**Differential Responses of Total and Active Soil Microbial Communities to Long-Term
Experimental N Deposition**

Zachary B. Freedman^a (zacf@umich.edu)

Karl J. Romanowicz^a (kjromano@umich.edu)

Rima Upchurch^a (rimaup@umich.edu)

Donald R. Zak^{a,b} (drzak@umich.edu)

^aSchool of Natural Resources & Environment, and ^bDepartment of Ecology and Evolution

University of Michigan, Ann Arbor, Michigan, USA 48109

#Corresponding author

Name: Zachary Freedman

Address: 440 Church Street

Ann Arbor, MI 48109

e-mail: zacf@umich.edu

tel: (734)763-8003

fax: (734)763-8395

Abstract

The relationship between total and metabolically active soil microbial communities can provide insight into how these communities are impacted by environmental change, which may impact the flow of energy and cycling of nutrients in the future. For example, the anthropogenic release of biologically available N has dramatically increased over the last 150 years, which can alter the processes controlling C storage in terrestrial ecosystems. In a northern hardwood forest ecosystem located in Michigan, USA, nearly 20 years of experimentally increased atmospheric N deposition has reduced forest floor decay and increased soil C storage. A microbial mechanism underlies this response, as compositional changes in the soil microbial community have been concomitantly documented with these biogeochemical changes. Here, we co-extracted DNA and RNA from decaying leaf litter to determine if experimental atmospheric N deposition has lowered the diversity and altered the composition of the whole communities of bacteria and fungi (*i.e.*, DNA-based) and well as its active members (*i.e.*, RNA-based). In our experiment, experimental N deposition did not affect the composition, diversity, or richness of the total forest floor fungal community, but did lower the diversity (-8%), as well as altered the composition of the active fungal community. In contrast, neither the total nor active forest floor bacterial community was significantly affected by experimental N deposition. Our results suggest that future rates of atmospheric N deposition can fundamentally alter the organization of the saprotrophic soil fungal community, key mediators of C cycling in terrestrial environments.

Keywords: N Deposition / microbial seed bank / saprotrophic microbes

1. Introduction

Our understanding of microbial communities is largely derived from environmental DNA; however, ~80% of cells, and ~50% of operational taxonomic units (OTUs) in soil may be inactive (Lennon and Jones, 2011). This “microbial seed bank” represents a reservoir of genetic diversity that could shape community composition in response to a changing environment. RNA has a much shorter turnover than DNA and can therefore serve as a tool to gain insight into active microbial community (Moeseneder et al., 2005). In soil, the subset of the microbial community that is putatively active at the time of sampling can be compositionally different than the DNA “seed bank”, and it may respond differently to environmental stress (McMahon et al., 2011; Baldrian et al., 2012; Barnard et al., 2013). Furthermore, fluctuations in active microbial community composition can disproportionally contribute to microbial community dynamics, as well as mediate essential ecosystem functions (Shade et al., 2014; Aanderud et al., 2015). The importance of the relationship between the composition of the total microbial community, and the subset that is putatively active, and how this balance can be affected by environmental change is providing novel insight into the dynamics of microbial communities (Lennon and Jones, 2011; Baldrian et al., 2012; Zhang et al., 2014), and is the focus of this study.

Anthropogenic modification of global biogeochemical cycles may be exacerbated by changes in the composition and structure of microbial communities (Falkowski et al., 2008; Zhao et al., 2014). For example, across much of the Northern Hemisphere, atmospheric deposition of biologically available nitrogen (N) has increased by an order of magnitude over the past 150 years (*e.g.*, from 0.5-1 to 15-20 kg N ha⁻¹y⁻¹), with this trend expected to continue through the next century (Galloway et al., 2004; Torseth et al., 2012). For twenty years, we

have applied chronic experimental N deposition ($30 \text{ kg N ha}^{-1} \text{ y}^{-1}$) to replicate northern hardwood forest stands at a rate expected in some locations by mid-century (Table 1 and Figure S1; Galloway et al., 2004). To date, experimental N deposition has increased net primary productivity (NPP) of wood, reduced litter decay, and has fostered soil organic matter (SOM) accumulation and dissolved organic carbon (DOC) leaching (Pregitzer et al., 2008; Zak et al., 2008). Experimental N deposition has also altered the composition of fungi (Edwards et al., 2011; Entwistle et al., 2013) and bacteria (Eisenlord and Zak, 2010; Freedman and Zak, 2014; Freedman and Zak, 2015b) in the forest floor. Furthermore, experimental N deposition has decreased the abundance and richness of functional genes mediating soil C and N cycling processes (Eisenlord et al., 2013; Freedman et al., 2013), decreased fungal lignocellulolytic gene expression, and increased the abundance of saprophytic bacteria that mediate the decay of polyphenolic compounds (Edwards et al., 2011; Freedman and Zak, 2014). These molecular-level responses appear to be linked to dramatic biogeochemical changes in soil that have fostered greater soil C storage (+10%), which we argue will lead to a potential mechanism reducing the accumulation of anthropogenic CO_2 in the Earth's atmosphere. However, we do not know whether they arise from a change in overall community composition or a change in the organisms actively metabolizing plant detritus into soil organic matter.

Soil fungi and bacteria exhibit different community dynamics (Prewitt et al., 2014), thus, to understand soil ecosystem processes it is essential to address the fungal and bacterial community simultaneously (Baldrian et al., 2012). Here, we sought to determine if experimental N deposition altered the composition of the total (DNA-based) and active (RNA-based) fungal and bacterial communities on decaying forest floor. We refer to the microbial

community as “saprotrophic” because we expect the majority of the organisms living in the Oe/Oa horizon are sustained by the metabolism of decaying organic matter. It is plausible that chronic N deposition can alter the composition of the total microbial community, as well as the subset of the community that are metabolically active, which may elicit a functional response consistent with our biogeochemical observations. To address our objectives, we i) determined the lignocellulolytic decay potential of the forest floor microbial community by quantifying the activity of two enzymes, cellobiohydrolase and peroxidase, that mediate the decay of common plant cell wall compounds, cellulose and lignin, respectively and ii) co-extracted genomic DNA and ribosomal RNA from forest floor, from which, we amplified and sequenced fungal 28S and bacterial 16S rRNA genes.

2. Materials and Methods

2. 1 Site description and sample collection

We investigated the influence of experimental N deposition on the total (DNA-based) and active (RNA-based) saprotrophic microbial community in four northern hardwood forest stands in Lower and Upper Michigan, USA (Table 1; Figure S1). The stands span the north-south geographic range of the northern hardwood forests in the Great Lakes region (Braun, 1950) and lie along a 500-km climatic and atmospheric N deposition gradient. All sites are floristically and edaphically similar and are dominated by sugar maple (*Acer saccharum* Marsh.). The thin Oi horizon is comprised of sugar maple leaf litter and the Oe/Oa horizons are interpenetrated by a dense root mat. The soils are sandy (85-90%), well-drained, isotic, frigid Typic Haplorthods of the Kalkaska series. Six 30-m by 30-m plots were established at each stand in 1994; three receive ambient N deposition and three receive experimental N deposition. Experimental N

deposition consists of NaNO_3 pellets broadcast over the forest floor in six equal applications during the growing season ($30 \text{ kg N ha}^{-1} \text{ y}^{-1}$); NO_3^- comprises ~60% of atmospheric N deposition (wet plus dry) in our study sites (Zak et al., 2008; Barnard et al., 2013).

Forest floor sampling occurred in late May to early June 2013. In this way, samples from all four sites occurred during a phenologically-similar period, a time at which ample moisture supports high rates of microbial activity. Within each 30-m-by-30-m plot, 10 random 0.1-m-by-0.1-m forest floor samples (Oa/Oe horizons) were collected by hand after removing the freshly fallen Oi horizon. All samples were composited within each plot and homogenized by hand in the field. A portion of the homogenized sample was immediately flash frozen on liquid N_2 for nucleic acid extraction and the remainder was kept on ice for enzyme analyses. Samples were transported to the University of Michigan within 48 hours of sampling, where they were stored at -4 or -80 °C for enzyme analysis and nucleic acid extraction, respectively. Enzyme analysis and nucleic acid extractions were initiated 72 hours after sampling.

2.2. Enzyme analysis

To determine if experimental N deposition reduced the lignocellulytic potential of the forest floor microbial community, we measured the activity potential of cellobiohydrolase (EC 3.2.1.91) and peroxidase (EC 1.11.1.7), extracellular enzymes that catalyze the degradation of two common plant litter compounds, cellulose and lignin, respectively. Enzyme assays were performed according to Saiya-Cork *et al.*, (2002). Briefly, cellobiohydrolase activity was determined using a fluorometric assay, in which methylumbelliferone-linked cellobioside was used as the substrate. We measured peroxidase activity after incubating the samples for 18 h at 25 °C using L-dihydroxyphenylalanine (L-DOPA). The fluorescence or optical density of the

oxidized reaction product was measured in 96-well plates using a Synergy HT microplate reader (Gen5, version 2.00.18, BioTek, Winooski, VT, USA). Cellobiohydrolase and peroxidase activity potential was expressed relative to the dry weight of forest floor, and defined as units, wherein 1 unit = 1 $\mu\text{m h}^{-1}$ of the oxidized reaction product. The effects of location, experimental atmospheric N deposition, and their combined interaction on cellobiohydrolase and peroxidase activity potential was determined by analysis of variance (ANOVA); means were compared with a protected Fisher's LSD (SPSS Statistics, Version 20, IBM Corp., Armonk, NY, USA).

2.3 Nucleic acid co-extraction and first-strand cDNA synthesis

Total nucleic acids were co-extracted in triplicate from 0.30 g (total fresh weight) of forest floor samples using a PowerLyzer PowerSoil DNA isolation kit with a PowerLyzer 24 homogenizer (MoBio Laboratories, Carlsbad, CA). The following protocol enabled us to co-extract DNA and RNA from forest floor. We modified the manufacturer's protocol by the initial addition of 250 μL phenol:chloroform:isoamyl alcohol (25:24:1; pH 6.7); bead beating at 4,000 rpm for 45 seconds; centrifugation at 4 °C; and overnight ethanol precipitation at -20 °C with linear acrylamide (20 $\mu\text{g/mL}$ final concentration). Extracted total nucleic acids were purified using a PowerClean DNA Cleanup kit (MoBio), and subsequently split into separate genomic DNA (gDNA) and RNA fractions. To prevent DNA contamination of the RNA sample, the RTS DNase kit (MoBio) was used. Following DNase treatment, the purified RNA fraction was reverse transcribed to complimentary DNA (cDNA) using the SuperScript III First-Strand Synthesis System for RT-PCR (Life Technologies, Grand Island, NY) with the universal fungal 28S rRNA gene primer LR3 (Vilgalys and Hester, 1990), and the universal bacterial 16S rRNA gene primer

27F (Lane, 1991). The RT-reaction master mix and thermocycling conditions followed manufacturer's protocols.

2.4 PCR amplification and high-throughput sequencing

PCR reactions were performed using the Expand High Fidelity PCR system (Roche, Indianapolis, IN) on a Mastercycler ProS thermocycler (Eppendorf, Hauppauge, NY). Barcoded universal fungal primers LROR and LR3 (Vilgalys and Hester, 1990) were used to amplify a ~700 bp region of the fungal 28S rRNA gene (D1 domain) from both the gDNA (total community) and cDNA (active community) pools. The master mix included 1× PCR Buffer, 400 μM primers, 0.01 mg BSA, 200 μM dNTP, and 2 U *Taq* polymerase per reaction. PCR conditions included an initial denaturation stage of 95 °C for 5 min followed by 20 cycles of 95 °C for 30 s, 54 °C for 30 s, and 75 s at 72 °C, with final extension at 72 °C for 7 min. Barcoded universal bacterial primers 27F and 519R (Lane, 1991) were used to amplify a ~500 bp portion of the 16S rRNA gene (V1-to-V3 region) from both the gDNA and cDNA pools from each plot. The master mix included 1× PCR Buffer, 400 μM primers, 1.5 mM MgCl₂, 200 μM dNTP, and 2 U *Taq* polymerase per reaction. PCR conditions included an initial denaturation stage of 95°C for 10 min followed by 25 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 20 min. All PCR amplifications were performed in triplicate and were pooled prior to purification.

PCR amplicons were purified using a MinElute PCR purification kit (Qiagen, Valencia, CA). Purified amplicons were pooled in equimolar concentrations within each plot and were sequenced on a PacBio-RS II system (Pacific Biosciences, Menlo Park, CA) using C4 chemistry and standard protocols (Eid et al., 2009). Barcoded PCR amplicons from four experimental plots were pooled on a SMRT cell and were sequenced using PacBio circular consensus technology.

2.5 DNA Sequence Processing and Microbial Community Structure

Fastq files used in this analysis have been deposited to NCBI under project accession numbers SRR1944476 and SRR1944477. Circular consensus sequences in fastq format were obtained using the pbh5tools package (Pacific Biosciences) and downstream processing was performed in mothur (Version 1.32.0; Schloss et al., 2009). Initial quality control measures removed any sequence with a consensus fold-coverage < 5, average quality score < 25 (50 bp rolling window), anomalous length (outside the range of 650 ± 50 bp and 500 ± 50 bp for fungal and bacterial assemblages, respectively), an ambiguous base, > 8 homopolymers, or a > 1 bp mismatch to either the barcode or primer (Freedman and Zak, 2015a). The remaining high-quality sequences were aligned with the Ribosomal Database Project (RDP) LSU training set (Version 7; Cole et al., 2014) for fungal 28S rRNA genes or the Greengenes database (Version 13.5; DeSantis et al., 2006) for bacterial 16S rRNA genes. Alignments were performed using k-mer searching (octamers) with Needleman-Wunsch global, pairwise alignment methods (Needleman and Wunsch, 1970). All sequences were then checked for chimeras using UCHIME (Edgar et al., 2011) and were taxonomically assigned using a Bayesian classifier (Wang et al., 2007) with a bootstrap cutoff of 80% against the RDP or Greengenes database for 28S and 16S rRNA gene assemblages, respectively.

Operational taxonomic units (OTUs) were generated using the average neighbor algorithm at 99% and 97% sequence similarity for fungal and bacterial assemblages, respectively. In this way, we investigated how experimental N deposition has altered the structure of the total and putatively active fungal and bacterial communities at a taxonomically similar level (*i.e.*, species). DNA sequences from the DNA and RNA-based communities were

clustered simultaneously into OTUs, thereby allowing OTU classifications to be shared between the total and active communities. OTU abundances were square root transformed to lessen the emphasis of the most abundant species in all downstream analyses.

Calculation of alpha- and beta-diversity indices and all subsequent analysis were executed in mothur and Primer (version 6, Primer-E Ltd., Plymouth, UK). A Bray-Curtis dissimilarity matrix (Legendre and Legendre, 1998) based on square-root transformed OTU abundance was generated, from which ordinations were obtained from principal coordinate analysis (PCoA). The significance of compositional differences between bacterial and fungal assemblages exposed to ambient and experimental N deposition were determined by permutational multivariate analysis of variance (PerMANOVA; Anderson, 2001) with assemblage (*i.e.*, total or active community) and site as factors. To determine if observed shifts result from differences in assemblage location or heterogeneity in multivariate space, a distance-based test for homogeneity of multivariate dispersions (PERMDISP; Anderson, 2004) was used. Additionally, differences in composition between the total and active communities were assessed by two-way PerMANOVA and PERMDISP with assemblage (*i.e.*, active or total community) and site as factors. Contributions of fungal or bacterial taxa to the total dissimilarity between ambient and experimental N deposition were determined using Similarity Percentage analysis (SIMPER; Clarke et al., 1993).

The effects of location, experimental N deposition, and their combined interaction on fungal and bacterial assemblage diversity (Shannon Index; H' ; Shannon and Weaver, 1963), and richness (Chao1 estimator; Chao, 1984) were determined by two-way analysis of variance (ANOVA); means were compared with a protected Fisher's LSD (SPSS Statistics, Version 20, IBM

Corp., Armonk, NY, USA). Additionally, differences in diversity and richness between the total and active communities were assessed by two-way ANOVA and Fisher's LSD with assemblage and site as factors.

3. Results

3.1 Extracellular Enzyme Activity

In the forest floor, peroxidase (-50%; $P = 0.01$) and cellobiohydrolase (-52%; $P = 0.02$) activities were substantively lower under experimental N deposition and varied across the four sites ($P < 0.01$; Figure 1). We observed no significant site by treatment interactions, indicating that experimental N deposition negatively affected cellobiohydrolase and peroxidase activity across sites.

3.2 Responses of total and active microbial taxa to experimental N deposition

After quality filtering, 209,530 28S rRNA gene sequences (DNA-based plus RNA-based) were distributed among 66,477 OTUs (99% similarity). Additionally, 288,285 16S rRNA gene sequences were distributed among 93,148 OTUs (97% similarity). For downstream analyses, all fungal and bacterial gene assemblages were rarefied to 2,200 sequences per plot. Bacterial and fungal communities were considered as either total (DNA-based) or active (RNA-based) communities.

The forest floor fungal community was dominated by OTUs attributable to the classes Leotiomyces (~35%), Sordariomyces (~17%) and Agaricomycetes (~17%; Figure S2). Several fungal classes were differentially abundant between the total and active community; however, this was not affected by the experimental N deposition treatment. For example, OTUs attributable to Leotiomyces were relatively less abundant in the active community as

239 compared to the total community under both ambient and experimental N deposition ($\sim 13\%$;
240 $P = 0.01$). Similarly, the relative abundance of Sordariomycetes was moderately lower in the
241 active community than the total community in both the ambient and N deposition treatments ($\sim 7\%$;
242 $P = 0.06$). Conversely, the relative abundance of Agaricomycetes, Microbotryomycetes
243 and Tremellomycetes were greater in the active community as compared to the total
244 community under both conditions ($P < 0.01$).

245 The effect of experimental N deposition on the relative abundance of fungal classes was
246 largely consistent between the total and active communities. In the total community, the
247 relative abundance of OTUs attributable to Leotiomyces was greater ($+4.6\%$; $P = 0.05$)
248 whereas the relative abundance of Dothideomycetes was lower (-1.9% ; $P = 0.01$) under
249 experimental N deposition. In the active community, the relative abundance of
250 Dothideomycetes (-2.2% , $P = 0.01$) was lower under experimental N deposition.

251 Bacterial communities in the forest floor were dominated by OTUs attributable to the
252 phyla Proteobacteria ($\sim 39\%$), Actinobacteria ($\sim 23\%$), Bacteroidetes ($\sim 12\%$), and Acidobacteria
253 ($\sim 11\%$; Figure S3). Similar to the fungi, some bacterial phyla were differentially abundant in the
254 total or active community, but this balance was not affected by experimental N deposition. For
255 example, Planctomycetes and Verrucomicrobia were more abundant in the active community
256 relative to the total community under both ambient and experimental N deposition ($\sim 2.0\%$; P
257 < 0.01). The relative abundance of bacterial phyla did not change between the ambient and
258 experimental N deposition conditions in both the total and active communities ($P > 0.15$).

3.3 Responses of total and active microbial community composition to experimental N deposition

The total fungal community was compositionally different from the active community under both ambient (Figure 2; Pseudo-F = 1.4; $P < 0.01$) and experimental N deposition (Pseudo-F = 1.5; $P < 0.01$). Both the total and active fungal community also differed in composition between sites (site effect; $P < 0.01$). The experimental N deposition treatment moderately altered the composition of the active fungal community (Pseudo-F = 1.1; $P = 0.06$), whereas, the total fungal community was unaffected by experimental N deposition (Pseudo-F = 1.0; $P = 0.21$). Among the active community, fungal assemblages under experimental N deposition were less heterogeneous than those exposed to ambient N deposition (ambient dispersion = 55.7 ± 0.7 , experimental N deposition dispersion = 58.1 ± 0.7 ; $P = 0.02$). The total fungal community heterogeneity did not differ between the ambient and experimental N deposition ($P = 0.42$). To determine the relative contribution of active fungal orders to compositional dissimilarity between ambient and experimental N deposition assemblages, SIMPER analysis was performed (Table 2). SIMPER determined that fungal orders Agaricales (8.2% of community dissimilarity), Helotiales (6.7%), Atheliales (6.5%), and Polyporales (5.5%) contributed the greatest to dissimilarity in the active community exposed to experimental N deposition.

Experimental N deposition did not cause a composition shift of bacterial assemblages in either the total (Figure 2; Pseudo-F = 1.0; $P = 0.35$) or active (Pseudo-F = 1.0; $P = 0.33$) communities. The total and active bacterial communities slightly differed in composition under the ambient condition (Pseudo-F = 1.1; $P = 0.07$), but there was no difference between total

and active communities under the experimental N deposition condition (Pseudo-F = 1.0; $P = 0.42$). Total and active bacterial assemblages differed across sites (site effect; $P < 0.01$).

3.4 Responses of total and active microbial diversity to experimental atmospheric N deposition

Fungal community richness (Chao1) was lower in the active than in the total community, and this was true under both ambient (-28% change from total community; $P = 0.05$; Table S2) and experimental N deposition (-40%; $P < 0.01$). Fungal diversity (H') was similar between the total and active communities under ambient and experimental N deposition ($P = 0.41$).

The experimental N deposition treatment did not affect the diversity or richness of the total fungal community (Figure 3 and Table S2; $P > 0.10$), and this response was similar across sites (site $\times$ treatment; $P > 0.60$). However, the active fungal community was significantly less diverse under experimental N deposition than in the ambient treatment (-6% change from ambient; $P < 0.01$). A similar response was observed for richness, although, sites responded to the experimental N deposition treatment in an independent manner (site $\times$ treatment interaction; $P = 0.01$). Active fungal communities were less rich under experimental N deposition in sites A and D (-57 and -42%, respectively; Fisher's LSD $P < 0.01$), whereas no response to experimental N deposition occurred in sites B and C ($P > 0.3$).

Bacterial diversity (H') and richness (Chao1) in the active community was less than that of the total community under both the ambient and experimental N deposition ($P < 0.01$; Table S2). The total bacterial community decreased in richness under experimental N deposition (-20% change from ambient; $P = 0.04$; Figure 3), and this change was consistent across sites (site $\times$ treatment; $P = 0.16$). This response was not observed in the active bacterial community ($P =$

0.28). Among both the total and active community, Shannon diversity (H') did not significantly change due to experimental N deposition ($P > 0.2$).

4. Discussion

4.1 A sustained biogeochemical response to experimental N deposition

Litter decomposition is complex process mediated by extracellular enzymes secreted by saprotrophic bacteria and fungi (Osono, 2007; Li et al., 2009; Ahmad et al., 2010; Edwards et al., 2011) and can be sensitive to N availability in the environment (Carreiro et al., 2000; Zak et al., 2008). In our long-term field study, experimental N deposition led to a decline in the activity of enzymes catalyze the degradation of two common plant litter compounds, cellulose and lignin, (DeForest et al., 2004; Waldrop et al., 2004; DeForest et al., 2005; Edwards et al., 2011); this trend continues to the present (Figure 1). In this study, cellobiohydrolase activity was lower under experimental N deposition as compared to the ambient treatment (Figure 1). It was previously demonstrated that experimental N deposition leads to lower cellobiohydrolase activity, albeit non-significantly (Edwards et al., 2011). Nearly two-decades of experimental N deposition has reduced litter decay in our long-term experiment, which has occurred concomitantly with reduced lignocellulolytic exo-enzyme activity potential (Zak et al., 2008). Here, we present evidence that a sustained decline in lignocellulolytic enzyme activity potential is consistent with increased soil C storage, which together suggests decreased microbial metabolism of plant litter and soil organic matter in our long-term field experiment.

4.2 Responses of fungal and bacterial total and active communities to future rates of N deposition

Changes in soil microbial communities are often associated with changes in the ecosystem processes they mediate (Strickland et al., 2009; Allison et al., 2013); thus, understanding mechanisms that affect microbial communities can be critical for understanding how ecosystem processes may respond to environmental change. In this experiment, experimental N deposition did not alter the total fungal community, but it did lead to a less diverse, compositionally different active fungal community, relative to the active community in the ambient treatment (Figures 2 & 3, Table S2). Arbuscular mycorrhizal and saprotrophic fungi, as well as fungal lignocellulolytic functional assemblages are sensitive to increased atmospheric N deposition (Edwards et al., 2011; van Diepen et al., 2011; Eisenlord et al., 2013; Entwistle et al., 2013). Nitrogen deposition also alters plant-fungi relationships (Dean et al., 2014). Furthermore, in forest soil, the active fungal community can differ from the total community (Baldrian et al., 2012), and similar dynamics have been observed among cellulolytic genes assemblages (Kellner et al., 2009). Our results indicate that forest floor fungal communities are fundamentally altered by elevated rates of N deposition, which may be one factor underlying the slowing of decay in our field experiment.

Previous observations revealed that experimental N deposition led to a taxonomically and phylogenetically distinct bacterial community, as well as a decline in the richness (~23%) of bacterial functional genes mediating C- and N-cycling processes (Eisenlord et al., 2013; Freedman et al., 2013; Freedman and Zak, 2014; Freedman and Zak, 2015b). However, in this study bacterial communities were not affected by experimental N deposition (Figure 2; $P = 0.35$

and 0.33 in total and active communities, respectively). We attempt to sample the microbial community in our long-term experiment interannually during a phenologically-similar period (*i.e.*, late-spring when ample soil moisture supports high rates of microbial activity). However, it is plausible that the bacterial response to future rates of N deposition, as well as other microbial responses to environmental change, may be seasonally or temporally dependent. Both bacterial and fungal communities exhibit seasonal community dynamics (Lipson and Schmidt, 2004; Bevivino et al., 2014; Voriskova et al., 2014; Matulich et al., 2015), as well as undergo successional changes during leaf decay (Torres et al., 2005; Chapman et al., 2013; Voriskova and Baldrian, 2013). To more definitively understand the microbial response to future rates of N deposition, we must gain a greater understanding of the seasonal and temporal robustness of the total and active microbial response to this pervasive agent of climate change.

In this study, the co-extraction of DNA and RNA from soil provided novel insight into the total and active fungal community response to this persistent agent of environmental change. Targeting both the total and active microbial communities to investigate how microbial composition and their ecosystem function (*i.e.*, decomposition) are affected by environmental change is crucial (Jones and Lennon, 2010; Baldrian et al., 2012; Wilhelm et al., 2014; Zhang et al., 2014). The co-extraction and PCR amplification of environmental DNA and RNA is conducive to unraveling complex dynamics of diverse microbial communities and may provide insight to how these communities respond to a changing environment. However, the concurrent analysis of rRNA and rRNA gene sequences also has its limitations; nucleic acid extraction, purification, reverse transcription and polymerase chain reaction (PCR) amplification are all potential

sources of biases (Lanzén et al., 2011; Blazewicz et al., 2013). The comparative, well-replicated approach to this study, as well as the taxonomic and multivariate similarity of total and active microbial communities (Figures 2, S2) indicates that our methods yielded consistent results.

4.3 Towards a microbial mechanism driving ecosystem C storage under future rates of N deposition

We documented molecular-level microbial community dynamics that underlie a biogeochemical response, as 20 years of experimental N deposition has reduced litter decay in our long-term experiment (Zak et al., 2008), indicative of a widespread phenomenon observed among terrestrial ecosystems exposed to simulated N deposition (Frey et al., 2004; Liu and Greaver, 2010; Maaroufi et al., 2015). Results presented here indicate that future rates of N deposition may not affect the composition of the saprotrophic fungal “seed bank” (*i.e.*, the total community), but can alter the subset of saprotrophic fungi that are active at a time of high microbial activity. Our observations add to a growing body of literature detailing ecosystem responses to long-term experimental N deposition in a northern hardwood ecosystem, wherein 20 years of experimental N deposition has increased total N in leaf litter, lowered fungal lignocellulolytic exoenzyme activity and fungal laccase expression, and favored bacteria with the genetic potential to degrade polyphenolic compounds, leading to incomplete lignin decay and increased SOM accumulation (Zak et al., 2008; Edwards et al., 2011; Freedman and Zak, 2014). Given the importance of saprotrophic fungi to litter decay (Valaskova et al., 2007; Osono et al., 2009; Snajdr et al., 2010), it is increasingly clear that a microbial mechanism is driving ecosystem C storage in our long-term field experiment.

A few key genotypes can play an important role for maintaining ecosystem functioning, thus, the autecology of microbial taxa can provide insight into how microbial communities will respond to a changing environment (Hallin et al., 2012). The greatest affect of our experimental N deposition treatment was on the active fungi. Specifically, the class Dothideomycetes emerged as potentially interesting because they i) increased in relative abundance in the active (~7%) versus the total (~5%) community, ii) were less abundant (~2%) under experimental N deposition, which was true in both the total and active communities, and iii) accounted for 3% of the dissimilarity between active fungal communities under the ambient and experimental N deposition condition (Table 2). Dothideomycetes is the largest class within the Ascomycota and encode almost 6,000 catabolic CAZyme catalytic domains; they also contain large variation in numbers of transposable elements between species (Ohm et al., 2012), and have been implicated in cell wall decay (Shrestha et al., 2011). Similarly, the Tremellomycetes were more abundant among the active community (~6%) than the total community (~2%), and in the active community were relatively less abundant under experimental N deposition than under the ambient condition (-0.3%; $P = 0.05$). Tremellomycetes are a class within the Basidiomycota that has been implicated in organic matter decay. Moreover, the orders Agaricales, Helotiales, and Atheliales together contributed 21% towards community dissimilarity in the active fungal community between ambient and experimental N deposition. The orders Agaricales and Atheliales are phylogenetically similar within the Basidiomycota and are closely associated with wood decay (Hibbett, 2006; Coelho et al., 2010; Floudas et al., 2015). Helotiales (Ascomycota phylum) are often found on decaying wood and have also been implicated in wood decay (Raja et al., 2008). Taken together, our results support the hypothesis that increased atmospheric N

deposition suppressed fungi implicated in lignocellulose decay, a plausible molecular mechanism underlying greater soil C storage in response to future rates of N deposition.

4.4 Conclusions

Twenty years of experimentally increased N deposition consistently and significantly altered the active saprotrophic fungal community in the forest floor, whereas the total fungal community was unchanged by this pervasive agent of climate change. The total bacterial community was less rich under experimental N deposition, but this response was not observed among the active community. The composition of the total and active bacterial communities was unaffected by experimental N deposition. From this, we suggest that chronic experimental N deposition has altered the composition of the active saprotrophic fungal community, whereas the soil bacterial community may be affected in a seasonally or temporally variable fashion. Results presented here supports the hypothesis that changes in the composition of active saprotrophic soil fungi is an important mechanism by which anthropogenic N deposition alters the cycling and storage of C in soil.

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655

Titles and Legends to Figures

Figure 1: The effect of experimental N deposition on cellobiohydrolase and peroxidase activity potential in forest floor. Values are expressed relative to the leaf litter dry weight. Mean $\pm$ standard error ($n = 12$) values are presented, eight analytical replicates were included.

* $P < 0.05$ by two-way ANOVA

Figure 2: The effect of experimental N deposition on the total and active fungal (A) and bacterial (B) communities. Ordinations were obtained from Principle Coordinates Analysis on based Bray-Curtis dissimilarity of OTU abundance tables. $P < 0.05$ by PerMANOVA

Figure 3: The effects of experimental N deposition on the total and active microbial community diversity (Shannon Index; H') and richness (Chao1 estimator). Values represent the mean $\pm$ standard error ($n = 12$) and are presented in Table S2. * $P < 0.05$ by ANOVA; Site $\times$ Treatment $P < 0.05$

Table 1

1 Table 1: Site, climatic, overstory, and ambient nitrogen deposition rates of four study sites
2 receiving experimental N additions.

Characteristic	Site A	Site B	Site C	Site D
Location				
Latitude (N)	46°52"	45°33"	44°23"	43°40"
Longitude (W)	88°53"	84°52"	85°50"	86°9"
Climate				
Mean annual temperature (°C)	4.8	6.1	6.5	7.7
Mean annual precipitation (cm)	91.9	93.3	92.8	86.6
Ambient N Deposition (Kg N ha ⁻¹ yr ⁻¹)	5.9	6.1	7.4	7.4
Vegetation				
Overstory biomass (Mg ha ⁻¹)	261	261	274	234
<i>Acer saccharum</i> biomass (Mg ha ⁻¹)	237	224	216	201
Environment				
<i>Leaf Litter (Oe/Oa horizons)</i>				
Litter C:N	63.7	57.1	52.9	43.4
Litter mass (g)	412	396	591	550
<i>Soil (0-10 cm)</i>				
Sand (%)	85	89	89	87
pH (1:1 soil/H ₂ O)	4.8	5.0	4.5	4.7
Base saturation, %	71	96	73	80

1 Table 2: Contribution of fungal orders to compositional dissimilarity as indicated by SIMPER
2 analysis.

Fungal Order	Average Dissimilarity	Diss./SD	% Contribution
Agaricales	2.26	1.34	8.15
Helotiales	1.85	1.29	6.68
Atheliales	1.8	1.42	6.49
Polyporales	1.53	0.71	5.51
unclassified	1.14	1.35	4.12
Diaporthales	1.13	1.17	4.07
Hypocreales	1.11	0.58	4.01
Sordariomycetes ^a	0.92	0.98	3.32
Cantharellales	0.92	1.04	3.32
Ascomycota ^a	0.9	1.23	3.23
Microbotryomycetes ^a	0.77	1.33	2.77
Dothideomycetes ^a	0.77	1.36	2.76
Tremellales	0.76	1.61	2.75
Pleosporales	0.74	1.41	2.66
Chaetothyriales	0.64	1.09	2.29

3 ^a *incertae sedis*

Figure 1

Figure 2

Figure 3