Title:

Do local-scale context dependencies shape how ectomycorrhizal fungal diversity structures with reduced or sustained experimental N addition?

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Abstract:

Atmospheric nitrogen (N) pollution visibly and rapidly changes forest systems, for example impacting the diversity of forest ectomycorrhizal (EcM) fungi. EcM fungi are measurable by components that vary in longevity, hence, reflecting different temporal scales of presence: EcM root-tips are viable for, at most, a couple growing seasons, compared to fungal structures that may persist in soil as DNA for decades or longer. In-growth mesh bags, in contrast, typically capture a single growing season of hyphal growth. The different components might portray different diversity responses by EcM fungi to large-scale N pollution, as well as any subsequent reductions from improved environmental standards. Within an established oak forest study system, we examined the impact of sustained and recently reduced experimental N addition on EcM fungal diversity and composition, measured in three main fungal components (root-free soil, root-tips, and mesh bag hyphae). We hypothesized that elevated soil N would reduce EcM fungal diversity, and that composition would change, but with differences among fungal components related to the temporal longevity of the components. Our expectations were largely met, in that richness primarily declined with increased soil N, and all trends were most pronounced with the soil EcM fungi (the only component potentially reflective of long-term fungal presence). We discovered that abatement of the experimental N treatment did not revert fungal trends to those of the same-site plots with ambient N treatment. Instead, the stochastic nature of local-scale disturbances, related to invasive earthworms and forest stand dynamics, likely impacted N levels and, thus, EcM fungal trends. Due to the context-dependency of localized disturbance(s), assessing the effects of reduced large-scale N deposition on EcM fungi can prove to be challenging.

Keywords: Nitrogen deposition, Nitrogen abatement, Ectomycorrhizal fungi, soil DNA, temporal longevity, invasive species

Introduction:

Ectomycorrhizal (EcM) fungi are essential contributors to the soil biodiversity of many terrestrial ecosystems, especially in temperate and boreal regions, due to the extent of EcM-associated tree species that dominate at these latitudes. The roles of EcM fungi in nutrient cycling are, likewise, functionally diverse. For example, within boreal forests with little atmospheric pollution, EcM fungi have been shown to contribute to all aspects of the nitrogen (N) cycle, from soil translocation and N uptake by tree symbionts to the enzymatics of decomposition (e.g., Sterkenburg et al. 2015, Högberg et al. 2017, Clemmensen et al. 2021). Natural variations in soil fertility gradients within these systems demonstrate how the diversity of EcM fungi changes, in association with vegetation composition, and that these variations impact the functionality of EcM fungi (e.g., Sterkenburg et al. 2015).

Increasing N pollution manifests in visible and rapid changes to the natural composition, growth and regeneration of forests (BassiriRad et al. 2015) along with their associated fungi (Arnolds 1991, Lilleskov et al. 2001, Peter et al. 2001, Avis et al. 2003). Nitrogen deposition disrupts the functioning of the EcM symbiosis, especially when forests are surrounded by urban, industrial, and/or agricultural sources of pollution (related to reactive N species and fertilization), and generally reduces EcM fungal diversity. The fewer species in higher N environments can also be different from those in lower N environments (as reviewed by Lilleskov et al. 2019). These responses are not, however, uniform. Some EcM taxa respond positively to increased N, termed nitrophilic (Avis et al. 2003, Avis 2012, Morrison et al. 2016), and EcM fungal responses may depend upon the background levels of N deposition (Moore et al. 2021). Recent research

suggests that alleviation of N addition might revert aspects of prior pollution on fungi, for example in terms of fruit body production (van Strien et al. 2017).

Most studies of N effects on EcM fungi have focused on one to two fungal components. For example, initial impacts of N deposition on EcM fungi were demonstrated by sporocarp fruiting patterns (Arnolds 1991, Lilleskov et al. 2001); this was supported by subsequent studies of EcM on root-tips (Peter et al. 2001, Lilleskov et al. 2002, Avis et al. 2003) and EcM mycelia from in-growth mesh bags (Nilsson et al. 2007, Wallander et al. 2013). Most recently, EcM fungi detectable in root-free soils have been investigated (Moore et al. 2021, Carrara et al. 2021). However, to our knowledge at this point, no study has simultaneously examined the responses of EcM as root-tips, as newly produced extramatrical hyphae (from within in-growth mesh bags), and as fungal structures within root-free bulk soils when exposed to sustained (i.e., 10-year) elevated N, nor abatement of the experimental treatment, a primary objective of ours.

In contrast to the regional- to continental-scale studies on N throughfall deposition and EcM fungi (Cox et al. 2010, Suz et al. 2014, van Strien et al. 2017, van der Linde et al. 2018), finer scale heterogeneity of soil properties can demonstrate more variability (Avis et al. 2008). The broader scale gradients in soil nutrients (e.g., N) match well to vegetation types, and each help to capture EcM fungal diversity and composition (Bahram et al. 2014, Tedersoo et al. 2014, Andrew et al. 2019). When research on EcM fungi and N amendment is conducted at finer scales, such as at the tree root level, the patchiness is considerably higher. Hence, the probability of identifying a specific EcM fungal taxon, no matter which component is investigated, is likewise variable – and different between the fungal components.

The probable influence of the fungal component to diversity studies initiates with the root-rhizosphere, a dynamic realm where EcM fungi must continuously contend with seasonal phases of root-tip growth, production and turn-over (McCormack et al. 2017) in order to maintain their symbioses. The distances that hyphae grow (i.e., exploration types) from the roots help explain the variability in the likelihood of quantifying species as hyphae in the soil, i.e., ranging from short distance (near the root) to longest distances as rhizomorphs extending through the soil (Agerer 2001). As the bulk soil is the primary substrate matrix of EcM fungi, this likewise contains a different representation of taxa than those as found on root-tips or actively growing hyphae (measured with in-growth mesh bags). In bulk soil beyond the root, taxa can be present due to a varied admixture of propagules (hyphae, dormant spores and sclerotia, even hypogeous fruit bodies) that are patchily distributed with accord to the soil environment and the existing root systems. Non-active remnants of prior fungal taxa in small pieces of environmental DNA (eDNA), for example bound to organic matter and other soil components, may demonstrate the earlier presence of a species in the bulk soil, but not necessarily its continued presence (Foucher et al. 2020). Thus, these three below-ground components (EcM root-tips, current-year hyphae and bulk soil beyond the root) can capture different taxa, and this is partly related to spatial patchiness as well as the temporal longevity of the components.

Our primary objective was to examine the impact of N on EcM fungi within an established study system that, unique to prior research (Avis et al. 2008, BassiriRad et al. 2015), contained plots with experimentally sustained as well as abated N addition. We simultaneously quantified taxonomic diversity and composition for three below-ground fungal components that represented

varied temporal scales: root-tips (current to prior growing season), hyphae that grew into mesh bags (current season), and all the fungal structures, active or not, found within bulk soil (current to decades past growing seasons). We hypothesized that by elevating soil N, richness of EcM fungi would decline overall and composition would change, but with differences among fungal components for the following two primary reasons:

1. EcM fungal richness, across soil N levels, would be greatest for fungal components that are representative of multiple seasons of activity (i.e., the bulk root-free soil). In contrast, lower EcM fungal richness would be measured in components reflecting current to prior (root-tips) or only the current (hyphae within in-growth mesh bags) growing season(s). This differentiation between fungal components is related to temporal aspects of the samples.

2. EcM fungal taxa with more explorative hyphal growth (i.e. long- and medium-distance exploration types, as categorized by Agerer 2001) will be relatively more frequent in hyphal in-growth bags than in the soil alone. They are most likely to grow into the mesh bags within the single growing season. In the bulk root-free soil, taxa can be identified that are not present in the in-growth mesh bags. In contrast, we expect lower-distance exploration types to be better represented on root-tips. This differentiation between fungal components is related to spatial aspects of the samples.

Regarding soil N levels, given that Avis et al. (2008) more frequently encountered on root-tips the long-distance *Boletus* spp. in ambient N treatments, and the shorter-distance fetid *Russula*

spp. more often in N fertilized conditions, now six years after this initial experiment, we expected that the response to soil N levels may be most clearly demonstrated by a shift in taxa between exploration types, and were curious to compare these trends between the fungal components.

Methods:

Site and Experimental Design

The current study was a modified long-term continuation of an original experiment (2003-2007) investigating the impact of N addition to remnant oak (*Quercus alba*, *Q. rubra*) forests. It took place at two natural areas within the greater Chicago metropolitan area that contained a high degree of similarity in tree species composition, size, age and density as well as fungal diversity, as described in Avis et al. (2008) and BassiriRad et al. (2015): Swallow Cliffs (SC) Forest Preserve in Cook County, Illinois, USA (41°40'35.8"N, 87°51'55.7"W; initial average annual NO_{3-} and NH_{4+} deposition of 19.27 kg ha^{-1} yr^{-1}), and Indiana Dunes (IND) National Lakeshore, Porter County, Indiana, USA (41°37'51.4"N, 87°05'22.0"W; initial average annual NO_{3-} and NH_{4+} deposition of 20.87 kg ha^{-1} yr^{-1}). The two sites are located approximately 60 km from one another. Background (non-treatment) atmospheric N deposition was fairly constant across the 20 initial years, as well at the time of this study in 2013, with levels of each corresponding to about 7 kg N ha^{-1} yr^{-1} in total. In each site (SC and IND), two of four 0.1 ha (40 m x 25 m each, containing 40 5 m x 5 m quadrats) plots received monthly (12 applications per year) experimental N fertilization by spray fertilizing three times the 5-yr average of nitrate and ammonium deposition as measured at a National Atmospheric Deposition Program (NADP; https://nadp.slh.wisc.edu/) site located close to each forest. The spray fertilizer solutions contained the site specific and monthly determined concentrations of potassium nitrate and ammonium sulfate (BFG Supply, Burton, OH, USA) dissolved in 6 liters of deionized water for each 0.1 ha treatment. This was considered a small to moderate increase in N, a specific goal of

the research, since others (e.g., Lilleskov et al. 2001, Avis et al. 2003) had up to that point conducted EcM-based research with much higher levels of N addition, despite that not all natural areas experienced such extremes to N pollution. Further, for the purposes of this study, using a moderate level of N increase rather than a large increase was advantageous as it allowed for us to see which measurable fungal component was more sensitive to increased N. If we had used massive increases in N levels such as in Avis et al. (2003), it is likely that all components would have responded substantially. In our study, the more moderate level provided us a window into the different levels of sensitivity of the different fungal components. The other two plots at each site were the ambient N treatments, with no N added, but simulating the treatment with an equivalent amount of water to that of the N additions.

The experimental design was modified following Avis et al. (2008) and BassiriRad et al. (2015), in an attempt to contrast how continued N addition at one site (IND) differed to abating (i.e., stopping) the experimental addition at the other site (SC). At the IND site, experimental fertilization occurred from 2003 until the time of our study in 2013, i.e., the treatment plots were N fertilized for 10 years. These were still paired with the ambient plots, which continued to receive no additional N above the background amounts. In contrast, at the SC site, treatment stopped in 2008, and all plots were only exposed to the background N amounts. Given the established similarity of the two sites in terms of proximity, vegetation, fungal diversity and soil type, that the two sites could not be modified while retaining the original balanced experimental design was a reduced issue. We nonetheless always present the results for treatment by each site, (i.e., Ambient IND, Always N IND, Ambient SC, Abated N SC). Especially the highly similar levels of fungal diversity previously measured at these sites (Avis et al. 2008) gave us confidence

that such a cross-site comparison would be timely to study, given that in some regions of the world, atmospheric pollution has decreased (e.g., van Strien et al. 2017).

Overview of 2013 Analyses

During the growing season of 2013, we collected soil cores and deployed hyphal in-growth mesh bags to determine the composition and diversity of multiple EcM fungal components to soil nutrients and N experimentation (sustained amendment or abatement). From the soil cores, we (i) harvested root-tips, the only fungal component measured in prior research at the site (Avis et al. 2008). Also, we (ii) extracted DNA from bulk soil from which we had removed the roots for the root tip analysis (termed "bulk root-free soil" in this paper) for molecular analyses to identify all fungal taxa within the soil, whether they were present from actively growing hyphae, as resistant dispersal propagules (spores and sclerotia), or remnant eDNA chemically bound to soil particles. Finally, we (iii) deployed in-growth hyphal mesh bags in the spring for the fungal hyphae to grow within until harvest at the end of the growing season.

Molecular identification techniques were utilized for all three fungal components. The root-tips were morphologically sorted within each sample to morphotype, and a representative root-tip for each single species was Sanger sequenced. To describe the EcM fungi in the root-free soil and in the hyphal in-growth bags, we used High Throughput Sequencing (HTS) in order to sequence multiple species simultaneously. The DNA sequence data from all three fungal components were processed to community and richness values for each core, scaling to the 0.1 ha plot-level for

213 statistical analyses as appropriate. We thereby identified the EcM taxa on (i) root-tips, the (ii)
214 bulk root-free soil, and those actively growing as (iii) hyphae within in-growth mesh bags.
215
216 *EcM fungi on root-tips and in soil: Soil cores*
217
218 In mid-July 2013, soil cores were taken (10 cm diameter by 14.5 cm depth) at ten sample points
219 within each plot (10 cores x 4 plots x 2 sites = 80 cores). We used the same soil sampling
220 strategy as in Avis et al. (2008): Cores were taken within a one-meter radius of an original target
221 oak tree while remaining in the same quadrat. Given that 6 years had passed since the original
222 study, in the very few cases that the original tree could not be located (e.g., dead), a substitute
223 tree in the plot was selected. All samples were up to 3 m (but as low as 1 m) from the locations
224 of the in-growth mesh bags (next section) in the soil, ensuring a high degree of spatial
225 autocorrelation (Lilleskov et al. 2004) while also minimizing any damage from taking a soil core
226 neighboring to the hyphae growing into the mesh bags. The soil cores were stored at 4 degrees C
227 and processed within two weeks of sampling for root-tips and soil.
228
229 The roots from each core were removed, floated in water, homogenized, and cut into ca. 2 cm
230 sections. Forty-eight root-tips were randomly selected per core, adding further root sections if the
231 first six did not contain enough root-tips (the maximum was nine). The root-tips were classified
232 into putative morphotype groups (Agerer 2001), as dead, and as non-ectomycorrhizal. A
233 representative of each morphotype was molecularly analysed (Meier et al. 2012, Andrew and
234 Lilleskov 2014). For the root-free soil samples, 250 mg of the homogenized soil was used for
235 DNA extraction (see below).

EcM fungi as hyphae: In-growth mesh bags

Hyphal in-growth nylon mesh bags were deployed early in June 2013 into soil at two depths (2-3 and 8-10 cm from the soil surface) for each of the soil 80 cores (above section). They were allowed five months for active hyphae to grow into them prior to harvest in mid- October of 2013. The mesh bags (6 x 6 cm) were filled with 30-35 g of washed, neutral sand lacking any fungi that, by limiting the carbon sources which saprotrophic fungi utilize for growth, selects primarily for EcM fungal growth (Wallander et al. 2004, Andrew et al. 2014, Phillips et al. 2013, Wallander et al. 2013). Once harvested, the bags were gently removed from the soil, adhering debris brushed off, and stored at 4 degrees C before, within days, extracting the hyphae.

For processing, hyphal bags were poured into 400 mL of water, making sure to rinse the hyphae from the mesh fabric into the solution. The mixture was swirled vigorously and decanted over mesh fabric, repeating four to five times. Hyphae, roots and sand debris on the mesh were rinsed into a petri dish. The hyphae balled together into a mass that was measured to a relative size class estimate; later, remaining sand particles were removed (through use of forceps) and the dry mass measured more specifically. The two quantifications of hyphal biomass correlated well, suggesting future studies could save effort with mass estimates from fresh hyphae; but for the purposes of this study, we only use the latter measurement for statistical analyses. The hyphae, being active when harvested, were kept frozen on ice while processing and transferred to -20 degrees C. Prior to DNA extraction, the hyphal samples were dried at 60 degrees C for 1-2 hours (Labconco), taking an average of 6 mg (range 0.1 to 125 mg).

Soil Nutrient Analysis

The remainder of each soil core was homogenized. The soils were air-dried and approximately 250 g per core were sent to A&L Great Lakes Laboratory (Fort Wayne, Indiana, USA) for the analysis of soil N (Dumas method; e.g., Bremner 1996), carbon (C), C:N, and mineral nutrients. There was one unexpected result in the SC soil N levels: they were overall greater at SC than at IND (Supplemental Fig. A.1). To reconstruct how this compared to prior N levels at SC, archived soils (dry and/or cold stored at Indiana University Northwest and The Field Museum of Natural History; from 2004, 2005, 2010) were similarly analyzed as possible. While those sample results were patchier, all available samples matched the 2013 soil levels for site and plot (see results). The soil variables N, C and C:N were included in model selection analyses as a standard check for basic soil nutrients' potential influences between the treatments, and those not collinear reported when statistically significant. As our focus was on N, this soil variable was favored over C in instances of collinearity.

DNA Analysis of Root-tips

Root-tip morphotypes were prioritized for Sanger sequencing, as they matched prior research (Avis et al. 2008), and this method was well established for single-species samples of EcM fungi (Meier et al. 2012, Andrew and Lilleskov 2014). Each morphotype root-tip was individually processed, extracting DNA (REDExtract-N-Amp Tissue PCR Kit; Sigma-Aldrich), and PCR amplifying the rDNA ITS region with the fungal-specific primers ITS1F (Gardes and Bruns

1993) and ITS4 (White et al. 1990), following standard cycling parameters (i.e., Andrew and Lilleskov 2014). Sequences were produced from an Applied Biosystems 3730 DNA Analyser at the Pritzker Laboratory and DNA Discovery Center, The Field Museum of Natural History. Sequence quality and identity was processed in Geneious version 7.1.2 at the University of Oslo, Norway.

Sequences were placed into one of five quality groups, based on the Highest Quality (HQ%) and length of the consensus sequence, which helped ascertain their taxonomic accuracies. Highest quality sequences had HQ ≥ 85% for sequences ≥ 250 bp, while lowest quality sequences had HQ < 25% and/or sequences < 250 bp. For each quality group, sequences were assigned to a taxonomic group based on percent matches to BLAST and UNITE vouchers (searches conducted in 2014). For the statistical analyses, we utilized sequences of medium quality (HQ ≥ 45% and ≥ 250 bp) and higher. BLAST matches were based on the percent of matching identical sites (Andrew and Lilleskov 2014). Given that shorter sequence reads were allowable (to 250 bp), the percent similarity of the top BLAST match to assign the taxonomic ranking was more stringent than when greater sequence lengths are used (e.g., Andrew and Lilleskov 2014). We applied rules similar to that as used by Nilsson et al. (2011): ≥ 97% similarity matched the OTU to the species level (i.e., likely conspecificity), 90% ≤ OTU < 97%, with a broader taxonomic ranking thereafter with each 10% increment decrease.

DNA Analysis of Hyphae and Bulk Soil Samples

The hyphae (from the in-growth mesh bags; the two depths combined) and the bulk root-free soil samples (matching the root-tip samples source) required HTS technology, as they contained multiple species. They were processed with the Illumina MiSeq, also at the Pritzker Laboratory and DNA Discovery Center. DNA from hyphal samples and bulk root-free soil were extracted using the MoBio soil DNA extraction kit (Qiagen) following manufacturer protocols.

The ITS1 rDNA subunit of each soil and hyphal sample, plus a mock community (Nguyen et al. 2015) and negative controls, were PCR amplified following Smith and Peay (2014). Specifically, KAPA HIFI Taq (Roche) with 10 uM (initial concentration) barcoded fungal-specific ITS1F-ITS2 primer set were combined with 12 ng (on average) of DNA per sample in a 25 ul total reaction volume. Reaction parameters were as recommended, except annealing temperature was 52 degrees C. DNA concentrations of amplified products were determined with a QuBit fluorometer (Life Technologies) and also with KAPA QPCR (Roche) following manufacturer recommendations. A library was constructed by combining 25 ng of each purified (AMPure XP Kit, Beckman Coulter) PCR reaction. The library was then sequenced using 250 bp paired end platform of the Illumina MiSeq.

Analysis of MiSeq data followed established protocols for EcM fungi (Nguyen et al 2016, http://cbs.umn.edu/kennedy-lab/protocols). Demultiplexed samples were quality filtered using cutadapt (Martin 2011), Trimmomatic (Bolger et al. 2014) and mothur (Schloss et al. 2009), including QIIME (Caporaso et al. 2010) processing of forward reads only. Using QIIME, we prefiltered for fungal sequences using UNITE as the reference. A two-step OTU selection occurred first with USEARCH and then UCLUST. A local BLAST search against the UNITE

fungal ITS database was used to identify OTUs. We conducted a stringency test to optimize the similarity of matches to the database, and found that removing sequences with less than a 95% match to the database resulted in comparable amounts of total OTU. We then removed any OTU that had less than 5 sequence reads, assuming that these were sequencing errors or minor contaminants.

Statistical analyses

Species matrices were created for the quality sequences of the EcM fungal OTUs (trophic guilds determined from FUNGuild; Nguyen et al. 2016), with results investigated both at the OTU and the genus level. Based on the separate sequence technologies between the root-tip sequences versus the hyphal and soil sequences, composition was considered separately for each fungal component. With the rarefied richness estimates, it was possible to compare directly between the three fungal components.

A linear mixed effects model with a random slope and random intercept were selected for regression analyses (Zuur et al. 2009, Crawley 2012). EcM fungal richness was predicted by the fixed effects of the soil parameters (N, C, C:N), hyphal biomass (combined 2-3 cm and 8-10 cm from the surface), and the treatment factors (Ambient IND, Always N IND, Ambient SC, Abated N SC), while accounting for the nested random effects of the spatial design (fungal component, quadrat, plot, site). The soil variables with the lowest pairwise Pearson correlation coefficients (below a threshold of 0.60; Dormann et al. 2013) as well as a variance inflation factor (VIF) value ≤ 5 (a conservative threshold; Zuur et al. 2009) were retained for model selection. Only C

was collinear (with N) and, hence, not included during selection procedures. Backward model selection of models that consecutively removed non-statistically significant variables was based on comparisons between the models with ANOVA and the marginal (fixed) and conditional (fixed and random) R^2 values (Nakagawa et al. 2013). The final selected model predicted richness by the interactions of soil N, the treatment and site, and the fungal component.

Compositional trends were investigated by the number of shared and unique OTUs, rather than a conventional ordination approach (i.e. gNMDS). These data were displayed as presences for each OTU aggregated by fungal component. The latter visualization could more explicitly allow the comparison of taxa by treatment and fungal component, and related to established knowledge on exploration types (Agerer 2001). Given the nature of the study design, i.e., site and treatment, alongside the three fungal components, to display community scores on a gNMDS provided no more information or insight than the results as presented.

Data processing and statistical analyses were conducted in R version 3.6.1 (2019-07-05) using the packages ggpubr (plotting), ggvenn (venn diagrams), ggVennDiagram (venn diagrams), MASS (regression analyses), mgcv (regression analyses), nlme (regression analyses), piecewiseSEM (R^2), reshape2 (formatting), stringr (formatting), vegan (rarefaction), wesanderson (plot colors).

Results:

Soil nutrient content

The sustained experimental N addition at the IND site paralleled a greater mean soil N content in 2013, compared to the ambient treatments (Supplemental Fig. A.1): Mean % N values (± standard deviation) were 0.16 ± 0.03 in ambient conditions, compared to 0.19 ± 0.05 with continued experimental N addition. Soil N levels at the SC site were, overall, higher: The ambient treatment soils contained 0.29 ± 0.07 %N, compared to the 0.22 ± 0.05 %N values in the abatement treatments. As we were curious to whether the difference in soil N between the two sites was a newer trend or from, at least, the time of original treatments, we contrasted the 2013 soil percent N results to those available from measurements in 2005. The change in values at IND were reasonably consistent between the plots by each treatment, increasing from 0.006 to 0.033 %N at the ambient locations and 0.038 to 0.064 %N at the elevated N locations. Fewer data were available for the SC site from earlier years. There was the suggestion of a greater soil %N increase at the SC site, given that the ambient conditions increased by a range of 0.051 to 0.097 %N. In terms of the non-modelled soil nutrients, the 2013 C and C:N results at the treatment level were mainly different in, perhaps, greater overlap between the two IND treatments for soil organic C than there was for soil N (Supplemental Fig. A.1, Supplemental Fig. A.2).

Comparisons of sequences between the fungal components and related methodologies

There was a total of 4032 root-tips sampled. Of those, 102 were classified as likely to be non-mycorrhizal during root sorting, with another 96 root-tips more challenging to discern. To maintain consistency, they were sequenced along with the 3,824 root-tips classified as EcM during microscopic root sorting. Analysable sequences were obtained for 1,618 of the root-tips (40.1%) with a mean of 640 bp (range of 5-1,212 bp) across all sequence qualities. Sequences were obtained, but were of too low quality for analyses, for 966 (24.0%) of the root-tips. No sequence was obtained for 1,448 (40.9%) of the root-tips. A total of 190 OTUs were generated from the root-tip sequencing. Of those 190 OTUs, our original designations of mycorrhizal status matched exactly to those obtained from FUNGuild for the non-mycorrhizal and mycorrhizal root-tips, and those that we had noted, based on microscopy, as possibly EcM were classified as such. 125 of the OTUs were EcM, of which 91 were at a quality to include in the data used for final analyses.

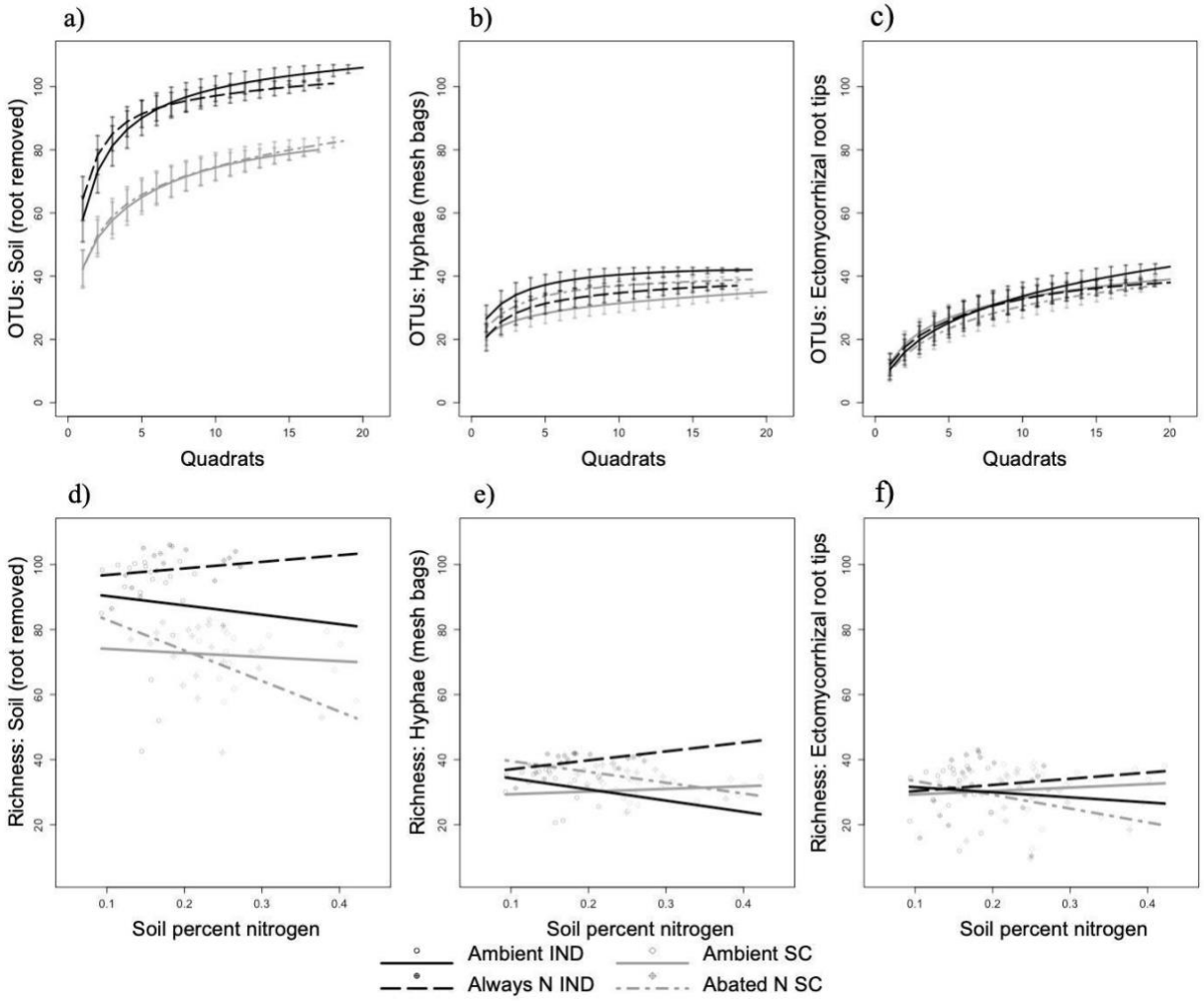
The Illumina MiSeq analyses for the root-free soil and hyphal samples produced 7,535,518 sequences with a range in sequence length from 35-251 bp and 46% GC content. Of those, 87,557 sequences passed quality control and filtering steps. They were clustered into 533 total OTUs, of which 212 were EcM OTUs with approximately 500 sequence reads per sample on average. For the soil and hyphal data used for analyses, they contained 166 and 75, respectively, of the EcM OTUs.

Richness trends

EcM fungal richness varied most clearly by the fungal component (Figure 1). The number of EcM fungal OTUs in bulk root-free soil samples was much higher (two to three times greater), with the maxima leveling off at 80 to 100 OTUs, versus those in the hyphal or root-tip samples which had maxima leveling off at 30 to 40 OTUs. Differences in accumulated richness were often equivocal (i.e., overlapping error bars), but some patterns did exist, and which were consistent with the expectation that higher N levels result in decreased diversity: richness was greater with the ambient treatments than the elevated N treatments at IND. The OTU accumulation curves displayed the treatment by site impacts to richness but could not include the site- and plot-level variations in soil parameters (Supplemental Fig. A.1, Supplemental Fig. A.2) that may likewise have influenced EcM fungal diversity.

The final selected LME model regressed the EcM fungal richness by the fixed effects of the fungal component, treatment by site, and soil percent N, the latter important to include as a proxy to the degree that the N treatment altered soil properties (Supplemental Table A.1). Specifying the fungal component as a fixed effect allowed the predictions to vary in combinations with the treatment by site factor, i.e., the random slopes and intercepts. Importantly, it was already clear that the fungal component substantially contributed to richness values of EcM fungi, so that it was primarily modelled as random effect, nested among quadrat, plot, and site. The model's marginal R^2 was 0.9074, the conditional R^2 was 0.9984, the AIC was 1653, and BIC was 1753. The two-way interactions of soil N, treatment by site, and the fungal component were all statistically significant (but with soil N:treatment by site approaching marginal significance), while the three-way interaction of all three variables was not statistically significant (Supplemental Table A.1). The interactions with the fungal component primarily signified the

437 exceedingly higher predicted richness of bulk root-free soil EcM fungi compared to the hyphal
438 and root-tip fungi. The significance of the interactions that included treatment by site suggested
439 differing possible trends, i.e., slopes, as visible in plotting the estimated EcM fungal richness by
440 soil N, for each treatment by site (Figure 1). The importance of regressing EcM fungal richness
441 by soil N was to understand how it impacted EcM fungal richness, especially important given
442 what were unanticipated differences in soil N at SC and IND sites (Supplemental Fig. A.1)
443



444 Figure 1. OTU accumulation curves for the three fungal components (top row; a-c) demonstrate
445 richness trends between the treatments. The LME model with model random slopes and

intercepts, for each fungal component plotted in separate panels to aid visualisation, predicts EcM fungal richness based on soil percent N content for each treatment by site level (bottom row; d-e). IND and SC abbreviate the two sites. Ambient IND, black shading for circle point and solid line; Always N IND, black shading for circle point with internal dash and long-dash line; Ambient SC, grey shading for diamond point and solid line; Abated N SC, grey shading for diamond point with internal dash and short-long dash line). Points are the data used for modelling, to reflect the variance.

Richness was primarily predicted to decline with increased soil N, as demonstrated for all three components for the ambient (IND) and abated N (SC) treatments, as well as the soil ambient (SC) treatment (Figure 1). The strongest decline was predicted for the abated N (SC) treatment. Interestingly, the relationship was not uniformly negative; a slight increase in richness along with soil N was predicted for the sustained exposure, always N (IND), treatment, as well as the ambient treatment for the hyphae and root-tip components (at SC). In terms of treatment effects, the differences at the IND site between the ambient and the experimental treatment (always N) became more pronounced with increased soil N. At the SC site, in contrast, an interaction between the ambient and abated N treatments across increased soil N rendered richness levels more contingent on soil N levels, i.e., the degree that richness differed between the two treatments was less consistent (Figure 1).

Compositional trends

Compositionally, most OTUs were shared across all treatments for each fungal component (Figure 2). However, there was an ordering based on the fungal component for the treatments: the treatment by site levels overlapped in OTUs, ranging from the least shared OTUs in the most diverse root-free soil samples, to the greatest shared and unique OTUs in the hyphae and root-tip samples, both of which contained less overall EcM fungal richness. There was greater uniqueness to the treatment by site levels for the root-tip OTUs than either of the other two components. In fact, root-tip OTUs were primarily either shared across all treatment by site levels, or unique to one of them. The two ambient treatments (at IND and SC) and the abated N SC each contained more unique root-tip OTUs, compared to the sustained N exposure with the always N IND treatment. The same trend partly existed for the hyphal OTUs, with the greatest number of unique OTUs for the ambient IND samples, and more homogenous overlap in the shared species. This trend was lacking in the bulk root-free soil samples. Instead, the amounts of shared and unique OTUs were fairly equal, except when shared across all of the treatment by site levels. At the genus level, even less distinction could be discerned between the fungal components by the treatment by site levels (Supplemental Fig. A.3), with nearly all genera shared across all the levels, with none unique to a specific treatment.

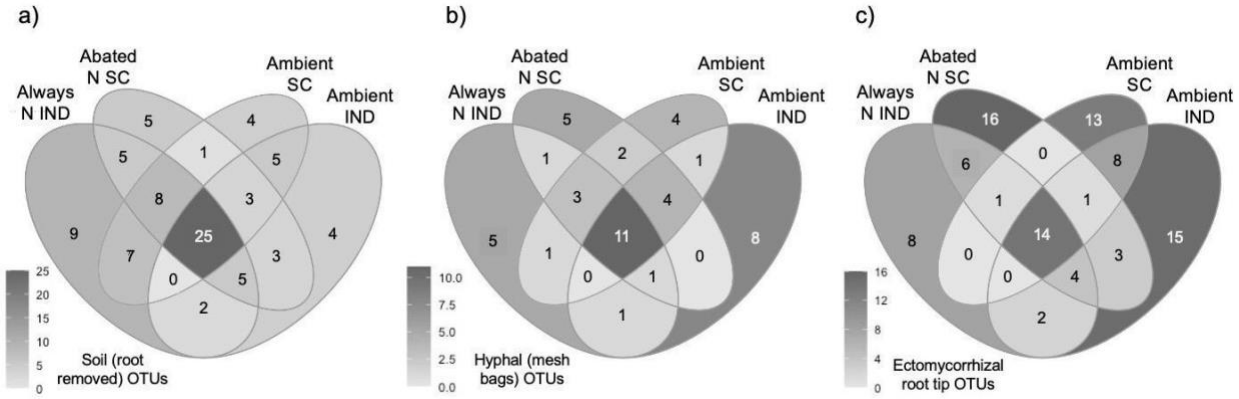
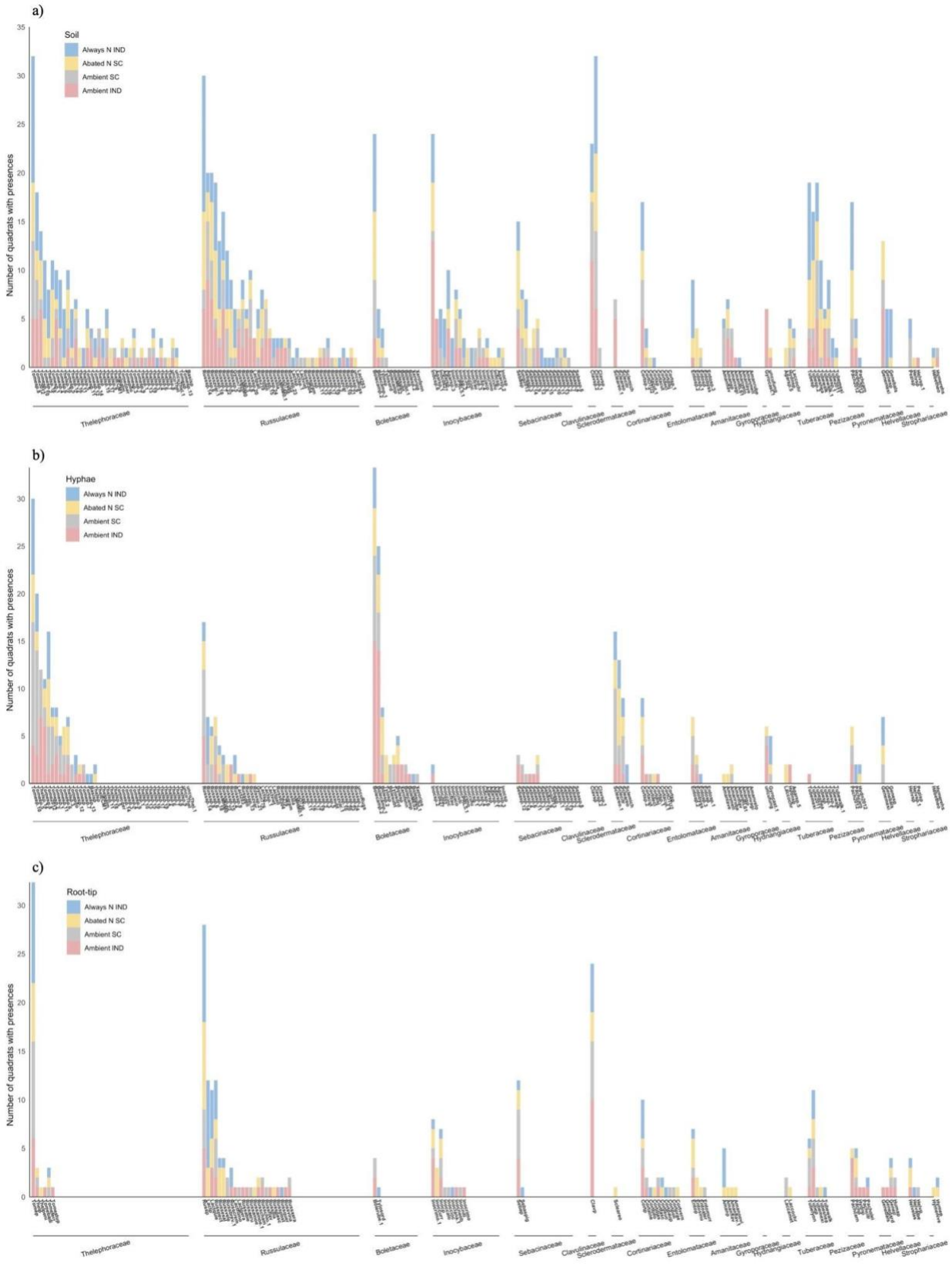


Figure 2: Venn diagrams of OTU richness for a) bulk root-free soil, b) hyphae from in-growth mesh bags and c) root-tip EcM fungal components. The shading grades from containing no shared species (lightest grey) to the maximum of shared species (darkest grey) possible for each fungal component. The treatment-site levels arrange, left to right: Always N IND, Abated N SC, Ambient SC, Ambient IND. IND and SC abbreviate the two sites.

Given that the hyphae and soil OTUs originated from the same molecular (HTS) procedures, this enabled us to compare the shared and unique OTUs for those specific components (Supplemental Fig. A.4). The root-free soil contained the vast majority of the OTUs, either unique to the soil (60.9%) or shared with the hyphae (25.0%), while far fewer were unique to the hyphal samples (14.1%). The least number of shared OTUs between the soil and hyphae was for the always N (IND) treatment (20; 16.8%), while the greatest shared amount was for the abated N (SC) treatment (26; 21.7%).

The most discernible compositional trends by taxa existed when comparing among fungal components, and not the experimental treatments (Figure 3). Specifically for the presence of fungal OTUs in bulk root-free soil compared to as hyphae within the in-growth mesh bags (Supplemental Fig. A.5), the dominating taxa in the soil (Russulaceae, Inocybaceae, Tuberaceae, Clavulinaceae), differed to those only found as hyphae (Thelephoraceae, Sebacinaceae, Amanitaceae, Cortinariaceae, amongst others), or those more exclusively (but not completely) found as hyphae (Boletaceae, Gyroporaceae, Sclerodermataceae). Comparing among all three fungal components, OTUs for *Cenococcum*, *Elaphomyces*, *Entoloma*, *Inocybe*, *Lactarius*, *Sebacina*, *Thelephora*, and *Tuber* were more common in the soil and on root-tips, and were less

commonly (to not) found as hyphae (Supplemental Fig. A.6). In contrast, *Boletus* and *Scleroderma* were more often present as hyphae and, for *Boletus* also in the soil, but they were not identified on the root-tips. *Clavulina* was only found in the soil, while *Hymenogaster* and *Piloderma* were exclusive to the root-tips. *Amanita*, *Cortinarius*, *Russula*, *Tomentella* and *Pachyphloeus* were represented in all three components. The hyphae of *Boletus* and the root-tips of *Cenococcum* were more common in ambient N levels than in either experimental treatment (Figure 3). Root-tips with the shorter-distance *Lactarius* and, in general for many of the *Russula* OTUs, were more often found with continued N addition.


a)
Soil
Always N IND
Abated N SC
Ambient SC
Ambient IND
Number of quadrats with presences
Thelephoraceae
Russulaceae
Boletaceae
Inocybaceae
Sebacinaceae
Clavulinaceae
Sclerodermataceae
Cortinariaceae
Entolomataceae
Amanitaceae
Gyroporaceae
Hydnangiaceae
Tuberaceae
Pezizaceae
Pyronemataceae
Helvellaceae
Strophariaceae
b)
Hyphae
Always N IND
Abated N SC
Ambient SC
Ambient IND
Number of quadrats with presences
Thelephoraceae
Russulaceae
Boletaceae
Inocybaceae
Sebacinaceae
Clavulinaceae
Sclerodermataceae
Cortinariaceae
Entolomataceae
Amanitaceae
Gyroporaceae
Hydnangiaceae
Tuberaceae
Pezizaceae
Pyronemataceae
Helvellaceae
Strophariaceae
c)
Root-tip
Always N IND
Abated N SC
Ambient SC
Ambient IND
Number of quadrats with presences
Thelephoraceae
Russulaceae
Boletaceae
Inocybaceae
Sebacinaceae
Clavulinaceae
Sclerodermataceae
Cortinariaceae
Entolomataceae
Amanitaceae
Gyroporaceae
Hydnangiaceae
Tuberaceae
Pezizaceae
Pyronemataceae
Helvellaceae
Strophariaceae

517

Figure 3. EcM fungal OTUs as found in the a) bulk root-free soil, b) hyphae from in-growth mesh bags, and c) root-tip samples. The taxa are ordered by OTU abundance across the families the same between panels, matching primarily with a left-right gradient from greater to lesser total family presences. Composition is visible along the y-axis for all OTUs (but requires zooming in). Comparisons are possible for the treatments between bar colors, and for the fungal components between the plot panels. Legend colors and sequence (from top to bottom of bars): blue (Always N IND), yellow (Abated N SC), grey (Ambient SC), and red (Ambient IND). IND and SC abbreviate the two sites.

Discussion and conclusions:

Soil nutrient content

The study design necessitated that we split, between the two original sites, treatments so that experimental N addition was sustained at one site (IND) while it was abated at the second site (SC), with both also containing the original ambient N treatments. The two sites had been initially selected for the uniformity of soils and tree composition between them (Avis et al. 2008). We were highly surprised to discover that soil N was greater at the SC site, despite the abated N treatment (Supplemental Fig. A.1). Thus, we found that we needed to consider, carefully, the potential effects of experimental N treatment, our primary goal, alongside the, now confounded, context dependencies between the sites, in relation to soil N. It added complexity to an already nuanced study design and complicated our analyses by reducing our capacities for statistical testing. Still, we believe the results are important to present in the manner in which we have discovered them, because, as we have found, an increase or decrease in N pollution from a particular source (e.g., reduction in localised industrial emissions, legislative actions against fertilization, and/or reductions from local farming), may *not* consequently reduce N levels within a given forested area, when compared to another regionally located forest - at least, not in terms of soil N. In addition, because we included the soil N values along with the treatment and site combinations within our regression analyses, in this manner we still achieved our goal of understanding how N impacts EcM fungi.

549 We suggest three context dependencies for why the SC site contained higher soil N levels,
550 despite SC being initially selected with IND for the uniformity of soils and tree composition:
551
552 1) The SC site is a forest fragment surrounded by urban and suburban infrastructure, with likely
553 non-point sources of N pollution that, despite proximity to the IND site, could have caused long-
554 term differences to soil N contents. In comparison to SC, the IND site is located in the Indiana
555 Dunes National Park, and thus it is surrounded by more continuous natural areas, and less
556 immediately by urban or suburban infrastructure. While IND may be downwind of major
557 industrial centers of the metropolitan area, and historically at the higher end of a 50-year N
558 pollution window, concerning the duration of this study, those levels of N deposition have been
559 relatively equal. Higher SC site N levels may be partly due to greater contemporary non-point N
560 pollution additions.
561
562 2) The consequences that earthworm invasions have on organic soil horizons (Hale et al. 2005)
563 are likely a main contributor to why much thinner soil O horizons have been observed at the IND
564 site across time of the experiment (PGA Pers. Observation). There is also circumstantial
565 confirmation of this in our 2013 soil nutrient analyses, given that soil organic C was reduced at
566 IND (Supplemental Fig. A.2). The thinner O horizon at IND could, thus, also help explain the
567 lower soil N values, when compared to SC.
568
569 3) Our final observation was the widespread presence of *Amphicarpaea bracteata*, an aggressive,
570 N-fixing viney herb legume, which has been noted in recent years at the SC site (and not at the
571 IND site). This has been a more sudden change, likely due to recent forest disturbance opening

572 up canopy gaps for its growth, i.e., not noted in the original experiment (PGA Pers.
573 Observation). Nitrogen fixation by this plant could have elevated the soil N levels at the SC site.
574
575 Combined, these probable contributors of 1) nonpoint N pollution, 2) the effects to soil from
576 invasive species spread, and 3) natural disturbance-related forest stand dynamics to understory
577 composition, likely all impacted the internal N cycling at the two sites individually, hence the
578 differences in soil N between them that we have found (Supplemental Fig. A.1). Nonetheless,
579 our results indicate that, even though IND now contains lower soil N when compared to SC, the
580 10 years of experimental N fertilization had increased soil N in the fertilized plots compared to
581 the ambient plots (Supplemental Fig. A.1). This was also supported by higher tree growth (tree
582 diameter, as measured by dendrometers, and annual leaf fall) in the fertilized plots at IND (PGA
583 Unpubl. data). And at the SC site, there was the indication of decreasing soil N in the abated,
584 compared to the ambient, treatments.
585
586 *Comparisons of sequences between the fungal components and related methodologies*
587
588 The most discernible differences in the richness and compositional trends of EcM fungi was
589 based on the fungal component and not the experimental N treatment, nor the percent of N
590 contained in soils or even the sites. Richness was remarkably higher for the bulk root-free soil
591 component than either the hyphal (from in-growth mesh bags) or the EcM on root-tips
592 components (Figure 1). The EcM fungi that were present were, also, taxonomically less unique
593 in the soil, and most unique on the root-tips (Figure 2), despite greater sequencing depth in HTS
594 technology applied to the soil samples versus the single-species Sanger sequencing approach

595 used for the root-tips. In terms of composition, the taxa were also most clearly arranged in
596 abundance by the fungal components (Figure 3).
597
598 To a degree, given the different processing and sequencing methodologies, we had expected that
599 the fungal component would affect our results. We kept the samples as spatially similar as
600 possible to reduce any sampling artifacts, and we purposefully included the components as a
601 random variable during statistical analyses (i.e., we expected greater richness in the HTS-derived
602 samples). What we did not expect was the clear indication that the differences between the
603 fungal components were most related to their actual ecological aspects (i.e., temporal
604 characteristics related to the fungal components) than to the expected molecular processing
605 aspects. If sequencing technology, and related processing, were the primary sources of richness
606 and compositional differences between the fungal components, then the results for the soil and
607 hyphae would have matched more than either component would have matched with the results
608 for the root-tips. In contrast, we found greater similarity in the hyphae and root-tips, with the soil
609 the exception in terms of total richness (Figure 1), greater taxonomic homogeneity (Figure 2),
610 and wider compositional coverage (Figure 3).
611
612 We compared further, and directly, to understand the results of the two fungal components
613 (hyphae and soil) analysed with the same sequencing technology (Supplemental Fig. A.4). The
614 bulk root-free soil component contained substantially more EcM fungal OTUs than did the
615 hyphae (from in-growth bags), as we had expected based on the temporal aspects of the fungal
616 components. Regarding sequencing technology, the EcM fungi from the hyphal in-growth mesh
617 bag samples actually matched more to those encountered on the root-tips (Figure 2) -

importantly, this pattern was found irrespective of the use of very different molecular approaches between the hyphae (HTS) and root-tips (Sanger). This indicates that we have measured tangible richness and composition trends between the fungal components and that are primarily related to the ecology, and not to laboratory processing necessitated for the study.

We note that the species composition on the root-tips were markedly different from the hyphal in-growth bags (in the most abundant taxa at least), and neither were as comprehensive as the bulk root-free soil EcM fungi (Figure 3). This result we believe, especially, is explained by the temporal scales of each measurement (next sections).

Richness and compositional trends

Our primary objective was to contrast experimental N addition with its abatement, in influencing the richness and composition of EcM fungi. Our primary hypothesis stipulated, based on earlier research (Avis et al. 2008, Lilleskov et al. 2019), that by elevating soil N - experimentally or otherwise - the richness of EcM fungi would decline, and the composition would change, especially related to taxa previously identified as more or less tolerant to N addition (Avis et al. 2003, Avis et al. 2008, Avis 2012, Morrison et al. 2016). In support of this, EcM fungal richness was greater for ambient than elevated N conditions at the IND site (Figure 1a-c), and the richness within ambient treatments was also predicted to decline with increased soil N (Figure 1d-f). The abatement of experimental N did not so clearly influence richness compared to ambient conditions, but the influence of other contributors to soil N (see above) likely weakened this relationship (Figure 1a-c). Importantly, richness was also predicted to decline as soil N increased

for the SC site abatement and ambient treatments (Figure 1d-f). The results of this study thus support our primary hypothesis, and are consistent with the general finding from other studies that elevating soil N leads to lower richness of EcM fungi. While this response is not novel to research knowledge (e.g., Lilleskov et al. 2019 and references within), we deepen the understanding of the N effect on EcM fungi by showing that such responses can be more evident in bulk root-free soil (Figure 1a, d) than as seasonally active hyphae from in-growth mesh bags (Figure 1b, e) or even EcM on root-tips (Figure 1c, f). Similarly, the fungal components varied in terms of their shared and unique OTUs (Figure 2) and composition (Figure 3), discussed next.

Our secondary objective focused on determining the extent that scale might be relevant to discerning the responses by EcM fungi to N effects. We arranged these expectations into two sub-hypotheses that related to the temporal aspects compared between the fungal components, as well as to the EcM mycelial growth traits (i.e., exploration types) of dominant taxa by experimental treatment.

Multiple seasons of fungal activity, from decades past to current, are represented by fungal structures and eDNA in bulk root-free soils (Benucci et al. 2020, Foucher et al. 2020), which most likely explains why richness for this component was so much greater than either of the other two components. We believe it is a result of the greater richness that the treatment differences and soil N responses were clearest for the bulk root-free soil EcM fungi (Figure 1). In contrast, the differences between treatments, when either the current growing season, as measured for the hyphae from in-growth mesh bags, or the current and past growing seasons, as measured for the EcM root-tips, were utilized were far less resolute. A future pathway to

664 resolving such discrepancies - given the fact that the taxa are ultimately a combination of root-
665 tips, active hyphae and other structures as found in the soil, combining “parts to parcel” would
666 greatly alleviate the taxonomic heterogeneity we found in composition (Figure 2, Figure 3).
667
668 We found distinct differences in the abundance and frequency of EcM fungi between fungal
669 components, as can be expected given that EcM fungi vary in the extent that they grow away
670 from colonized root-tips. Exploration types are reflective not only of active growth distances, but
671 also the capacity of fungi for nutrient scavenging. Taxa with extensive extraradical mycelia
672 and/or rhizomorphs are capable of distant scavenging, while those that invest little in extraradical
673 mycelium instead remain proximal to the root-tips. Could short-distance types be more capable
674 of taking up soluble and mobile soil resources, such as labile forms of N in the rhizosphere, than
675 longer-distance types? In our study sites, the Boletales were very common in the hyphal in-
676 growth mesh bags (Figure 3; it was previously found on roots, i.e., Avis et al. (2008) but rarely
677 in 2013; perhaps sequencing technologies interfered with its presence in 2013 on roots, i.e.,
678 Andrew and Lilleskov 2014). In contrast, taxa in the Russulaceae produce shorter-distanced
679 hyphae from the root-tips, but fruited often and abundantly in these sites (P. Leacock Unpubl.
680 data); the importance of this is that, presumably, the soils would be rich with spores from
681 *Russula* and *Lactarius*, which we do find in terms of fungal propagules within the bulk root-free
682 (Figure 3).
683
684 In returning to our primary objective of N effects to EcM, the finding that the Russulaceae, at the
685 root-tip level, tended to be common with sustained experimental N addition (Figure 3) is
686 consistent with previous studies (Avis et al. 2003, Suz et al. 2014, Morrison et al. 2016, Carrara

et al. 2021). Although the nitrophilic mechanism is not well understood, one hypothesis is that these taxa are very strong root colonizers (A.F.S. Taylor pers. comm.), likely dominating whole root sections. It is conceivable that, as other EcM taxa reduce root-tip colonization due to increased N, this creates a feedback for Russulacean taxa, being "nitrophilic" and strong root competitors, to colonise more roots. This can be contrasted to taxa in the Boletaceae, who perhaps by expending energy in long-distance exploration, reduce their root-tip colonisation. These are some likely reasons that the hyphae of *Boletus* were more common in ambient treatment than in either experimental form, and why root-tips of *Lactarius* and *Russula* OTUs were more often found with continued N addition (Figure 3), as we had expected.

Conclusion

Increased soil N levels have consequential impacts to EcM fungi that are demonstrated differentially between fungal components. There are temporal (duration and longevity of seasonally produced components) aspects which interact with taxon-specific fungal traits to, ultimately, reduce EcM fungal richness with elevated soil N. Importantly, the abatement of N pollution from a known source may not mediate all issues of N, should other sources continue to add pollution, or modifications due to species invasion and forest stand disturbance reverberate to soil N levels. The resoluteness of a N pollution pattern by EcM fungi in aggregate form can quickly unravel into realistic, but complex trends. These signify the necessity to unify fungal trends across fungal components, related to the mechanistic causations of local-scale context dependencies.

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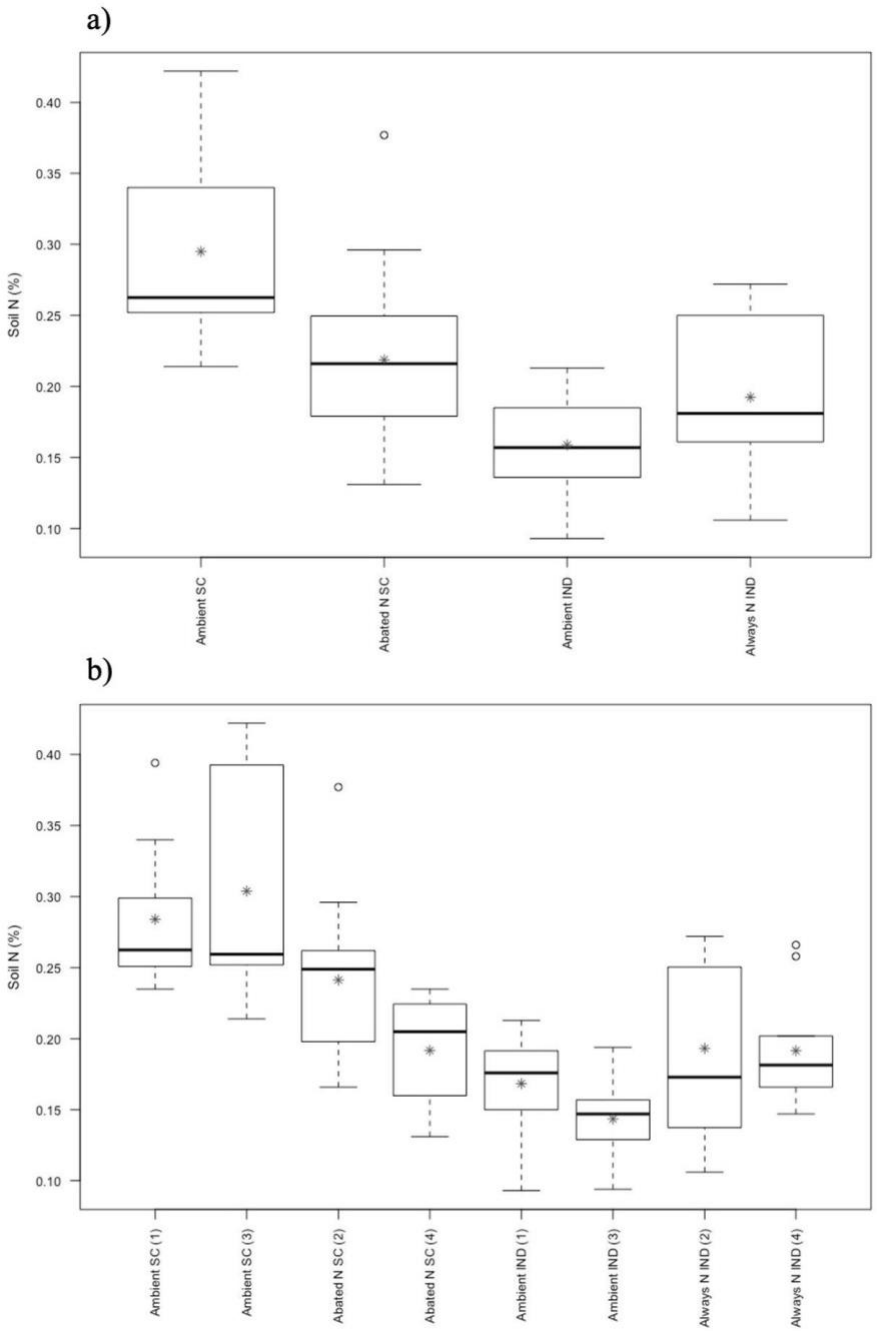
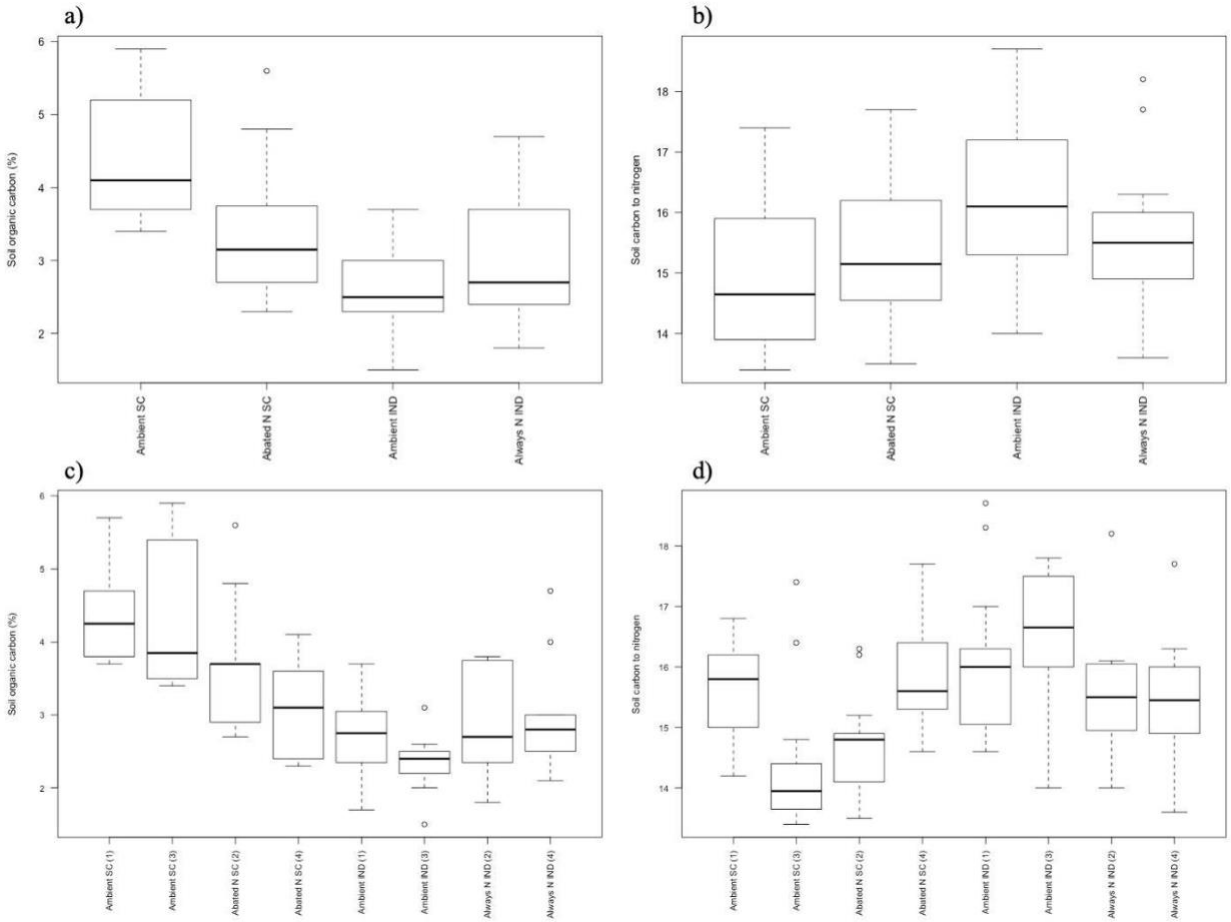
Appendix A. Supplementary material (Fig. A.1 - Fig. A.6, Table A.1 - Table A.2).



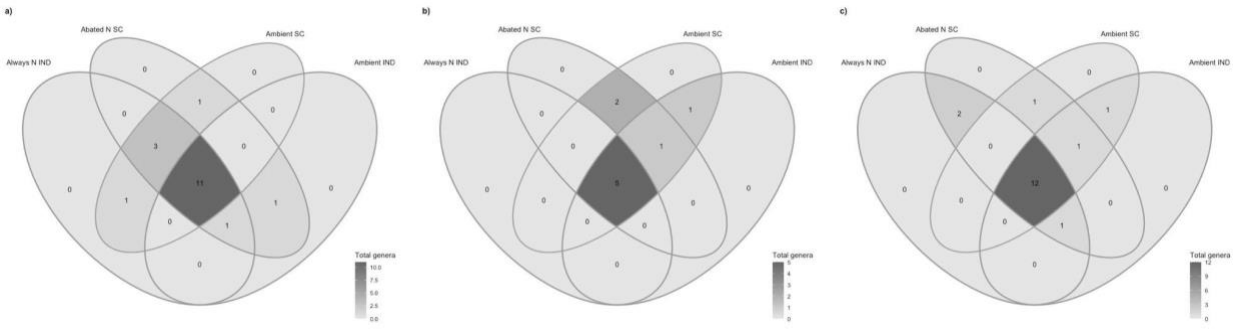
Supplemental Fig. A.1: Percent soil N in 2013 at the (a) treatment (including site abbreviation) and (b) treatment at the plot (as numbers) level. Boxplots demonstrate the interquartile range

906 (IQR; 25-75%) and the solid bar is the median. The ranges are minimum and maximum for the

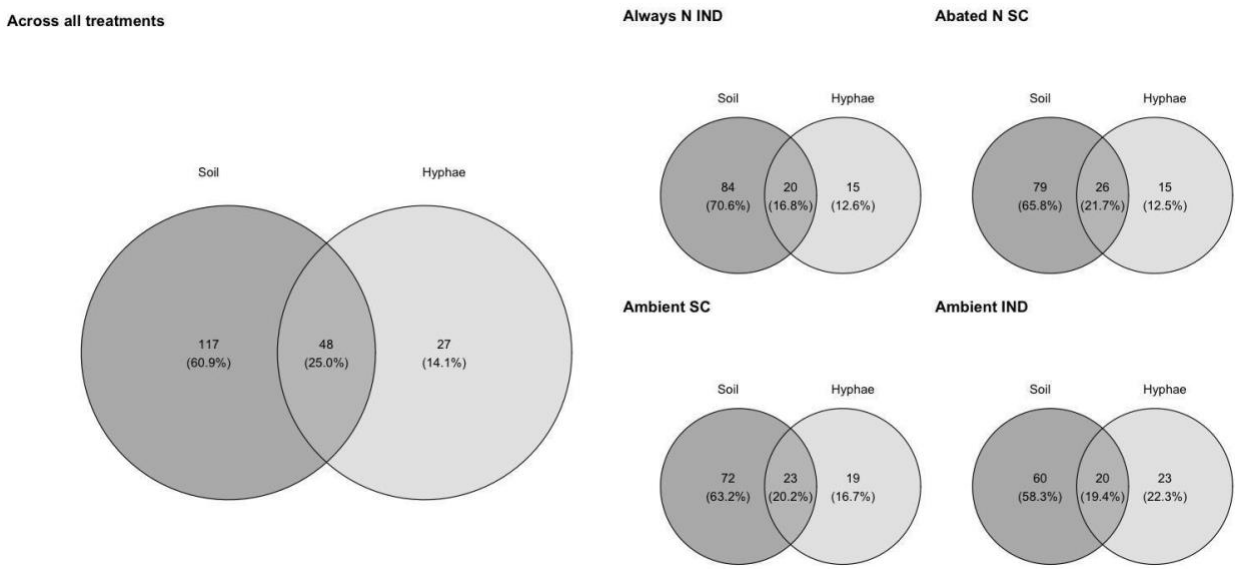
907 IQR, with any circle points considered outliers. The asterisks are the mean values.



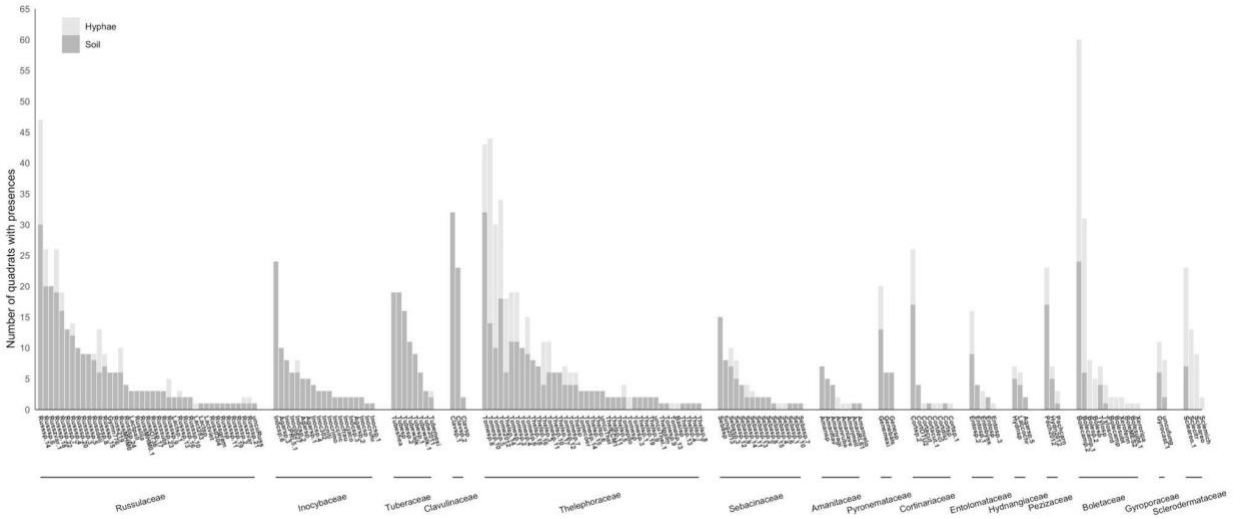
Supplemental Fig. A.2: Percent soil organic carbon (a, c) and the soil carbon to nitrogen ratio (b, d) at the (a, b) treatment (including site abbreviation) and (c, d) treatment at the plot (as numbers) level. Boxplots demonstrate the interquartile range (IQR; 25-75%) and the solid bar is the median. The ranges are minimum and maximum for the IQR, with any points considered outliers.



Supplemental Fig. A.3: Venn diagrams of OTU genera for a) soil, b) hyphae and c) root-tip EcM fungal components. The shading grades from no shared species (lightest grey) to the maximum of shared species (darkest grey) for each fungal component. The treatment-site levels arrange, left to right: Always N IND, Abated N SC, Ambient SC, Ambient IND.



Supplemental Fig. A.4. Venn diagrams for OTU richness compared between the soil and hyphae fungal components, along with the treatments by sites.



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929 Supplemental Fig. A.5: Total presences of EcM fungal OTUs, presented across the treatments

930 and sites in order to focus on how the hyphae (light grey) compared to the soil (dark grey)

931 samples.

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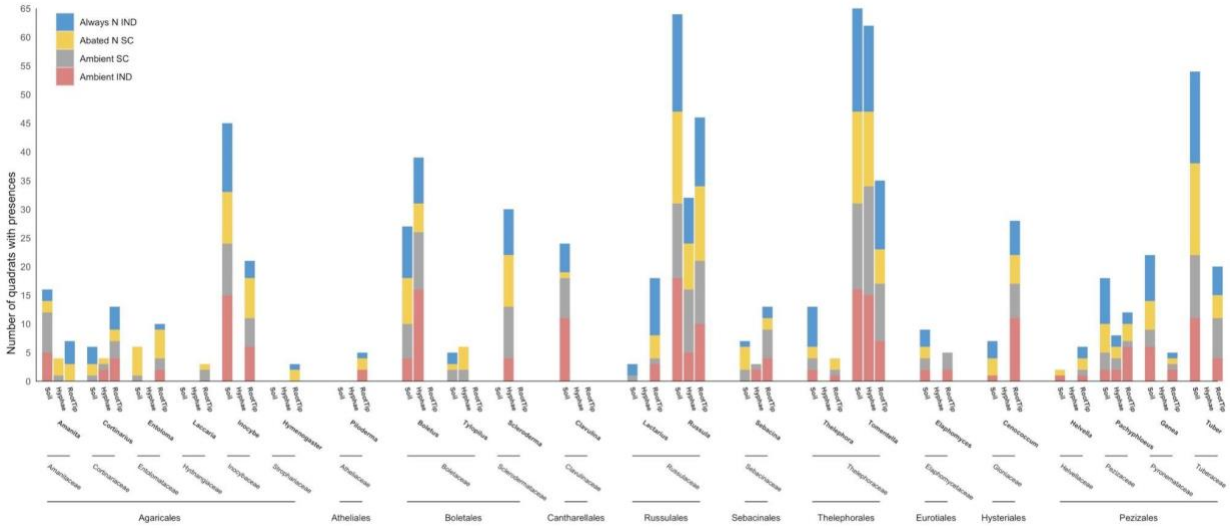

Supplemental Fig. A.6: Total genus-level presences of each EcM fungal component by treatment and site. The taxa are ordered by phylum and alphabetically by order, family and genus. Legend colors and sequence (from top to bottom of bars): blue (Always N IND), yellow (Abated N SC), grey (Ambient SC), and red (Ambient IND).

	numDF	denDF	F-value	p-value	Level of statistical significance
(Intercept)	1	205	7848.363	< 0.0001	***
Soil N	1	205	29.486	< 0.0001	***
Treatment by site	3	205	17.202	< 0.0001	***
Fungal component	2	0	914.11	NA	NA
Soil N : Treatment by site	3	205	2.719	0.0456	*
Soil N : Fungal component	2	205	14.789	< 0.0001	***
Treatment by site : Fungal component	6	205	8.791	< 0.0001	***
Soil N : Treatment by site : Fungal component	6	205	0.162	0.9864	

Supplemental Table A.1: ANOVA table of the LME regression for the fixed-effects. Note that, since values were established as different between fungal component, hence it was modelled as a random effect, it was also included as a fixed effect in order to allow for an interaction of treatment and soil N effects between the components.