a taxonomic identity with genus and species. Sequences with values of 94%–95% similarity between the query sequence and top hit were assigned a taxonomic identity at the genus level only. Rarefaction was performed to 40,000 reads per sample with the *rrarefy* function in the Vegan package of R (Oksanen et al., 2013). Species were identified

as EM, ericoid/unknown endophyte, or saprotrophic based on the most current knowledge of the ecology of known close relatives (genera or species) according to Tedersoo, May, and Smith (2010). Read abundances for all OTUs for each sample were summed and the abundance of each OTU expressed as the relative abundance per sample. A matrix containing the rarefied relative abundance of each OTU per sample was then used to analyze differences in community composition.

2.8 | Statistical analyses

The change in top soil organic matter stocks and pH with N addition rate was analyzed using linear regression (Figures S1 and S2). The effects of N treatments (0, 3, 6, 12, 50 kg N ha^{-1} $year^{-1}$), trenching (trenched or untrenched), substrate type (litter or humus), and their interactions on organic matter mass loss were tested using mixed effect models. Nitrogen addition treatment, trenching, and substrate type served as fixed factors, with trenching nested within N addition treatment. Because this analysis showed some interactions between substrate type and the other main factors, we followed this analysis with investigating the effects of N treatments, trenching, and their interaction on each substrate separately. When a significant main N treatment effect was found, we performed *post hoc* regression analysis with N treatment serving as the independent variable, and substrate mass change serving as dependent variables. PLFA functional groups were compared using two-way mixed models with N addition treatment, and trenching serving as main factors, and with trenching nested within N addition treatment. *Post hoc* analyses comparing differences between N addition levels (0, 3, 6, 12, 50 kg N ha^{-1} $year^{-1}$) were tested using one-way ANOVA, or nonparametric Kruskal–Wallis test when necessary.

The PLFA marker and fungal sequence data were analyzed using multivariate approaches. For PLFA, a detrended correspondence analysis was first performed in order to indicate whether linear or unimodal methods should be utilized. The gradient length was 0.70 *SD* units, suggesting linear methods should be used. We then performed Monte Carlo permutation tests (n = 999, α = 0.05) to evaluate whether multivariate differences occurred in the overall PLFA signatures in response to the N addition treatments. Secondly, we described multivariate PLFA signatures using redundancy component analysis (RDA) to determine how N addition and trenching treatments affected microbial community variability. For fungal sequence data, OTUs were converted to relative abundances, and two-factor PERMANOVA comparisons were conducted using Bray–Curtis similarity matrices, with N addition rate and trenching serving as fixed factors. When these analyses revealed significant N treatment effects, we then performed one-way PERMANOVA pairwise tests to reveal treatment differences. Significant differences revealed by these analyses were graphically displayed using canonical analysis of principal coordinates (CAP), which is a constrained ordination approach used to graphically display multivariate comparisons with a priori group designations (i.e., N treatments).

We further performed correlation analyses between total humus, total litter, C, N, and P mass losses; and fungal PLFA biomasses where the replicates are the different N addition treatments (n = 30) using two-tailed Pearson's correlation tests. By examining the difference in mass loss (total, C, N, and P) between outside (SAP and EM fungi) and inside (SAP) the trenched plots we estimated the decomposition and nutrient removal by EM fungi.

All univariate analyses were performed using SPSS (v. 22.0; Chicago, USA), while all multivariate analyses were performed using CANOCO (v. 5.0; Biometris, Wageningen, the Netherlands) and PRIMER with PERMANOVA add on package (v. 7.0; Plymouth, UK).

3 | RESULTS

3.1 | Soil C accumulation and decomposition

Our estimates of organic horizon C stocks showed that C accumulated at a rate of 21.2 kg C per kg N addition (Figure S1). The pH of the organic horizon significantly declined by 0.22 pH units in response to the highest N addition rate (Figure S2). Our three-way mixed model showed a significant main effect of N addition treatments on total, C, and N mass changes, a main effect of trenching on N and P mass changes, and a main effect of substrate type on total, C, N, and P mass changes during the incubation period (Table 1). The main effect of N addition was a reduction in total and C mass losses (Figure 1a,b), and an increase in N mass gained as N addition rates increased (Figure 1c). Follow-up two-way mixed models showed that the main effect of N addition on total mass change occurred because litter was responsive to N addition treatments, whereas humus was not (Table 2; Figure 1e). Furthermore, the responsiveness of litter to N treatments was stronger inside trenches than outside trenches (Figure 1f). There was also a significant three-way interaction between N addition, trenching, and substrate on total and C mass changes (Table 1; Figure 2a,b). In addition, there was a substrate by trenching interactive effect on total and N mass changes (Figure 2a,c). The two-way mixed models further showed that trenching increased N and P (at p < 0.1) mass gain for litter, and decreased N and P mass losses for humus (Table 2; Figure 2c,d).

3.2 | Microbial community responses of soil phospholipid fatty acids

We found that N addition had a significant effect on most of the PLFA response variables except for the fungi: bacteria ratio (Figure 3; Table 3). Significant effects were due to lower concentrations in the high N plots (50 kg N ha^{-1} $year^{-1}$) than in the low N and control plots (Figure 3a–e) of total PLFAs, total bacteria, fungi, gram positive, and gram negative, while actinomycetes significantly increased in the high N treatment relative to the control and low N treatments (Figure 3f). All PLFA variables also responded significantly to trenching, except for gram negative PLFAs. The trenching treatment had

TABLE 1 Results from a three-way ANOVA evaluating the main and interactive effects of nitrogen (N) treatment (0, 3, 6, 12, and 50 kg N^{-1} ha $year^{-1}$), trenching (trenched or not trenched), and substrate type (litter or humus) on mass loss variables, including total mass loss, as well as loss of specific elements including carbon (C), N, and phosphorous (P)

	Total mass		C mass		N mass		P mass	
	F-value	*p*-value	*F*-value	*p*-value	*F*-value	*p*-value	*F*-value	*p*-value
Nitrogen treatment (N)	**3.970**	**0.005**	**2.585**	**0.041**	**3.439**	**0.011**	0.673	0.612
Trenching (T)	2.091	0.151	0.220	0.640	**27.734**	**<0.001**	**6.583**	**0.012**
Substrate type (S)	**1,253.777**	**<0.001**	**1,413.872**	**<0.001**	**219.374**	**<0.001**	**206.720**	**<0.001**
N × T	1.367	0.251	0.341	0.850	0.503	0.733	0.559	0.693
N × S	1.496	0.209	0.787	0.536	0.887	0.475	0.371	0.829
T × S	**9.184**	**0.005**	2.717	0.102	**15.784**	**<0.001**	0.471	0.494
N × T × S	**2.917**	**0.025**	**3.564**	**0.009**	0.570	0.685	0.511	0.728

Note: F- and *p*-values in bold indicate significant effects at $p < 0.05$.

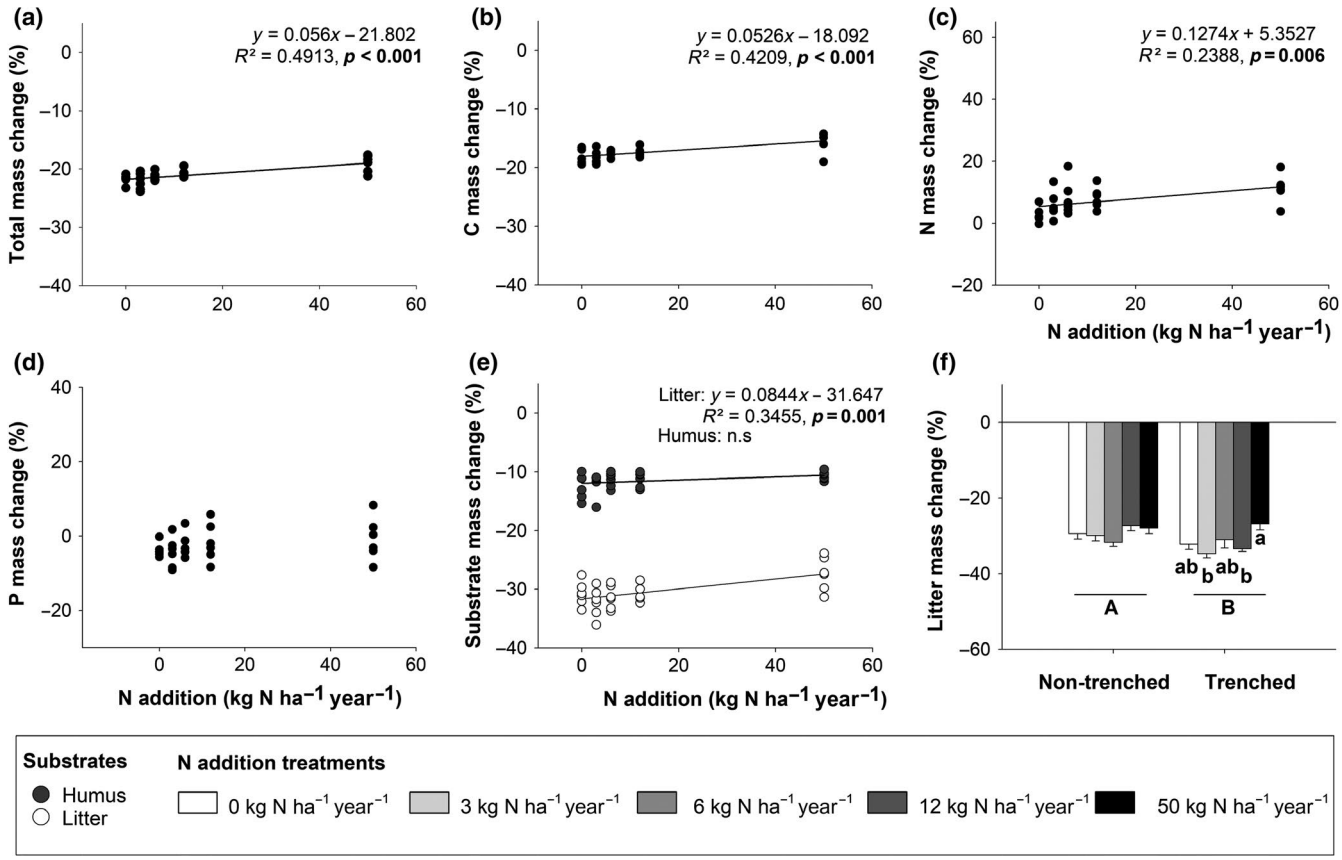

FIGURE 1 Change in total (a), carbon (b), nitrogen (c), and phosphorus (d) mass of decomposition bags in response to nitrogen addition treatments on average across two substrate types (humus and litter) and trenching treatments (trenched or not trenched); total mass for each substrate type separately averaged across trenching treatments in response to nitrogen addition treatment (e) and total mass change for litter in response to nitrogen treatment inside or outside trenches (f). Negative values indicate a loss in mass over the incubation period. The data points are the average of the other factors that are left out of that figure at the plot level. Each regression analysis has 30 data points in when other factors were ignored. For panels (a–e), a regression line and equation is plotted only for significant relationships. For panel (f), significant differences are indicated between trenching treatments with upper case letters (A or B) and within each trenched and not trenched treatments with lower case letters (a or b) below bars determined by *post hoc* analyses

contrasting effects on the different PLFA-response variables, with lower concentrations of total PLFAs, fungi, and the fungi: bacteria ratios in the trenched plots, and higher concentrations of gram positive, actinomycetes, and total bacteria PLFA in the trenched plots. There were no interactive effects of N addition and trenching on any PLFAs.

TABLE 2 The *F*-values and *p*-values from two-way mixed model ANOVAs evaluating the main and interactive effects of nitrogen treatments (N) and trenching treatment (T), and their interactive effects, on mass loss variables for two different substrates (litter and humus) the percent change in total mass, and mass of carbon (C), nitrogen (N), and phosphorous (P) from two substrates (litter or humus) that were decomposed for 1 year. Trenching consisted of permanent exclosures of roots and their mycorrhizae, and paired untrenched plots. The nitrogen treatment consisted of five addition rates (0, 3, 6, 12, and 50 kg N ha^{-1} $year^{-1}$)

	Nitrogen treatment (N)		Trenching (T)		N × T	
	F-value	*p*-value	*F*-value	*p*-value	*F*-value	*p*-value
Litter						
Total mass	**3.353**	**0.017**	**7.001**	**0.011**	**2.569**	**0.049**
C	2.345	0.067	1.836	0.182	2.065	0.099
N	2.138	0.090	**23.567**	**<0.001**	0.498	0.737
P	0.522	0.720	2.971	0.091	0.476	0.753
Humus						
Total mass	1.172	0.334	2.207	0.144	1.068	0.382
C	0.653	0.627	0.892	0.349	1.776	0.148
N	2.401	0.062	**4.428**	**0.040**	0.910	0.466
P	0.520	0.721	**8.023**	**0.007**	1.010	0.411

Note: Values in bold indicate statistical significance at $p < 0.05$.

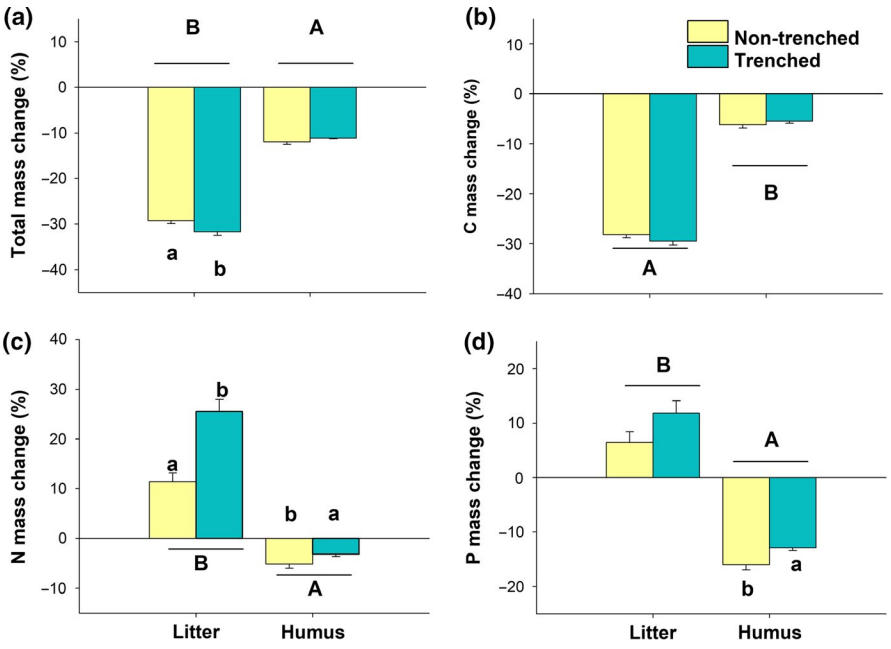

FIGURE 2 Change in total (a), carbon (b), nitrogen (c), and phosphorous (d) masses in response to trenching treatments for litter and humus substrates on average across nitrogen addition treatments. Negative values indicate a loss in mass over the incubation period. Significant differences are indicated between substrates with upper case letters (A or B) and within each trenched and nontrenched treatments with lower case letters (a or b) determined by *post hoc* analyses

The multivariate analysis using RDA and Monte Carlo permutation tests confirmed that significant differences in PLFA signatures were present in response to N addition and trenching treatments (P-1st axis = 0.002, P-2nd axis = 0.002; Figure S3). The first RDA axis explained 47.8% of the variation among PLFA markers, while the second RDA axis explained 7.0% of the variation. Furthermore, the first RDA axis was associated with trenching treatment, while axis 2 was associated with N addition treatments. Positive loading scores on the first axis were influenced by the 18:2ω6 fungal PLFA, which was relatively more abundant in the nontrenched control and low N treatments (3, 6, and 12 kg N ha^{-1} $year^{-1}$). All the remaining PLFA markers had negative loading scores on the first axis, with positive loading scores on the second axis mostly influenced by the 10me16:0, 10me17:0 actinomycete PLFAs, and i-16:0 gram-positive PLFA, which were the most abundant in the trenched high N treatment (50 kg N ha^{-1} $year^{-1}$). Negative loading scores on the second axis were mostly influenced by the cy-19:0, 16:1ω7, 16:1ω9 gram negative PLFAs, the a-15:0, i-17:0 gram-positive PLFAs, which were the most abundant in the trenched control and low N treatments (Figure S3).

Correlation analyses using all N addition treatments combined showed significant correlations between mass losses inside trenches, mass loss differences (i.e., the difference between outside and inside the trenched plots) and fungal PLFA marker biomasses, and no significant correlations outside trenches (Table 4). Total litter mass loss inside trenches was positively correlated with fungal PLFA values inside trenches (i.e., saprotrophic fungi). Total humus mass loss difference and P humus mass loss difference were both positively correlated with fungal PLFA marker difference (i.e., EM fungi).

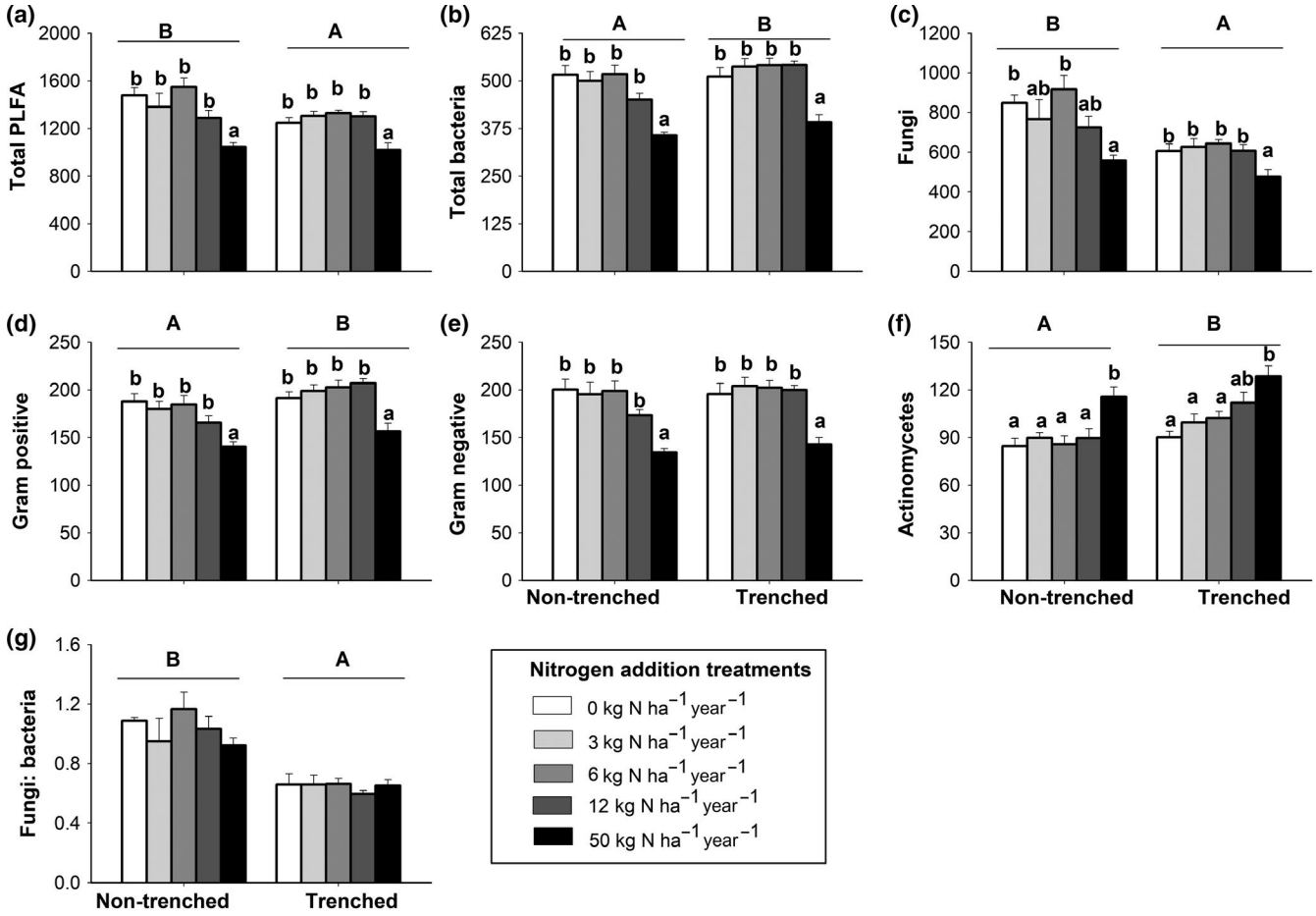

FIGURE 3 Mean (±*SE*) response of phospholipid fatty acid analysis (PLFA) expressed in nmol PLFA/g organic matter of the soil organic horizon outside and within the trenched plots for 0, 3, 6, 12, and 50 kg N ha^{-1} $year^{-1}$ for total PLFA (a), total bacteria (b), fungi (c), gram positive (d), gram negative (e), actinomycetes (f) and the fungi:bacteria ratio (g). Different capital letters (A or B) on top of each group of five bars indicate significant differences between trenching treatments regardless N addition treatments, while lower case letters (a or b) above each bar indicate significant differences between N addition treatments determined by *post hoc* analyses

3.3 | Fungal mycelial community from ingrowth mesh bags

Illumina sequencing of fungal DNA extracted from mesh bags produced 4,223,598 sequences. After quality filtering, clustering, and OTU identification, and rarefaction to 40,000 reads per sample, the molecular dataset consisted of 2,513,268 reads corresponding to 722 fungal OTUs. The proportion of sequence reads belonging to EM fungi was not significantly affected by N addition, but there was a significant interaction between trenching and N addition (Table 3). In general, trenching was associated with significantly lower EM fungal relative abundance (Table 3; Figure 4a). In the untrenched plots, N additions were associated with a significant increase in EM fungal reads (F = 4.83, p < 0.037), while in the trenched plots EM fungal reads decreased, although not significantly, with N additions (Figure 4a). The relative abundance of saprotrophic fungal reads did not respond significantly to N additions or trenching but was higher in the trenched plots and tended to increase with higher N in trenched and decrease with higher N in untrenched plots (Table 3; Figure 4a). Ericoid fungal relative abundance was unaffected by N addition, but was significantly greater in trenched plots (Table 3; Figure 4a). The proportional abundance of ascomycetes did not respond to N additions in the untrenched plots, but increased significantly (F = 4.96, p < 0.035) in response to N addition inside trenched plots, and in response to trenching (Figure 4b). Basidiomycete abundance was unaffected by N addition, but strongly decreased in response to trenching. Zygomycete abundance decreased in response to N additions and increased in response to trenching (Table 3; Figure 4b).

Both EM and saprotrophic fungal community compositions were responsive to N treatments and trenching (Table 3). For EM fungi, N treatment effects were due to community differences between the 50 kg N ha^{-1} $year^{-1}$ treatment with all other treatment levels (Figure 5a). For SAP, N treatment effects were due to the community in the 50 kg N ha^{-1} $year^{-1}$ treatment differing from that of the 0, 3, and 6 kg N ha^{-1} $year^{-1}$ treatment, while that of the 12 kg N ha^{-1} $year^{-1}$ treatment was intermediate (Figure 5b). Species contributing to community differences in EM and saprotrophic fungi among N treatments are reported in Tables S3 and S4, respectively. Among the OTUs that contributed most to the N response of the EM fungal community, *Xerocomus ferrugineus* and *Tylospora asterophora* were consistently

TABLE 3 The *F*-, pseudo-*F*, and *p*-values from two-way mixed model ANOVAs and PERMANOVAs evaluating the effect of nitrogen addition treatments (0, 3, 6, 12, and 50 kg N ha^{-1} $year^{-1}$), trenching treatment (trenched or not trenched), and their interactive effects, on phospholipid fatty acid (PLFA) data and fungal sequencing data. PLFA data were grouped into different microbial functional groups, each of which was analyzed using ANOVA. Fungal sequencing data were grouped into ectomycorrhizal (EM) or saprotrophs, and community composition differences were evaluated using PERMANOVA, whereas relative abundance (RA) of each group were compared using ANOVA

	Nitrogen addition treatment (N)		Trenching treatment (T)		N × T	
	F-value	*p*-value	*F*-value	*p*-value	*F*-value	*p*-value
PLFA data (nmol PLFA/g soil organic matter)						
Fungi	**8.62**	**<0.001**	**25.68**	**<0.001**	1.25	0.304
Gram positive	**15.01**	**<0.001**	**15.36**	**<0.001**	1.81	0.141
Gram negative	**18.74**	**<0.001**	1.86	0.179	0.80	0.532
Actinomycetes	**10.45**	**<0.001**	**12.04**	**0.001**	0.72	0.583
Total bacteria	**22.27**	**<0.001**	**6.73**	**0.012**	1.47	0.225
Fungi:bacteria	1.17	0.333	**47.26**	**<0.001**	0.84	0.510
Total PLFA	**14.08**	**<0.001**	**6.53**	**0.014**	1.43	0.238
Fungal sequencing data						
EM composition	**1.77**	**0.003**	**8.16**	**0.001**	0.85	0.809
EM RA	2.11	0.15	**23.3**	**<0.0001**	**6.56**	**0.013**
Saprotroph composition	**1.60**	**0.009**	**1.74**	**0.050**	0.77	0.908
Saprotroph RA	0.05	0.821	1.83	0.182	3.08	0.085
Ericoid RA	1.1	0.298	**8.00**	**0.0065**	0.12	0.729
Ascomycete RA	1.55	0.219	**12.59**	**<0.001**	2.72	0.105
Basidiomycete RA	0.08	0.780	**29.92**	**<0.0001**	1.62	0.208
Zygomycete RA	**9.00**	**0.004**	**4.012**	**0.05**	0.08	0.780

Note: There were 4,59 *df* for the nitrogen treatment and nitrogen treatment × trenching interaction, and 1,59 for the trenching treatment.
Values in bold indicate statistical significance at $p < 0.05$.
EM and Saprotroph composition data were analyzed using PERMANOVA, pseudo-*F*-values are reported instead of *F*-values.

more abundant in the highest N treatment, while *Tomentellopsis echinospora* and Sebacinales Group B SH197475.07FU were consistently less abundant in the highest N treatment. Furthermore, among the OTUs that contributed most to the N response of the saprotrophic fungal community, *Ascocoryne cylichnium* and *Trichoderma viride* were consistently more abundant in the highest N treatment, while *Stereopsis vitellina* was consistently less abundant in the highest N treatment compared to the control and the 6 kg N ha^{-1} $year^{-1}$ treatment.

4 | DISCUSSION

4.1 | Decomposition response

Our aim was to investigate how N enrichment impacts humus and litter decomposition, and furthermore, to evaluate whether the responses to N addition could be related to the abundance and composition of soil microbial communities.

In partial support for our first hypothesis, we found that on average across both substrates, trenching treatments and N addition caused a reduction in mass loss (Figure 1a). However, our data also showed that litter mass loss was more responsive than that of humus to N enrichment during our 1-year incubation (Figure 1e). Discrepancies between substrate mass loss may be due to differences in substrate stoichiometry. We found that humus and litter

TABLE 4 Correlation analyses between change in total, carbon, nitrogen, and phosphorus mass of decomposition bags for humus and litter; and fungal phospholipid fatty acid (PLFA) marker biomasses where the data points are replicates of the different N addition treatments ($n = 30$) using two-tailed Pearson's correlation tests

	18:2ω6 fungal PLFA marker biomass		
	Outside trenching (O)	Inside trenching (I)	Δ = O − I
Humus mass loss			
Total	0.35	0.16	**0.44**
Carbon	0.34	0.05	0.22
Nitrogen	0.32	0.31	0.23
Phosphorus	0.42	−0.21	**0.38**
Litter mass loss			
Total	0.14	**0.37**	0.04
Carbon	0.14	0.31	0.05
Nitrogen	0.16	−0.02	0.17
Phosphorus	0.09	−0.04	0.13

Note: The symbol delta in the third column corresponds to data calculated as the difference between the value outside and inside trenching treatments.
Values in the table are the *r* coefficients, with ones in bold indicating significant correlations ($p < 0.05$).

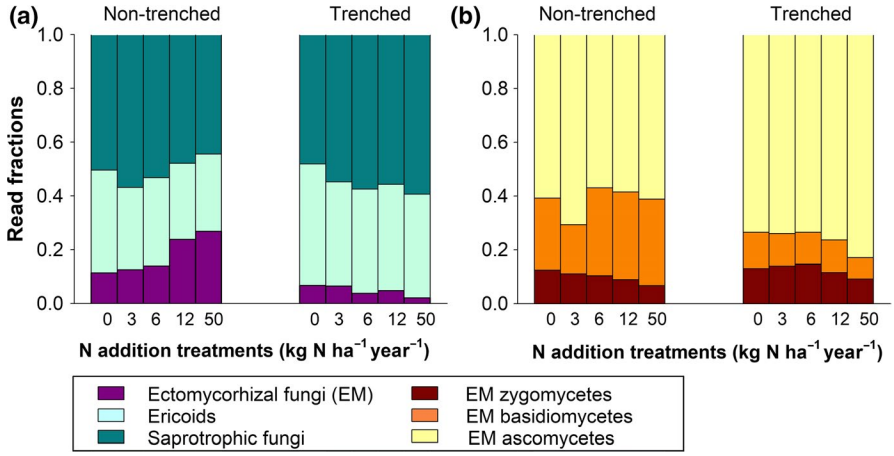

FIGURE 4 Proportion of total operational taxonomic unit reads belonging to ectomycorrhizal, ericoid and saprotrophic fungi (a), and the proportion of ectomycorrhizal zygomycete, basidiomycete, or ascomycete reads (b) in response to nitrogen and trenching treatments

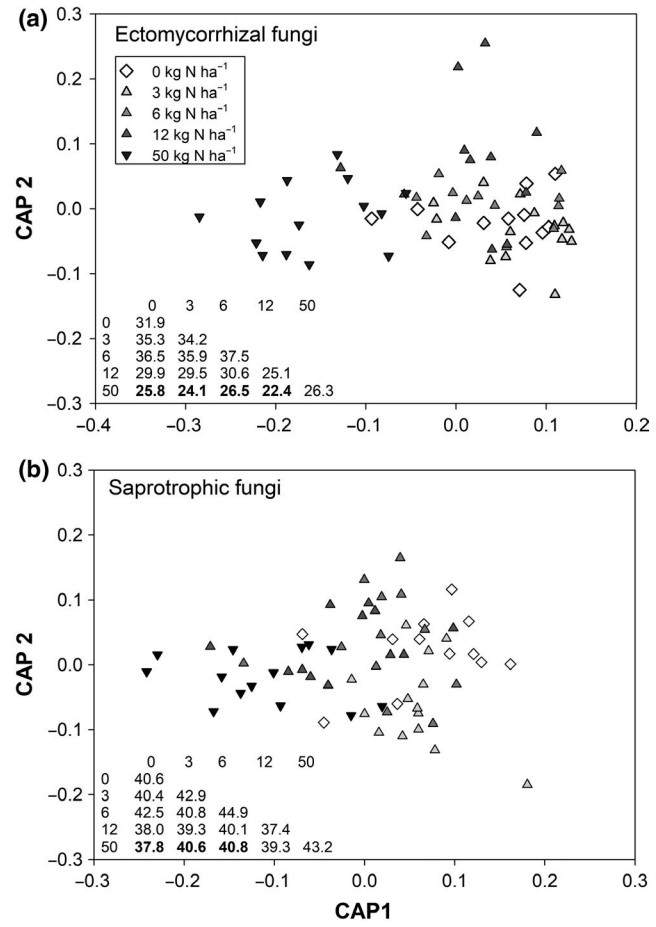

FIGURE 5 Canonical analysis of principal coordinates of ectomycorrhizal (a) and saprotrophic (b) fungal communities in response to long-term (11 year) nitrogen treatments (0, 3, 6, 12, and 50 kg N ha^{-1} year^{-1}). In the lower left of each panel are the within and between group similarities (%), with values in bold indicating a significant difference between groups. Data correspond to the results of PERMANOVA analyses reported in Table 3

had C:N:P ratios of 577:15:1 and 2694:20:1, respectively. Previous studies have shown higher decomposition rates of litter compared to humus, which is likely the result of litter having higher concentrations of labile C compounds (e.g., cellulose, hemicellulose) that are easier to decompose. In contrast, more recalcitrant C compounds remain in humus (e.g., lignin) with lower C:N ratios which may help explain lower decomposition rates of humus even with no N addition (Zhang, Hui, Luo, & Zhou, 2008). Furthermore, our data are in agreement with previous studies that have shown reduced litter decomposition rates in response to anthropogenic N enrichment (Hobbie et al., 2012; Knorr, Frey, & Curtis, 2005; Maaroufi, Nordin, Palmqvist, & Gundale, 2017). Moreover, another N addition study (10 years, 30 kg N ha^{-1} year^{-1}) showed that recalcitrant soil organic matter compounds, such as lignin- and other suberin-derived compounds, were selectively preserved in the soil in response to N relative to unfertilized deciduous forests (VandenEnden et al., 2018).

We also did not find support that decomposition would be enhanced instead of impaired at low levels of N addition (3, 6, or 12 kg N ha^{-1} year^{-1}), since decomposition rates in the low N treatments generally followed a linear relationship up to the high N treatment. This result contrasts some previous studies that have shown relatively low levels of N addition (20 kg N ha^{-1} year^{-1}) over short durations (5 years) can stimulate fungal activity (Hasselquist & Högberg, 2014; Hasselquist et al., 2012). It could be that these stimulatory effects that have been reported are short-lived, and no longer present after 11 years of N addition when our sampling was conducted. Furthermore, our study is unique in that we included much lower N addition treatments than any other experiment of this type. Previous research from our study system has shown that nonvascular understory plants sequestered a significant amount of these low N inputs in their tissues, which suggests little N is initially available to actually impact soil processes at low addition rates (Gundale et al., 2011), which may explain why low N addition rates did not have any impact on decomposition.

Trenching significantly decreased the concentration of fungal PLFA markers (Figure 3) as well as the proportion of fungal sequence reads that were EM fungi (Figure 4), suggesting our trenching treatments were successful in achieving their objective of excluding most of EM fungi. This allowed us to isolate the role of EM fungi and SAP in driving responses to N, as well as understand how EM fungi and SAP interact with each other. In support of our second hypothesis, we found that the responsiveness of litter decomposition to N additions differed depending on whether root-associated microbes were included or excluded. Specifically, decomposition of litter declined in

response to high N enrichment more strongly inside trenches compared to outside (Figure 1f). Given that trenches excluded most EM fungi, this finding suggests that saprotrophic decomposition activity is more sensitive to N additions, that SAP may be relatively more important than EM fungi for driving decomposition of fresh litter, and that changes in either saprotrophic microbial abundance, composition, or activity are likely key for explaining reductions in litter decomposition in response to N (which we discuss further in the section below). Our trenching treatment showed that competitive interactions between EM fungi and SAP occurred, regardless of N addition rate. The data showed that litter, but not humus, decomposed faster on average inside trenches compared to outside. This response is consistent with the so-called 'Gadgil effect', whereby EM fungi are proposed to reduce decomposition rates through competition with SAP, whereby EM fungal exclusion releases SAP from this competition which subsequently enhances saprotrophic activity and litter decomposition rates (Averill & Hawkes, 2016; Gadgil & Gadgil, 1975).

This competition between EM and SAP may be for a variety of resources (Almeida, Rosenstock, Forsmark, Bergh, & Wallander, 2019; Averill & Hawkes, 2016; Fernandez & Kennedy, 2015; Koide & Wu, 2003; Kyaschenko, Clemmensen, Karltun, & Lindahl, 2017), and our data suggest that N or P acquisition may be key for this interaction. Specifically, we found that both litter and humus had more total N and P mass at the end of the incubation inside compared to outside trenches, which suggests that EM fungi were either effective at exporting N and P from substrates during the incubation period relative to when these substrates were primarily colonized only by SAP (Figure 2), or for litter that EM fungi were effective at excluding SAP from colonizing. This reduction in N and P from substrates in the presence of EM fungi (i.e., outside of trenches) compared to when they are excluded (i.e., inside trenches) is in line with the Gadgil hypothesis, and may thus explain the enhanced decomposition of litter we observed inside trenches compared to outside. This interpretation is further consistent with several other studies showing that EM fungi export N and P from decomposing substrates. For example, Bending and Read (1995) reported a decline of N and P concentrations in humus colonized by the EM fungi *Suillus bovinus*, and Nicolás et al. (2017) reported a decline of N compounds in maize litter bags colonized by EM fungi in a Northern coniferous forest.

4.2 | Microbial community response

In support of our third hypothesis, we found that N treatments also caused substantial changes in microbial community abundance and composition, and many of these effects occurred independently of trenching, and despite a minor decline in soil pH (0.22 pH units). Nitrogen enrichment caused a reduction in total PLFA concentrations, including fungi and several bacterial PLFA groups, except for the actinomycetes which increased in response to N. Nitrogen enrichment further caused significant changes in both the EM and saprotrophic fungal community composition (Figure 5), indicating that different species that are likely more N demanding became dominant as plots became enriched with N. While many species contributed to the compositional differences that emerged in response to N, the EM fungi *X. ferrugineus* and *T. asterophora* increased the most in response to N, whereas a variety of other OTUs decreased (e.g., *T. echinospora* and Sebacinales_Group_B_sp_SH197475.07FU; Table S1). Other studies also reported an increase in *Xerocomus sp.* in response to N addition (Almeida et al., 2019; Hasselquist & Högberg, 2014). The genus *Xerocomus* belongs to the long distance exploration type (i.e., EM fungi foraging strategy) that produces larger hyphal biomass contributing to fungal necromass with slow turnover compared to short-distance exploration type (Agerer, 2001; Koide, Fernandez, & Malcolm, 2014) suggesting it may be more efficient at foraging for P, when N is not the main limiting nutrient (Almeida et al., 2019). Our results are also in agreement with Toljander, Eberhardt, Toljander, Paul, and Taylor (2006) and Kjøller et al. (2012) who reported an increase in *T. asterophora*, a short-distance exploration type (Agerer, 2001), along natural N gradients. Short-distance exploration types have been suggested to increase when tree C allocation to EM fungi declines, which is proposed to occur in response to N enrichment (Hasselquist & Högberg, 2014; Lilleskov, Hobbie, & Horton, 2011). Likewise, many OTUs contributed to the changes in saprotrophic community composition that emerged in response to N, with some OTUs increasing (e.g., *A. cylichnium* and *T. viride*), and others decreasing (*S. vitellina* and *Mycosymbioces mycenaphila*).

We also found that bacterial abundances declined, similarly to fungi, in response to N additions, including gram positive and gram negative bacteria (Figure 3). In contrast, one particular bacterial group, the actinomycetes, consistently increased in response to the high N treatment. One mechanism that may explain the increase in actinomycetes is that they are faster growing and more N demanding (i.e., referred to as copiotrophs or r-strategists) and are able to outcompete microbes with more conservative growth strategies (i.e., referred to as oligotrophs or K-strategists) such as fungi (Andrews & Harris, 1986; Koch, 2001) when N is highly available. This suggestion is consistent with Fierer et al. (2012) who found an increase in actinomycetes relative to oligotrophic microbial groups in response to a gradient of N addition (0–291 kg N ha^{-1} $year^{-1}$). Actinomycetes are also known to be antagonists of soil fungi through the secretion of antibiotic compounds (Jayasinghe & Parkinson, 2008), and are unable to completely degrade lignin as is done by some soil fungi (Berrocal, Rodríguez, Ball, Pérez-Leblic, & Arias, 1997; Godden, Ball, Helvenstein, Mccarthy, & Penninckx, 1992; Zak, Pregitzer, Burton, Edwards, & Kellner, 2011), which may help explain the observed overall reduction in litter decomposition in response to N additions. This is also in line with Zak et al. (2011) who suggested that an increased role of actinomycetes in litter decay in response to N enrichment may cause a reduction in litter decay rates.

Our data also showed that trenching caused numerous shifts in microbial community abundance and composition, some of which could help explain the decomposition response to trenching that we observed. The PLFA data showed that trenching greatly reduced fungal biomass (Figure 3); whereas, trenching increased the abundance of PLFA markers for gram-positive bacteria, and actinomycetes, suggesting a negative interaction between EM fungi and these bacterial groups. The sequencing data indicated that trenching was associated with decreased relative abundance of EM fungi and increased relative

abundance of ericoid fungi; saprotrophic fungal relative abundance also increased (~5%), but not significantly so. Additionally, trenching was associated with increased zygomycetes and ascomycetes and decreased relative abundance of basidiomycetes (Figure 5). Thus, in addition to reduced abundances of EM fungi, the enhanced litter decomposition we observed inside trenches (i.e., the Gadgil effect) may have been due to changes in saprotrophic fungal composition, as well as an increase in other free-living SAP (i.e., bacteria).

Also consistent with our third hypothesis, these shifts in microbial community abundances and composition in response to trenching were significantly correlated with substrate mass loss variables in several cases. For litter, we found that decomposition was positively correlated with the fungal PLFA marker inside trenched plots. This further highlights the important role of saprotrophic fungi in litter decomposition. It is plausible this relationship occurs because N addition directly impairs enzymatic activities of SAP (Deforest, Zak, Pregitzer, & Burton, 2004; Freedman, Romanowicz, Upchurch, & Zak, 2015; Freedman, Upchurch, Zak, & Cline, 2016) which may reduce their ability to both decompose litter and accrue biomass (Waldrop, Zak, Sinsabaugh, Gallo, & Lauber, 2004; Zak, Holmes, Burton, Pregitzer, & Talhelm, 2008). Despite the lack of significant change in total humus mass loss in response to N addition, we did find that the differences between trenched and untrenched plots in total and P mass loss from humus (i.e., estimation of EM fungal contribution to mass loss) were positively correlated with the difference in fungal PLFA concentrations between trenched and untrenched plots (i.e., EM fungal PLFA), which suggests that EM fungi are important for P acquisition from humus. Our results are consistent with Bending and Read (1995) who reported a 22% increase in humus P loss in response to the colonization of EM *S. bovinus* in a mesocosm experiment. However, no P was removed from humus by EM *Thelephora terrestris* in the same study demonstrating that this capacity is not general within the EM community. We further note that humus N and P mass loss was greater outside compared to inside trenches during the incubation; and furthermore, that humus N mass change was nearly significantly reduced ($p = 0.062$) in response to N enrichment. These relationships taken together suggest that EM fungi primarily exploit humus as an N and P rather than a C source, which may explain why total humus mass loss (which disproportionately consists of C mass loss) was unresponsive to our N treatments. If humus is primarily colonized by EM fungi as an N and P source, associated C losses may be secondary and more subtle to detect, and may emerge over longer time scales than our incubation time (1 year) as EM fungi shift toward extracting increasingly recalcitrant N that is more tightly bound in complex C skeletons (Berg & McClaugherty, 2008; Martínez et al., 2005).

4.3 | Implications

Our results have substantial implications for better understanding the mechanisms through which anthropogenic N addition can influence soil C sequestration in boreal regions. First, we show that low N addition treatments simulating current atmospheric N deposition rates do not appear to have positive effects on decomposition as we hypothesized, but instead appear to follow a negative linear response that extends to our highest N addition rate (50 kg N ha^{-1} $year^{-1}$), where we also observed reduced abundances of bacteria and fungi, and altered composition of saprotrophic and EM fungal communities. Furthermore, actinomycetes were the only microbial PLFA markers that increased in response to N additions, but only significantly so in the highest N treatment, suggesting their relative importance as decomposers increases in response to high levels of anthropogenic N enrichment. Secondly, the trenching treatment in our study also revealed a different role of saprotrophic and EM fungi in driving decomposition, that is, SAP were more important for driving mass loss of litter (decomposition); whereas, EM fungi appeared more important for reducing N and P concentrations in humus and litter (Lindahl et al., 2007) rather than respiring organic matter, as there was always less N and P in humus and litter at the end of the incubation when EM fungi were included than when they were excluded. Our data further indicate that the responses of saprotrophic fungal activity may be relatively more important in response to N addition, and more direct than EM fungal activity. Our study does not exclude the possibility that additional mechanisms may contribute to the buildup of soil C that is frequently reported in response to N enrichment; however, our study does clearly show that reduced saprotrophic activity is a key mechanism driving this response.

Our findings linking litter and humus decomposition rates to soil microbial community structure and abundance provides important mechanistic insights into how anthropogenic N enrichment can influence ecosystem C balances, which may help improve efforts to model the impact of anthropogenic N deposition on the global C cycle (Averill & Waring, 2018). For example, a rapidly expanding research area in the field of global C modeling (e.g., Dynamic Global Vegetation Models) is to couple the C cycle with the N cycle in order to represent the impacts of N inputs and internal cycling on net ecosystem C exchange. Many of these models emphasize that anthropogenic N deposition will lead to enhanced soil C stocks due to increased organic matter input; however, our data suggests these models should also emphasize that anthropogenic N leads to functional changes in the microbial community that drive organic matter decomposition, and that these responses are highly dependent on N input rates.

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