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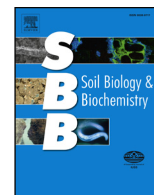

Impacts of experimentally accelerated forest succession on belowground plant and fungal communities

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ABSTRACT

Understanding how soil processes, belowground plant and fungal species composition, and nutrient cycles are altered by disturbances is essential for understanding the role forests play in mitigating global climate change. Here we ask: How are root and fungal communities altered in a mid-successional forest during shifts in dominant tree species composition? This study utilizes the Forest Accelerated Succession ExperimenT (FASET) at the University of Michigan Biological Station (UMBS) as a platform for addressing this question. FASET consists of a 39-ha treatment in which all mature early successional aspen (*Populus* spp.) and paper birch (*Betula papyrifera*) were killed by stem-girdling in 2008. Four years after girdling, neither overall fungal diversity indices, plant diversity indices, nor root biomass differed between girdled (treated) and non-girdled (reference) stands. However, experimental advancement of succession by removal of aspen and birch resulted in 1) a shift in fungal functional groups, with significantly less ectomycorrhizal fungi, 2) a trend toward less arbuscular mycorrhizal fungi, and 3) a significant increase in the proportion of saprotrophs in girdled stands. In addition to shifts in functional groups between treated and untreated stands, ectomycorrhizal fungi proportions were negatively correlated with NH_4^+ and total dissolved inorganic nitrogen (DIN) in soil. This research illustrates the propensity for disturbances in forest ecosystems to shift fungal community composition, which has implications for carbon storage and nutrient cycling in soils under future climate scenarios.

1. Introduction

Determining how carbon (C) and nitrogen (N) cycles are altered by disturbances is essential to our understanding of the future states of the world's forests. Soil processes are among the least well-known components of these cycles (Carney et al., 2007; Norby and Zak, 2011). Nitrogen is particularly important in forest ecosystems, as it is most often the nutrient limiting primary production (Lebauer and Treseder, 2017; Norby and Zak, 2011; Vitousek and Howarth, 1991). Because N availability (N_{avail}) has differential effects on tree species growth and functional groups, it strongly influences plant community composition and forest C sequestration (Norby et al., 2010). Plant and fungal communities play key roles in soil processes impacted by disturbances and successional shifts (Chapman et al., 2005; Courty et al., 2010a; Horton and Bruns, 2001; Kaye and Hart, 1997; Lilleskov and Bruns, 2001).

As plant community composition shifts through time (*i.e.*, during succession), competition for N among individuals of different plant species in N-limited systems increases (Tilman, 1990; Vitousek and Howarth, 1991). This competition can be exacerbated as N becomes more limited and plants reliance on previously "inaccessible" N increases (Luo et al., 2004). Plants have evolved various strategies to compete for nutrients, including varying above ground vs. below ground allocation. Previous research has shown that the proportion of carbon allocation to roots can remain constant across a nitrogen gradient, but that fine root turnover rates increase as N_{avail} increases (Hendricks et al., 1993; Nadelhoffer et al., 1985).

An additional adaptation manifests in mycorrhizal associations between plant roots and fungi that enables plants to acquire more nutrients and water than they might otherwise attain without a fungal symbiont (Kirk et al., 2004; Smith and Read, 2008). Mycorrhizal associations can enhance plant competitive abilities and accelerate belowground nutrient cycling. Two dominant forms of mycorrhizae in forests are ectomycorrhizal fungi (EM) and arbuscular-mycorrhizal fungi (AM). AM fungi are the most ancient form of mycorrhizal symbiont, have plant symbionts belonging to all phyla, and are characterized by the formation of arbuscules in plant roots, which function to transfer nutrients and carbon between the mycorrhizal symbiont and plant host (Smith and Read, 2008). EM fungi form a mantle, or sheath, enclosing the plant root and grow within the root cortex to form a Hartig net where nutrient transfer occurs. EM mine soils for inorganic

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nutrients and assimilate amino acids and amino sugars from soil solution (Schimel and Bennett, 2004). Controversy exists, however, as to how widespread phylogenetically and to what extent EM may function to break down soil organic matter (SOM) via protease, lignocellulase and peroxidase enzymes, and transfer nutrients to the plant host (Averill et al., 2014; Bödeker et al., 2014; Pellitier and Zak, 2018; Talbot et al., 2013).

Field studies have shown that EM can vary temporally, spatially, and along gradients of both species richness and enzymatic activity (Courty et al., 2010a,b; Peay et al., 2010; Taylor et al., 2014). Studies of EM fruiting bodies have shown declines in fungal diversity and abundance with increasing N_{avail} at regional scales (e.g. Lilleskov and Bruns, 2001). New molecular techniques can provide finer resolution of EM community structure, revealing highly diverse and patchy belowground distribution and poor associations between sporocarp dominance and EM dominance on roots (Gardes and Bruns, 1996; Horton and Bruns, 2001). These distribution patterns may have important implications for nutrient cycling as research has shown that shifts in fungal community structure can influence soil C sequestration via differences in melanin production and mycelial physiology (Clemmensen et al., 2015; Fernandez and Kennedy, 2015; Fernandez and Koide, 2014; Siletti et al., 2017).

Fungal saprotrophs, a third functional group, secrete enzymes including glucosidase, hemicellulases, and phosphatases that serve to break down SOM (Talbot et al., 2013). Decomposition of SOM by saprotrophs makes N available in soil and produces CO_2 (Högberg et al., 2003; Štursová et al., 2012). Fungal saprotrophs compete for N with plants and their mycorrhizal symbionts as they break down SOM (Averill et al., 2014; Cairney and Meharg, 2002; Högberg et al., 2003). EM fungi and their plant symbionts may inhibit saprotrophic decomposition rates by removing available N from soils as well as by forming thick hyphal mats that prevent saprotroph growth (Averill et al., 2014; Cairney and Meharg, 2002; Lindahl et al., 2013). This competition between EM and saprotrophic fungi for N may lead to increases in soil C stocks if saprotrophic decomposition is inhibited as a result.

Contrary to EM presence possibly resulting in competition with decomposers, AM fungi presence has the potential to increase decomposer activity in forest soils by increasing substrate availability (Averill et al., 2014). AM fungi may increase substrate availability to saprotrophs by facilitating access to SOM patches as they preferentially grow towards the N-rich substrates (Bonfante and Anca, 2009; Cheng et al., 2012). They can also prime saprotroph-mediated decomposition of SOM by releasing labile C (Carney et al., 2007; de Graaff et al., 2010; Phillips et al., 2011). These interactions can have implications for decomposers, which when released from competitive restraints with EM due to increased N_{avail}, can break down organic C stocks accumulated in soil since the last major disturbance.

Measuring the effects of plant community succession on saprobic fungi and mycorrhizal associations is difficult on short time scales (*i.e.*, 1–10 years) and is more often investigated using long-term monitoring or chronosequence studies (Gartner et al., 2012). The Forest Accelerated Succession Experiment (FASET, described in METHODS) provided us with an opportunity to study relationships between changes in plant physiological responses, interspecific competition for soil resources, and fungal community structure in a large-scale, experimental increase in the rate of forest community succession.

Here, we address this question concerning impacts of a non-stand replacing disturbance in a temperate forest ecosystem: How do root and fungal communities differ in a mid-successional mixed hardwood forest following an intermediate disturbance? We hypothesized that girdling and subsequent mortality of mid-successional forest tree species affect below ground plant and fungal community composition by removing two dominant mid-successional EM associated species. This hypothesis leads to the following predictions:

1. Given previously observed increases in maple leaf production and the removal of two EM associated tree species (aspen and birch), the relative abundance of maple roots will increase in girdled stands.
2. The proportion of EM in the fungal community will decrease in treatment plots two tree species with ectomycorrhizal symbionts removed via girdling.
3. The proportion of saprobic fungi will increase in the treatment plots due to less competition for N.
4. The proportion of AM will increase in treatment plots and under higher N_{avail} as an AM plant symbiont becomes a canopy dominant.

2. Methods

2.1. Study site

The study was conducted at the University of Michigan Biological Staion (UMBS) in northern Michigan, USA (45°35′N 84°43′W). Mean annual temperature is 5.5 °C and mean annual precipitation is 817 mm. The bounds of the overall study site (including treatment and reference footprints and their plots) is ~140 ha and lies on a high-level sandy outwash plain and an adjacent gently sloping moraine and includes 17 unique landscape ecosystem types (Lapin and Barnes, 1995; Pearsall, 1985). The landscape ecosystems are generally similar in vegetation (northern mixed forest) and soils (coarse-textured), but vary locally (1–10 ha) in their topography and parent material, soil subgroup, and dominant tree and understory taxa. Across 60–65% of the area, soils are excessively well-drained Entic Haplorthods of the Rubicon series (Soil Survey Staff, 1991). The typical morphology of this series consists of an Oi and Oe horizons 1–3 cm thick, a bioturbated A horizon 1–3 cm, an E horizon 10–15 cm thick, and Bs and BC horizons of sand with occasional gravel and cobble (Nave et al., 2014). Approximately 30% of the study area is on more productive landscape ecosystems, where Lamellic and Alfic Haplorthods of the Blue Lake and Cheboygan series predominate. These soils differ from the Rubicon chiefly in the presence of stratified gravel, clay or loamy sand E′ and Bt horizons. The remaining 5–10% is underlain by Alfic Haploquads of the Riggsville series which are located in lower landscape positions (specifically, surrounding Treatment Replicate Stand #2; Fig. 1) and as a result have a seasonal water table and generally higher soil moisture status than the well-drained Haplorthods. Across all of these soils, approximately half of the fine root biomass is located in the upper 20 cm of soil and the forest floor C mass is approximately 5–15 Mg C ha^{-1}.

The main FASET treatment area occupies 33 ha of forestland within an eddy-covariance tower footprint, located within a larger area of more or less homogenous aspen-dominated, mixed, mid-successional forest. The treatment involved stem girdling of all mature *Populus tremuloides, P. grandidentata*, and *Betula papyrifera* trees (~6700 stems) in 2008, followed by mortality of nearly all girdled stems during the ensuing 3 years (Nave et al., 2014). Girdled trees were the dominant species in this mid-successional forest and represented ~30% of pre-treatment foliar biomass. In addition to the main treatment area, 3 replicated 2-ha experimental units located on other, nearby landscape ecosystems were also subjected to aspen and birch girdling. These plots served as independent replicates of the girdling treatment on sites with different ecosystem properties.

2.2. Field sampling

We installed 12 plots, consisting of 6 paired plots, with of each pair being a plot (16 m radius) within the (girdled) treatment area and a nearby plot located ~50 m outside the treatment area (Fig. 1). The 6 paired locations were selected on the basis of landform-productivity relationships at UMBS (Nave et al., 2017), which likely (based on variation in productivity) occupied a N_{avail} gradient. This allowed for analyses of both treatment and nutrient gradient effects. Each plot contained at least 1 canopy and sapling tree of each of *Pinus strobus* (white pine), *Quercus rubra* (northern red oak), and *Acer rubrum* (red

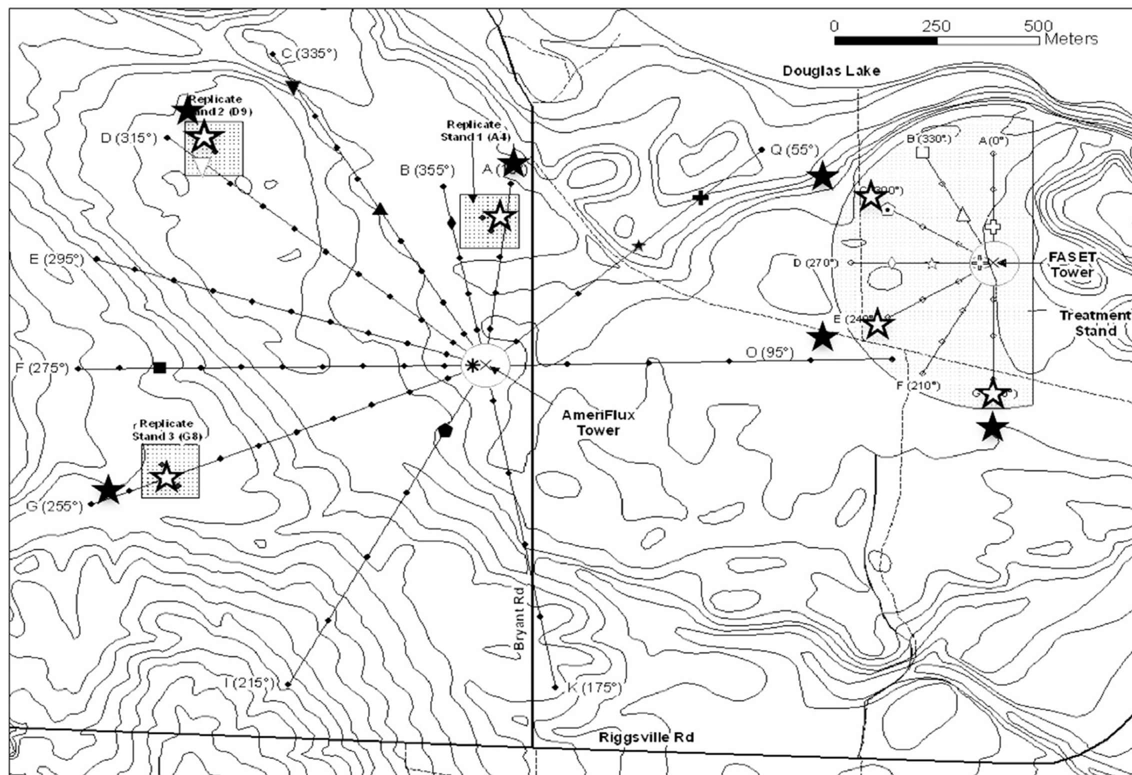

Fig. 1. Mixed deciduous forest at the University of Michigan Biological Station (UMBS). 12 paired plots; white stars indicate plots in the girdling treatment and black stars indicate plots in the reference forest. The paired plots were located in the main treatment footprint as well as 3 replicate sites that vary in soil fertility and landscape ecosystem type.

maple). We focused on plots containing these 3 tree species, as they are the co-dominant taxa with aspens and birches at UMBS, and are likely to replace the shorter-lived species as succession progresses at our site, and regionally across large forested areas of the upper Great Lakes. One plot pair contained no white pine (treatment and reference D9) and another pair (treatment and reference G8) had only sapling white pines. The canopy and sapling trees within the pairs had were of similar diameter at breast height (DBH) distributions. A reference point for each plot was placed centrally within the grouping of study trees and a 16 m-diameter plot was established from that point.

We measured DBH of all the trees in each 16 m plot in June 2012, and calculated stand basal area by summing basal areas (BA = π(DBH/2)) of all stems in each plot. Five A-horizon soil cores (5 cm diameter) were collected at random locations in each plot for molecular analyses of root and soil fungal communities, root and soil C and N concentration and stock measurements. Soil cores were stored in a −80 °C freezer until processing.

Four ion exchange resin bags (IERBs) per plot were deployed at random locations during June 2012 to determine available nitrogen (N), Ca^{2+}, and PO_4^{3-} in each plot using methods of Nave et al. (2011). The IERBs were placed beneath intact flaps of the O + A horizons. Each IERB consisted of a nylon foot stocking (MacPherson Leather, Seattle, WA, USA) containing ~30 mL of acid-washed, rinsed Dowex Marathon MR-3 mixed bed IER beads (Dow Chemical, Midland, MI, USA), packed into a PVC ring (5 cm diameter, 2 cm height). IERBs were collected from soils in September 2012. Resins were extracted using 2 M LiCl and the solution was analyzed on a SmartChem 200 (Westco Scientific Instruments, Crookfield, CT, USA). Ammonia, nitrate and ortho-phosphate were analyzed using the U.S. Environmental Protection Agency (EPA) standard laboratory methods EPA 350.1, EPA 353.2 and EPA 365.1, respectively (U.S. EPA, 1993a, 1993b; 1993c). Calcium was analyzed with the chlorophosphanazo-III vanadate method (Noda et al., 2010). Analyte concentrations were scaled by extract volume, resin mass, and PVC ring area to calculate indices of surface soil NH_4^+, $NO3^-$ and PO_4^{3-} and Ca^{2+}. All 4 resins from each plot were averaged to represent plot level means.

2.3. Sample processing for DNA analysis

Roots were extracted by hand from the soil cores. Only roots that were less than 5 mm diameter and up to the third order of branching were kept for further analysis. The roots were then cut into 2 cm sections and homogenized. Once sorted, the soil and roots were lyophilized for 24 h and pulverized in a SPEX Certiprep 8000D Mixer/Mill (Metuchen, New Jersey, USA). Subsamples of each of the 5 cores for each plot were combined to make one sample per plot. This was done for both roots and soils. A total of 2 g of soil was subsampled from each soil core; totaling 10 g of soil for the plot sample and 0.2 g of pulverized roots per soil core were subsampled. Combined samples were then stored at −80 °C until DNA analyses were conducted.

2.4. DNA extraction, PCR amplification and sequencing of 28S rRNA: soil fungi

DNA analysis of fungal communities was conducted on homogenized soil samples from soil cores. Genomic DNA was extracted from the soil samples using a Powermax Soil DNA Isolation Kit (MoBio). The 28S region was amplified from purified genomic fungal DNA using forward primer LROR (5′- AACCGCTGAACTTAAGC) (Vilgalys and Hester, 1990) and a modified verstion of the reverse primer LF402 (5′-TTCCCTTTCAACAATTTCAC) (Tedersoo et al., 2015). Each plot had its own reverse primer (LF402) with a 6 base pair barcode appended to the 5′ end for multiplexing. PCR reactions were carried out using ExTaq proofreading DNA polymerase (Takara) under the following conditions: 2.9375 µl PCR grade H_2O, 1.25 µl 10X ExTaq buffer, 2 mM $MgCL_2$, 0.2 mM dNTPs, 0.5uM primer LF402, 0.5uM primer LROR, 0.0625 µl of

5 units/uL ExTaq (.025 U/uL) and 5 µl genomic DNA. PCR cycle parameters were as follows: 3 min of initial denaturation at 94 °C, 35 cycles of 1 min denaturation at 94 °C, 30 s annealing at 54 °C, and 2 min at 72 °C with a final extension at 72° C for 7 min. Fungal PCR products were ~350–400 base pairs long and were purified using a QIAquick PCR Purification Kit (Qiagen). Purified PCR products were then analyzed for DNA amount using a NanoDrop 2000 (Thermo Scientific, Wilmington, DE, USA). An average of approximately 9.5 ng DNA per sample were pooled together to a final concentration of 114 ng/ul. The pooled sample was then used to generate a P4-C2 library using a DNA Template Prep Kit 2.0 for sequencing on a single SMRT cell of a Pacbio-RS II at the University of Michigan Sequencing Core.

2.5. DNA extraction, PCR amplification and sequencing of chloroplast DNA from roots

Genomic DNA was extracted from the root samples using a Dneasy Plant Maxi Kit (Qiagen). The genomic DNA extractions were then purified using a PowerClean® DNA Clean-up Kit (MoBio Laboratories, Inc.). The chloroplast region was amplified sequencing the *trn*L (UAA) intron from purified genomic root DNA using forward primer C (5′-GGGGATAGAGGGACTTGAAC) and reverse primer D (5′- CGAAATCG GTAGACGCTACG) (Taberlet et al., 1991). Each plot had its own forward primer (C) with a 6 base pair barcode attached for multiplexing. PCR reactions were carried out under the following conditions: 2.9375 µl PCR grade H_2O, 1.25 µl 10X ExTaq buffer, 2 mM $MgCL_2$, 0.2 mM dNTPs, 0.5 µM primer C, 0.5 µM primer D, 0.0625 µl of 5 units/uL ExTaq (.025 U/uL) and 5 µl plant genomic DNA. Negative and positive controls were run with each set of amplifications to monitor reagents and procedures. Cycle parameters are as follows: 3 min of initial denaturation at 94° C, 35 cycles of 1 min denaturation at 94° C, 30 s annealing at 54° C, and 2 min at 72° C with a final extension at 72° C for 7 min. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen). Purified PCR products were then analyzed for DNA amount using a NanoDrop 2000 (Thermo Scientific, Wilmington, DE, USA). An average of approximately 8 ng DNA per sample were pooled together to a final concentration of ~100 ng/ul DNA and sent to the University of Michigan Sequencing core for PacBio analysis.

2.6. Sequence analyses

Fungal and root sequences were processed using Mothur v.1.32.0 software (Schloss et al., 2009). Before processing in Mothur sequences were filtered using the bash5tools.py python script of the pbh5tools package, excluding all circular consensus sequences (ccs) that didn't complete 6 full passes. Sequences were then trimmed of barcodes and primers and then filtered with a Qaverage = 65. Possible chimeras were filtered using UCHIME in Mothur (Schloss et al., 2009). During chimera filtering, settings were set to self-reference so that more abundant sequences served as the reference sequences. Fungal sequences were rarefied to 1373, representing the least abundant sample, with an average Good's coverage of 96.57%. Root sequences were not rarified due to significantly lower sequence counts for reference plot A4. Good's coverage was calculated with an average coverage of 99.26%, with a Good's coverage for reference A4 of 98.24%. Distance matrices were created in Mothur using the pairwise.seqs command. A cutoff of 97% similarity was used while clustering sequences to determine OTUs and singletons were then removed. Sequences were then classified to genus level using BLAST option on the National Center for Biotechnology Information site (http://blast.ncbi.nlm.nih.gov/) and were assigned to genus if base pair matches met or exceeded 97% similarity. OTUs were then assigned to ecological niches based on a literature review of their genus level phylogenetic classification. The most predominantly representative functional group for each genus was used to assign ecological guilds. If no predominant ecological group emerged from the literature review we classified the functional group as unknown.

2.7. Data analysis

A principal coordinate analysis (PCoA) was conducted using distance matrices for fungal and plant communities using the Bray-Curtis method (Borcard et al., 2008). Overall community differences were tested with a Mantel test using distance matrices. Predictions regarding the fungal communities were tested in SigmaPlot and R, using regressions of the proportions of fungal functional groups against nutrient availability and beta regressions to test for differences in fungal proportions between girdled and reference pots. Regressions were also used to test for root biomass/relative proportions relationships with NH_4^+ NO_3^-, Ca^{2+} and PO_4^{3-} availability. Due to our samples size (n = 5) and the high within group variation across our data set, we have low power to detect average observed effect size of 0.6. Therefor, the absence of support for our hypotheses must be considered only weak support for the null. We accept as significant statistical tests with $\alpha \le 0.05$ and ascribe marginal significance for $0.05 < \alpha < 0.10$.

3. Results

3.1. Fungal and plant root communities estimated using sequences

DNA sequencing of plant root and fungal soil community composition revealed a total of 600 fungal Operational Taxonomic Units (OTUs) spanning most major fungal phyla and subphyla Ascomycota, Basidiomycota, Mucoromycota, Chytridiomycota). The mean number of fungal OTUs per plot was 120 (~33 s.d.). The number of fungal OTUs

Table 1
Fungal and plant diversity indices by treatment and reference plot. OTU Richness indicates the number of observed taxonomic units based on genomic sequencing of LSU sub unit and trnL-U intron from fungi and plants, respectively. No differences in diversity were detected between treatment and reference plots.

Treatment/Plot	Fungal			Plant		
	OTU Richness	InvSimpson	Shannon	OTU Richness	InvSimpson	Shannon
TREATMENT A4	157	0.95	3.86	9	9.39	2.32
TREATMENT C3	127	0.92	3.42	6	9.38	2.31
TREATMENT D9	142	0.89	3.22	8	10.94	2.39
TREATMENT E3	87	0.90	3.06	6	9.45	2.33
TREATMENT G3	112	0.88	2.95	6	9.37	2.31
TREATMENT G8	126	0.91	3.17	8	10.44	2.37
REF A4	131	0.93	3.44	7	10.48	2.37
REF C3	57	0.94	3.27	6	9.84	2.34
REF D9	175	0.95	3.91	9	10.96	2.40
REF E3	95	0.92	3.22	5	9.43	2.32
REF G3	94	0.85	2.71	7	9.76	2.34
REF G8	145	0.96	3.87	9	10.57	2.33

ranged from 57 in reference plot C3 to 157 fungal OTUs in treatment plot A4 (Table 1). Sequencing of plant roots reveal 14 plant OTUs, which approximately corresponds with the observed aboveground diversity at this site. The mean number of plant OTUs per plot is 7 (± 1.4 s.d.). The number of plant OTUs range from a low of 5 OTUs in reference plot E3, to a high of 9 in treatment A4, reference D9 and reference G8.

The 50 most abundant of the 600 fungal OTUs are listed with tentative genera/species identification, functional classification into ecological guild, the proportion of base pairs matched using BLAST, and proportion of plots containing the OTU (Supplemental table 1). Operational Taxonomic Units were identified using BLAST searches. Each OTU was placed into one of 5 functional groups: ectomycorrhizal, arbuscular mycorrhizal, saprobic, plant pathogen (PP), or other/unknown based on literature searches and taxonomic relatedness. Of the top 50 most highly represented OTUs, 25 were saprobic. *Umbelopsis dimorpha* was the most abundant OTU and is a generalist, saprobic fungus (Šturšová et al., 2012) that was found in all 12 plots. Ectomycorrhizal (EM) species constituted 34% of the most abundant OTUs with *Lactarius, Russula* and *Cenococcum* as common genera found in all 12 plots. Only one plant pathogen, *Ramularia* sp. and one arbuscular mycorrhizal OTU, *Glomus* sp. was found among the 50 most abundant OTUs. The remaining 6 OTUs were unknown soil fungal species. The putative uncultured *Ramularia* plant pathogen is the 8th most highly represented fungal OTU and was found predominately in both D9 plots. There was no effect of forest treatment (aspen and birch girdling) on overall fungal community diversity as indicated by either the Shannon-Wiener ($p = 0.970$) or Simpson indices ($p = 0.842$).

A total of 14 different plant OTUs were identified across the 12 plots (Supplement Table 2). The three most abundant species were *Acer rubrum*, *Pinus strobus* and *Quercus rubra*. *Acer rubrum* is the most well represented of any plant species in both treatment and reference plots. *Acer rubrum* root proportions were lower in 5 of the 6 paired treatment plots relative to reference plots. *Pinus strobus* showed an opposite trend with higher root proportions in 5 of the 6 paired treatment plots (Supplement Table 2). Of the 14 OTUs, 9 are canopy or subcanopy tree species, 4 are herbaceous plants and one is a grass. It should be noted that reference plot A4 has low plant counts relative to the other plots, presumably due to a poor chloroplast PCR amplification efficiency.

A PCoA analysis of the fungal community showed the D9 treatment and reference plots, both of which are located in an area of unique soil and ecosystem properties, as outliers, clustering outside of the other plots in the ordination (Fig. 2A). Another PCoA of the fungal community was performed removing D9 paired plots illustrating that paired plots cluster together (Fig. 2B). Similar to the fungal communities, the plant root PCoAs reveal that the treatment and reference D9 plots clustered apart from the rest of the plant communities (Fig. 3A). An additional PCoA removing D9 plots from the plant community analysis was also performed, with paired plots clustering together similar to fungal communities (Fig. 3B). There were several of the 50 most abundant fungal OTUs including an uncultured *Ramularia* sp. *Tomentella batryoides*, *Sebacina epigaea*, *Russula cf. pectinata*, *Chaetomium iranianum* and *Scabropezia flavovirens* that are predominately found only in D9 plots as well as the PP previously mentioned. Moreover, several plant OTUs including *Fraxinus* sp., *Rubus sp.*, *Fagus grandifolia*, *Tilia americana* and *Shizachne sp*. are also found almost exclusively in D9 plots and were responsible for the plots clustering away from the other plant communities. Based on this obvious separation of the D9 plot pair in ordination space we excluded this plot pair from further analysis.

3.2. Soil nutreint concentrations

Available phosphate (as indicated by IERB extractions) was significantly greater in treatment than in reference plots (Fig. 4D, $p = 0.046$). However, we detected no differences in NH_4^+, NO_3^-, DIN (dissolved inorganic nitrogen; $NH_4^+ + NO_3^-$) or Ca^{2+} between reference and treatment plots (Fig. 4).

3.3. Root chemistry

Treatment plots had significantly lower percent carbon in the roots (Fig. 5A), lower C:N ratios (Fig. 5B), and lower overall root C pools (Fig. 5C) than did reference plots ($p = 0.012$, $p = 0.045$, $p = 0.050$). Root percent N and total N stock in the roots did not differ between treatment and reference. There was no detectable difference in root biomass of maples, oaks or pine along the dissolved inorganic nitrogen DIN gradient as indicated ion exchange resin bag values (Fig. 6). No significant difference in root relative abundances were observed for any species along the N_{avail} gradient. We did not find support for the prediction that maple relative abundances will increase in girdled stands or under higher N_{avail}. In contrast, we found support for the opposite trend; maple relative abundance tended to decrease in girdled stands relative to treatment (Supplemental Table 2).

3.4. Fungal functional proportions

The most abundant functional group was saprotrophs, followed by EM fungi, Unknown/other taxa, plant pathogen, and AM (Table 2). The proportion of EM taxa tended to be lower in treatment plots than in reference plots (beta regression, $p = 0.013$). Ectomycorrhizal proportions also decreased significantly along NH_4^+ (Fig. 7A), and DIN (Fig. 7C) gradients ($p = 0.01$, $r^2 = 0.58$ and $p = 0.01$, $r^2 = 0.55$ respectively). EM proportions tended to decline as NO_3^- and PO_4^{3-} increased, but not significantly. Prediction 3 was supported as saprotroph proportions increased significantly with NH_4^+ availability (Fig. 7A, $p = 0.02$, $r^2 = 0.54$) and approached a significant relationship along the DIN gradient (Fig. 7C, $p = 0.07$). The mean proportion of saprobic fungi was ~10% higher in treatment (62.6%) than in reference (52.2) plots, and differed significantly between reference and treated plots (beta regression, $p = 0.044$). No trends were found between arbuscular-mycorrhizal abundances and any nutrient availability (Fig. 7). Contrary to predictions, we found a (non-significant) trend of lower AM relative abundances in girdled than in reference plots, similar to our observation for maple roots abundances.

Table 2
Proportion of sequences belonging to fungal functional groups by treatment. DNA sequences were matched using BLAST and assigned to a functional group based on life history. P-values are reported from a one tailed *t*-test with p-value < 0.05 reported as significant.

Proportion of Fungal Functional Groups by Treatment							
Functional Group	Treatment mean %	Treatment std %	Treatment n	Ref mean %	Ref std %	Ref n	p value
Ecto-mycorrhizal (EM)	26.38	8.7	5	39.19	6.26	5	0.058
Arbuscular Mycorrhizal (AM)	0.84	0.59	5	1.3	1.11	5	0.21
Saprotrophs (S)	62.63	10.0	5	52.15	6.3	5	0.1
Plant Pathogens (PP)	2.73	0.73	5	3.07	1.66	5	0.36
Other	7.42	3.66	5	4.28	1.15	5	0.09

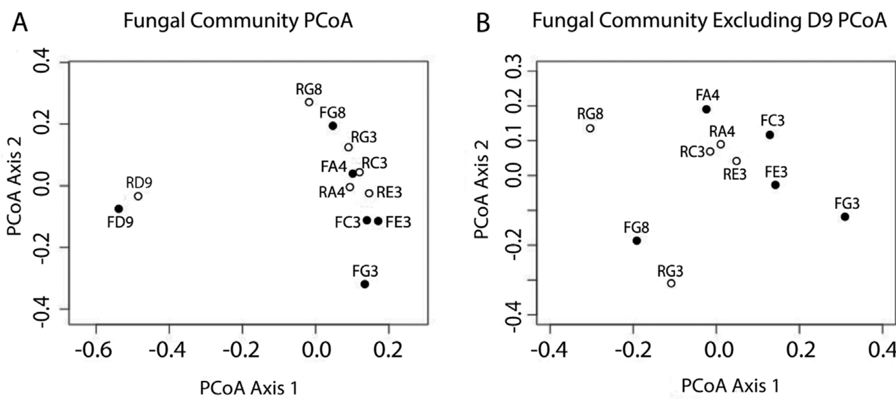

Fig. 2. Principal Coordinate Analyses for fungal communities. Community data are derived using Bray-Curtis distance matrices. Open circles are associated with reference plots. Closed circles represent treatment plots. A) Fungal community ordination with all 6 paired plots. The plot pair D9 cluster is distinct from the rest of the communities. B) Fungal community ordination without D9 plots.

4. Discussion

Previous FASET results showed differential responses in leaf area by species that likely reflect their shifting competitive responses following disturbance that removed early successional dominants. Relative to pre-girdled (2007) conditions, maple (*Acer rubrum* and *A. saccharum*) leaf area increased by 61% and oak (*Quercus rubra*) leaf area increased by 13% as of 2011 (Nave et al., 2011) following mature aspen and birch mortality. Additionally, increases in AM hyphal relative abundance were positively correlated with maple leaf production (Nave et al., 2013). Despite these previous findings we did not find support for increasing belowground maple root dominance at this site. As such, the prediction that maple proportions will increase in girdled stands was not supported. In contrast, we found a trend toward decreasing maple proportions ($p = 0.07$) compared to *Pinus spp*. (mostly eastern white pine, *P. strobus*) in treatment vs. reference paired plots (Supplemental table 2). This finding, coupled with previous results indicating an increase in leaf N concentration and leaf area (Nave et al., 2011), suggests an overall repartitioning of resources aboveground by maples as they responded to the aspen and birch die offs. In contrast, eastern white pine tended to increase in below ground root dominance at the site ($p = 0.02$) despite a trend towards decreasing EM proportions. Lower C:N ratios in bulked fine root samples in treatment than in reference plots suggest that fine roots may be turning over more rapidly (*sensu* Nadelhoffer, 2000) overall in the treated than reference plots. Lower C:N ratios have previously been associated with faster root turnover (Hendricks et al., 1993; Nadelhoffer, 2000; Nadelhoffer et al., 1985). Although increased root turnover was observed in girdled FASET stands (Nave et al., 2011), the ratio in this case is driven by decreases in root C concentration and thus could be due to more decomposed roots from girdled trees.

No discernible trends in root proportions from oak or pine were detected along any nutrient availability gradient despite previous finding of decreasing overall root biomass with increasing nitrogen (Nave et al., 2014). Relative abundances of maple sequences did tend to decrease with increasing N_{avail}, again suggesting a repartitioning of resources aboveground by maples, but the trend was not significant (Fig. 6). Moreover, total root biomass was not affected by girdling. While the lack of a significant result in the present study is not interpretable, this contrast with prior published findings is an important point for consideration.

We found no support for our fourth prediction that AM relative abundances will increase with succession; however, a slight decrease in AM proportions was detected in girdled plots. Maples are the only canopy level species that have AM associations at our site, and were shown to have increased in leaf area more than other species (oak and pine) that are replacing aspen and birch trees at our site (Gough et al., 2013). However, there may not have been an increase in AM abundance along a nutrient gradient because the trees can acquire the nutrients needed to sustain growth as soil nutrients become more readily available (Egerton-Warburton and Allen, 2009; Smith et al., 2009). Tree species may invest in short-lived roots acquiring nutrients rather than an investment of carbohydrates into a fungal symbiont. Our results offer evidence to support this, as there is a trend toward less maple and AM presence in treatment stands.

Although overall fungal diversity did not differ between treated (girdled) and reference (non-girdled) plots, fewer EM fungal taxa were detected in the girdled plots (Table 2). We also found a negative relationship between the proportion of EM and NH_4^+ and DIN (Fig. 7AC), confirming our second prediction. Nave et al. (2014) found in this same system that the N cycle is shifting from an NH_4^+ dominated to a NO_3^- dominated system. Given prior research on N_{avail} and successional

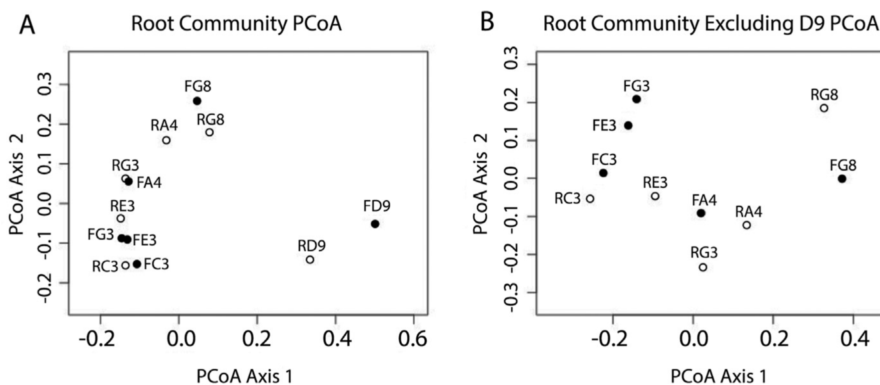

Fig. 3. Principal Coordinate Analyses for fungal communities. Results are calculated using Bray-Curtis distance matrices. Open circles are associated with reference plots. Closed circles represent treatment plots. A) Root community ordination with all 6 paired plots. The plot pair D9 cluster is distinct from the rest of the communities. B) Root community ordination excluding plot pair D9.

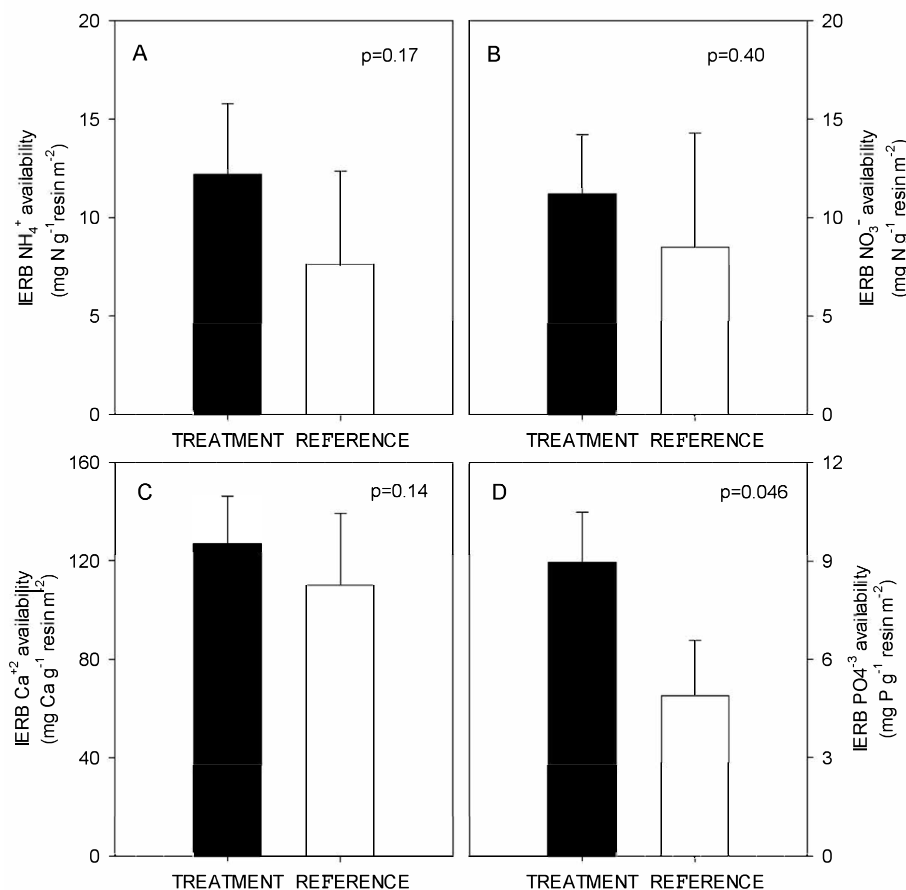

Fig. 4. Ion exchange resin bag (IERB) nutrient availability in paired TREATMENT/REFERENCE plots. For each analyte and treatment, the bar indicates the mean and standard deviation of n = 5 plots; p values are for paired *t*-test comparisons of paired TREATMENT/REFERENCE plots.

stages and our data indicating a negative relationship between NH_4^+ and EM fungi, we expect EM fungi presence to decline with ongoing successional birch and aspen mortality in the aging reference forest. Although EM proportions declined, relative proportions of eastern white pine tended to increase in girdled stands. A reduction in EM abundance and potential increase in pine root production could lead to a shift in saprobic communities, if they are released from competition with EM and provided a nutrient rich resource as roots turnover (Averill et al., 2014; Hendricks et al., 1993). Additionally, EM fungi may produce organic N degrading enzymes allowing for greater access to and increased uptake organic N (Näsholm et al., 1998; Read and Perez-Moreno, 2003) which could place them in direct competition with saprotrophs for N (Averill et al., 2014). As EM fungi decline in their abundance, this could reduce competition between EM fungi and saprotrophs accordingly, due to EM ability to take up both organic and inorganic N.

Our results also showed a non-significant trend of saprobic fungal increase in treated plots, with ~7% more saprobic abundance in fungal communities of girdled than in communities of reference stands. We did find support for the hypothesis that the proportion of saprotrophs in the fungal community increase with N availability as evidenced by the positive relationship between the proportion of the fungal community that is saprobic and soil NH_4^+, and by the positive trend between saprotrophs and DIN (Fig. 7C). Saprotrophs could increase in the late successional stands for several reasons. First, the successional stage of a forest is often indicated by the proportion of dead trees in a stand (Nave et al., 2014). As masses of dying and dead trees increase with stand age, more substrate becomes available for saprotrophs to utilize (Gough et al., 2007; Sturtevant et al., 1997). Historically, stand carbon accumulation has been predicted to increase as forests increase in age through a peak at mid-successional stages (Odum, 1969). Recent research, however, suggests that forests continue to increase in carbon uptake into later successional stages (Gough et al., 2013). In deciduous forests this leads to increases in leaf and woody litter inputs to soils, thereby providing more substrate for saprotrophs. Across a nearby 87-year experimental chronosequence at UMBS litterfall traps reveal over two-fold (2.2) increases in leaf litter fall from the youngest to oldest stands (Auclerc, *unpublished*).

Increasing root turnover in older stands could also provide rapidly dying, N-rich root resources for saprobes. We did not measure root turnover directly, however the results of lower C concentrations in the roots, narrower root C:N ratios and overall lower root C stocks in the treatment plots (Fig 5) are consistent with what would be expected if faster root turnover were occurring as a result of our experimental disturbance. Additionally, previous work found that root N concentrations increased in treatment plots relative to references (Gough et al.,

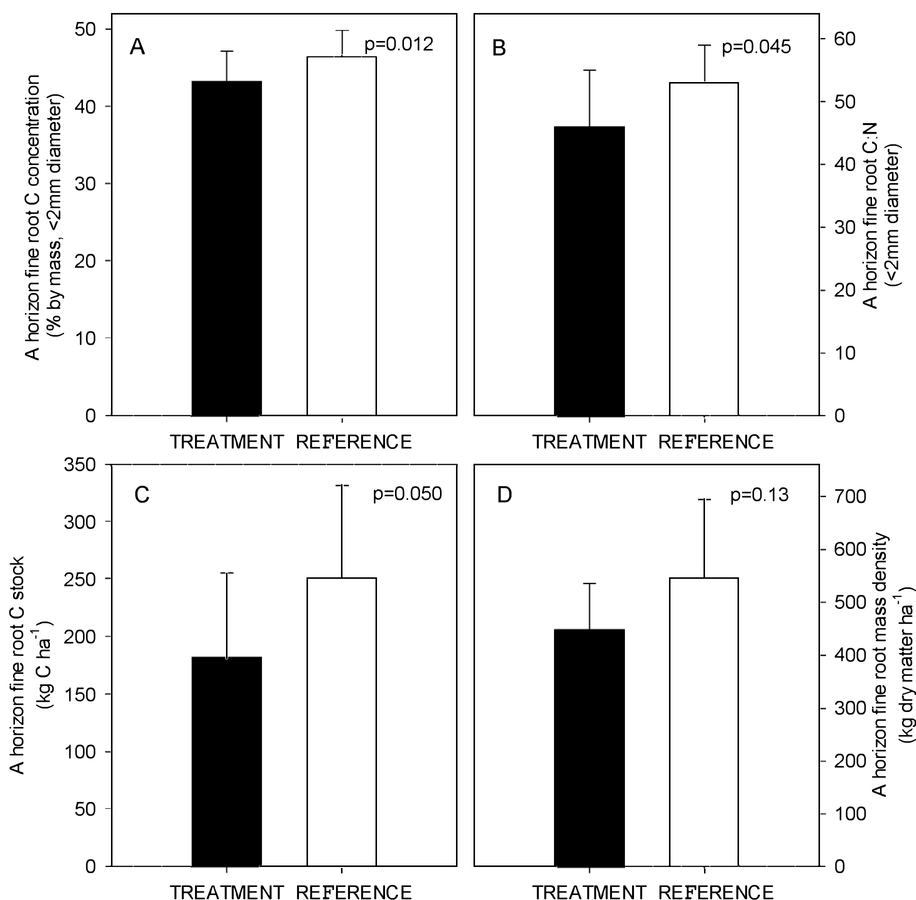

Fig. 5. A horizon fine root tissue chemistry, C stock, and mass density in paired TREATMENT/REFERENCE plots. For each panel, the bar indicates the mean and standard deviation of n = 5 plots; p values are for paired *t*-test comparisons of paired TREAETMENT/REFERENCE plots.

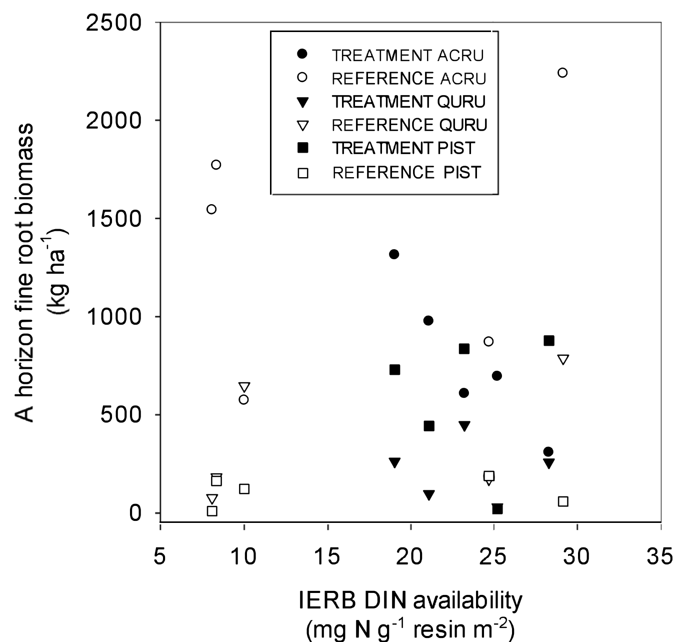

Fig. 6. Regression of A horizon fine root biomass and ion exchange resin bag inorganic N availability across plot pairs. Points plotted show plot-level mean values for root biomass (by tree species) and IERB DIN availability; filled points represent treatment plots and open points represent reference (non-girdled) plots. Tree species codes are ACRU (*Acer rubrum*), QURU (*Quercus rubra*), and PIST (*Pinus strobus*).

2013). Previous research using ecosystem N budgets to estimate fine root production showed positive relationships among litterfall, belowground C and N allocation to fine roots, and annual net primary production (Nadelhoffer and Raich, 1992), which could provide increasing substrate for decomposers. Increased N availability, being linked with increased root turnover and net primary production, coupled with a decrease in EM fungi that compete directly with decomposers, could create favorable environments for saprobic fungi. A release in competition due to lower EM, coupled with an increase in substrate (i.e. dead trees, increased leaf litter and increased root turnover) for saprotrophs, could stimulate saprobic activity and thereby impact the amount of carbon stored in soils (Averill et al., 2014; Orwin et al., 2011).

4.1. Conclusion

Four years after experimental stem girdling of all birch and aspen trees in a mid-successional north temperate mixed forest, overall fungal diversity indices did not differ between experimental and reference stands with no stem girdling. However, we found evidence of shifts in fungal functional groups, as there were significantly fewer EM fungi in girdled than in reference plots and greater proportions of saprobic fungi in girdled than in reference plots. Our results suggest that as forests at our site and similar forests across the northeastern United States mature, relative abundances of mycorrhizal fungi in soil fungal communities in these forests will decrease while saprobic fungal abundances increase. Our study shows the propensity for disturbances in forested ecosystems to drive shifts in fungal community composition both taxonomically and functionally as nutrient availabilities change.

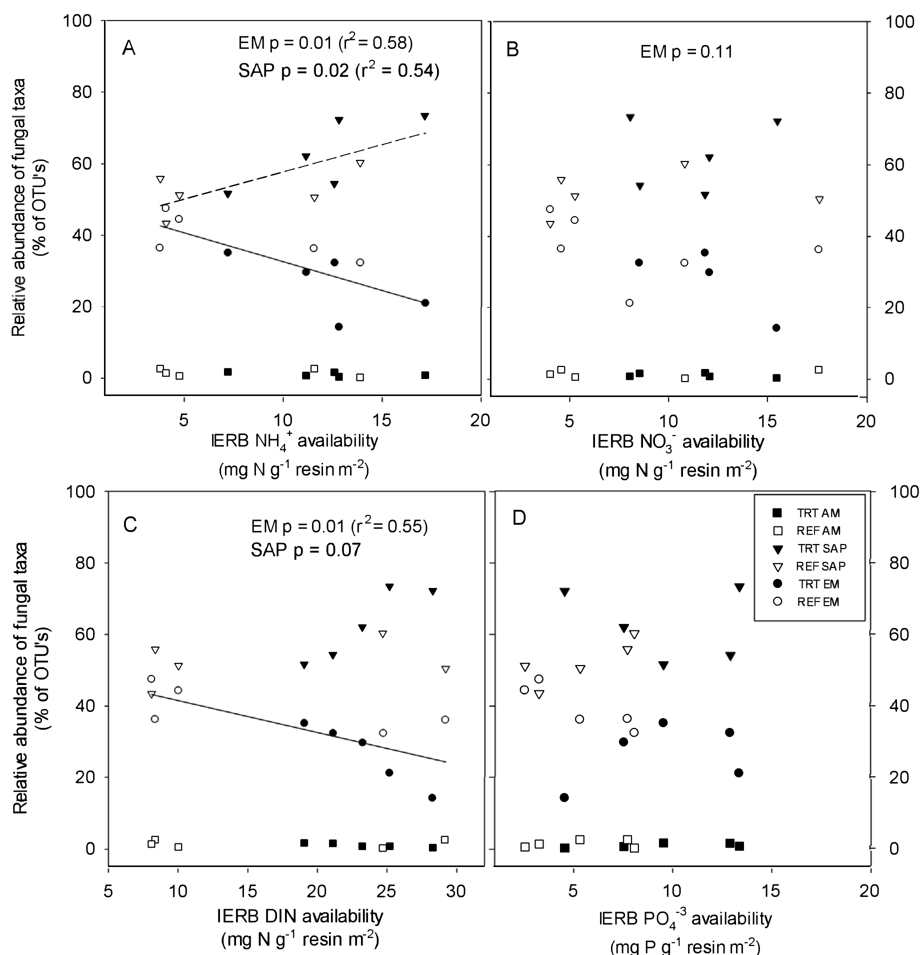

Fig. 7. Distributions of IERB-available nutrients and the relative proportions of fungal functional groups, across treatment and reference plots. Each point represents a plot, in which the IERB-available nutrient concentration is the mean of n = 4 IERBs and the mean proportion of OTUs in each fungal functional group across n = 5 soil samples. A) Ectomycorrhizal proportions decreased significantly ($p = 0.01$, $r^2 = 0.58$, solid line) and saprotrophic proportions increased significantly ($p = 0.02$, $r^2 = 0.54$, dashed line) with increasing NH4+ availability. B) Functional groups vs. NO_3^- showed no significant trends in fungal proportions and N availability. C) Ectomycorrhizal proportions decreased with an increase in DIN ($p = 0.01$, $r^2 = 0.55$) and saprotrophs showed a trend of increasing proportions with DIN ($p = 0.07$). D) Fungal functional groups vs. PO_4^{3-} showed no significant trends.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2018.06.022.

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