Effects of urbanization and nitrogen addition on ectomycorrhizal fungal communities

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Abstract

The relationship between ectomycorrhizal fungi (EMF) and their hosts represents an essential facet of multiple element cycles in terrestrial systems. Anthropogenic nitrogen deposition, however, disrupts the symbiosis between ectomycorrhizal fungi and their plant partners, restructuring the ectomycorrhizal fungal community. I investigated the effects of nitrogen addition and urbanization on ectomycorrhizal fungal communities along an urbanization and nitrogen addition gradient by characterizing the ectomycorrhizal community associated with Red oak (*Quercus rubra*) via DNA extraction and amplicon sequencing. With species data, exploration type data, and percent colonization, I calculated extramatrical mycelial biomass (EMM) along the urbanization and nitrogen addition gradients. My data indicate that EMF communities are restructured by both urbanization and nitrogen addition, and that EMM biomass generally declines with increasing soil nitrogen levels. This finding supports the hypothesis that the increase of accessible nutrient supply undermines the transactional relationship between EMF and their plant hosts.

Key Words

Ectomycorrhizal fungi, nitrogen deposition, urbanization, soil fungal communities, terrestrial carbon cycle, exploration type, EMM biomass.

Introduction

Mycorrhizal associations are central to the evolution and survival of terrestrial vascular plants (Brundrett 2002, Malloch et al. 1979). Mycorrhizal fungi enhance the ability of plants to take up relatively scarce, essential nutrients, such as phosphorus and nitrogen, in return for sugars from photosynthates. These nutrients are exchanged in the plant root via distinct, polymorphic mechanisms. Mycorrhizal associations also protect plants against pathogens and drought (Duchesne et al. 1987, Boyle and Hellenbrand 1991). The oldest bryophyte-like plants discovered in the Devonian fossil record showed evidence of a mycorrhizal symbiosis much like vesicular-arbuscular mycorrhizae, the oldest and most abundant form of mycorrhizae, even before roots, themselves, evolved (Brundrett 2002). Currently, only 20% of plant species are non-mycorrhizal (Tester et al. 1987).
Mycorrhizal fungi, in addition, are important mediators of terrestrial carbon cycling (Ekblad 2013). Plants transfer up to 10-20% of their photosynthate to mycorrhizal fungi (Treseder and Allen 2000). The fate of this flux of carbon underground and into mycorrhizal fungi is relatively understudied.

Ectomycorrhizal fungi, comprising 5000-6000 species (Agerer 2006), associate with about 2% of plant species (Tendersoo et al. 2010), specifically, trees and shrubs in temperate forests (Malloch et al. 1979). Ectomycorrhizal fungi represent an important, yet understudied, carbon sink because much of the biomass produced by these fungi, sourced from plant partners, is not readily decomposed, contributing substantially to soil organic matter (Treseder and Allen 2000). The parameters of ectomycorrhizal fungal production and turnover, however, are largely unknown.

Anthropogenic nitrogen deposition disrupts the symbiosis between ectomycorrhizal fungi and their plant partners, restructuring the ectomycorrhizal fungal community (Treseder and Allen 2000, Lilleskov et al. 2002). When large amounts of labile nitrogen are available to plants, ectomycorrhizal colonization and species richness often decreases and community composition changes dramatically (Treseder and Allen 2000, Lilleskov et al. 2002, Morrison et al. 2016). Species of ectomycorrhizal fungi grow at different rates, produce biomass of varying magnitudes that varies in recalcitrance, rendering their capacity for carbon sequestration community specific (Treseder and Allen 2000). As ectomycorrhizal fungal community composition changes in response to nitrogen deposition, its capacity and rate of carbon sequestration changes, altering the magnitude of this belowground carbon sink. Understanding how ectomycorrhizal fungal communities respond to nitrogen deposition, therefore, is central to predicting how this important carbon sink will react to accumulating nitrogen pollution (Treseder and Allen 2000).

Ectomycorrhizal fungi can be partitioned into distinct ecophysologies according to hyphal exploration type (Agerer 2001). Hyphal exploration type is characterized by the length of extramatrical, radiating hyphae (contact, short, medium, long) and texture (smooth, fringe, mat). Generally, hyphal exploration types are associated with nutrient foraging strategies. Long types, such as *Boletus*, are long-distance foragers, while short and contact types, like *Cenococcum* and some *Russulas*, respectively, gather nutrients adjacent to the root (Agerer 2001). Hyphal exploration types are not plastic, and are generally genus specific (Agerer 2001, Hobbie and...
Agerer 2010). It is important to note that only 5-10% of ectomycorrhizal fungal species have been classified according to exploration type (Wallander et al. 2013).

Weigt et al. (2012) generated standard values for extramatrical mycelial (EMM) biomass according to exploration type, given total ECM mantle length and percent colonization. Extramatrical mycelial biomass increases as hyphal exploration type lengthens. Given ectomycorrhizal species data, which yields relative abundance of exploration type, percent root tip colonization, and average mantle length, it is possible to estimate EMM biomass in an ecosystem.

I investigated the effects of nitrogen addition and urbanization on ectomycorrhizal fungal communities by characterizing the ectomycorrhizal community associated with Red oak (Quercus rubra) via DNA extraction and amplicon sequencing along a nitrogen amendment gradient and an urbanization gradient. With species data, exploration type data, and percent colonization, I calculated EMM biomass along the urbanization and nitrogen addition gradients.

As nitrogen deposition and urbanization intensifies, I expected dominant hyphal exploration type to shorten, and EEM biomass to decrease, decreasing fungal inputs to soil organic carbon.

**Materials and Methods**

Urbanization Gradient, Harvard Forest to Boston

The urbanization gradient was established and is maintained by Dr. Lucy Hutyra and Dr. Pamela Templer of Boston University (Rao et al. 2014). We selected four sites along this gradient. Two represent rural forests and two represent suburban-low urban forests.

Chronic Nitrogen Amendment Experiment, Harvard Forest

We used the three plots maintained for the Chronic Nitrogen Amendment Experiment at the Harvard Forest since 1988 – control, low-N (50 kg/ha/yr), and high-N (150 kg/ha/yr). These plots are treated with NH₄NO₃ six times each month. Morrison et al. (2016) investigated the effects of chronic nitrogen addition on soil fungal communities in these plots. I will compare these findings with my own.

Sample Collection
Throughout the urbanization gradient I randomly selected three individual Red oak (Quercus rubra) trees at each site that are roughly equidistant (60 m) from the area of disturbance to control for edge effect. I also collected soil cores from three randomly selected Red oak (Quercus rubra) trees in the nitrogen addition plots. Within 1 m of the trunk of each tree, I extracted three soil cores for each Red oak (Quercus rubra) tree, which are known to partner with ectomycorrhizal fungi, that extended 10 cm under the surface of the soil. I removed the top layer of litter to bias the sample against saprophytic fungi. I stored the soil cores at 4°C until further processing.

Sample Processing

I pooled and processed each tree-specific triplicate by dry sieving them through a 3 mm sieve that was rinsed with ethanol and deionized water between triplicates. I collected the fine roots and suspended them in deionized water. I homogenized the throughfall of each triplicate and extracted a soil subsample, according to the advice of Dr. Jie Wei and Joseph Vineis (Wei, Vineis pers. comm. 2018). I produced 21 soil subsamples.

DNA Extraction, Amplification, and Sequencing

I extracted DNA from the soil subsamples via the MO BIO DNeasy PowerSoil kit, following the manufacturer’s protocol. I tested the extracted DNA for content and quality with a NanoDrop. I then transferred my samples to Dr. Morrison at the Bay Paul Center for PCR amplification, using the ITS1 primer to selectively amplify fungi. Generally, the ITS1 primer alone is not sufficient to determine relative abundance of fungal taxa, yet I used it as a rough indicator of the soil fungal community. The amplified sequences were sequenced at Dr. Hillary Morrison’s lab at the Bay Paul Center using MiSeq Illumina Next-Gen sequencing. The sequences were run through VAMPS (The Visualization and Analysis of Microbial Population Structures, https://vamps.mbl.edu) yielding species data. I excluded all sequences not identified to the species level. I also excluded all operational taxonomic units (OTUs) under 1% relative abundance, which are generally regarded as unreliable indicators of actual relative abundance (Lundberg et al. 2012, Smith and Peay 2014). I used the distribution of OTUs determined to the species level as a proxy for the distribution of the greater ectomycorrhizal community.

Biomass Calculation
I calculated the average total number of root tips by extrapolating the average number of root tips per 2 cm root segment (n=20) to the total number of root tips per unit volume given total root length per unit volume, provided by Emma Conrad-Rooney. I calculated the number of colonized root tips by multiplying the site-specific rate of colonization, also provided by Emma Conrad-Rooney, by the average total number of root tips per unit volume. Given sequencing data, I assigned exploration type to my species data using multiple literature sources, including Agerer and Rambold (2004-2011: An information system for characterization and determination of ectomycorrhizae), and Tedersoo and Smith (2013). When I could not assign exploration type to the species level, I assigned it the exploration type associated with the genus (Tedersoo and Smith 2013). For the Russula and Lactarius genera, I assigned medium exploration type to unknown species, although exploration type varies across these genera. This assignment is imperfect, adversely affecting the validity of my biomass estimates, yet I would rather assign an exploration type that dominates most of the genus than exclude these prevalent species entirely. These estimates, therefore, are subject to change given species-specific determination of exploration type. I determined the relative abundance of each exploration type from these exploration type assignments and the relative abundance of each species per site. I multiplied each group of colonized tips by the average mantle length (6 mm) which I estimated from literature values (Agerer and Rambold, 2004-2011: An information system for characterization and determination of ectomycorrhizae). I calculated EMM biomass via the methods proposed by Weigt et al. (2012). I multiplied the total mantle length of each exploration type by the corresponding standard value that relates EMM biomass to mantle length. I then extrapolated EMM biomass per unit volume to a square meter at a depth of 10 cm, which I used to compare EMM biomass across sites.

**Results**

**Sequencing**

Out of all the sequences I received, about 60% were ectomycorrhizal (Fig. 1). Only 50% of the ectomycorrhizal fungal sequences were identified to species level. I identified 121 species of ectomycorrhizal fungi from 25 families. The most abundant families are Russulaceae, Amanitaceae, and Elaphomycetaceae, although only Russulaceae (~80%) was present throughout all the sites (Fig. 2).
Community

Species richness, determined from OTUs with relative abundance greater than 1%, like in Morrison et al. (2016), does not change over the urbanization gradient except for a slight decrease in SW, an urban site (Fig. 3). Species richness is lowest in the low nitrogen addition plot and highest in the control. Species richness is slightly decreased in the high nitrogen addition plot.

Evenness, or the relative distribution of OTUs (Hill 1973), is generally greater in the urban sites than the rural sites (Fig. 4). Evenness in the nitrogen addition plots is highest in the control plot, although only slightly higher than the high addition plot. Evenness in the low addition plot, however, is greatly reduced.

Shannon Weiner diversity, used in Morrison et al. (2016) to examine changes in diversity in the soil fungal community, is comparable between rural and urban sites, although HF1, a rural site, demonstrated particularly low diversity (Fig. 5). The control plot has the highest diversity over the nitrogen addition gradient, slightly higher than that of the high addition plot. The lowest diversity across both gradients occurs in the low nitrogen addition plot.

The nitrogen addition gradient yielded high relative abundance of Lactarius and Russula species, both of the Russulaceae family (Fig. 6). In particular, Lactarius imperceptus was dominant in the control and low addition plot, while Russula atropurpurea was dominant in the high addition plot.

Total Mantle Length

Total mantle length does not vary significantly over the urbanization gradient (Fig. 7). Mantle length decreases over the nitrogen addition gradient, reflecting changes in percent root tip colonization (Conrad-Rooney pers. comm. 2018).

Exploration Type Distribution

Medium exploration type dominates most of the sites across both gradients (Fig. 8). In the urban sites, however, HW exhibits about 40% of short distance EMF. The other urban site, however, is composed almost entirely of medium distance EMF. One of the rural sites also contained low levels of short distance EMF. The control and low nitrogen addition plots are
entirely dominated by medium distance EMF. The high nitrogen addition plot, however, is
dominated by contact EMF.

Biomass

Biomass of EMM is slightly greater in the rural sites than the urban sites (Fig. 9). Biomass
decreases across the nitrogen addition gradient, with a dramatic drop between the low
and high nitrogen addition sites.

I found a significant (P<0.05) negative relationship between nitrogen mineralization and
EMM biomass (r^2 = 0.5051) (Fig. 10). I found a significant (P<0.05) negative relationship
between soil nitrate and EMM biomass (r^2 = 0.8672) (Fig. 11).

There is a strong, positive, and significant (P<0.05) relationship between EMM biomass
and soil molar C:N (provided by Maggie Anderson) along the nitrogen addition gradient (r^2 =
0.9961) (Fig. 12). This dramatic relationship, however, is not replicated in the urbanization
gradient (r^2 = 0.0031).

Discussion

I used operational taxonomic units (OTUs) specified to species level that represented
more than 1% of the ectomycorrhizal OTUs as a proxy for the ectomycorrhizal fungal
communities throughout the gradients. Additionally, due to logistical restraints, I extracted DNA
from soil subsamples rather than pooled root samples. The species data, therefore, represents the
soil fungal community rather than the root fungal community. I did not capture the plant-
mediated filtering that occurs between the soil and the root fungal communities, although in the
nitrogen addition gradient it is likely insignificant (Morrison et al. 2016). Nevertheless, I treated
the resulting sequences as representative of the potential ectomycorrhizal fungal colonizers of
trees in mixed New England forests, much like Morrison et al. (2016).

My findings support the hypothesis that nitrogen addition restructures ectomycorrhizal
fungal communities. Although no distinct trend was found across the urbanization gradient
regarding species richness, evenness, or diversity, a pattern emerged in the nitrogen addition
plots. The low addition plot contained the lowest species richness, evenness, and diversity, due to
the dominance of one species of fungi, *Lactarius imperceptus*. In contrast, species richness,
evenness, and diversity were only slightly decreased in the high nitrogen addition plot. This finding is contrary to that published in Morrison et al. (2016), wherein richness was highest in the low nitrogen addition plot and lowest in the high addition plot, while diversity declined from the control plot to the high addition plot. Additionally, while Morrison et al. (2016) determined that *Russula vinacea* increased in abundance across the nitrogen addition gradient, I found that *Lactarius imperceptus* increased from the control to the low addition plot, while *Russula atropurpurea* dominated in the high addition plot. I did not find *Russula vinacea* in either of the gradients. This inconsistency could be due to my use of the ITS1 primer alone, or my exclusion of sequences that were identified at genus but not species level. If not, this finding prompts further investigation into the dynamics and plasticity of the EMF community following chronic nitrogen addition.

Dominant hyphal exploration type did not change over the urbanization gradient. Only in the high addition plot did the dominant exploration type switch from medium distance to contact type. This data support previous studies that have reported decline in medium distance types associated with nitrogen addition (Lilleskov et al. 2011). However, this finding must be treated with caution until all species of *Russula* and *Lactarius* captured in my data have been officially assigned to an exploration type.

It is important to note that my estimate of EMM biomass is weakened by the framework from which the standard values were conceived, wherein the relationship between mantle length and biomass was derived from cultures devoid of microbial competition and herbivory (Weigt et al. 2012). Additionally, many ectomycorrhizal fungal species reduce mycelial production in response to nitrogen addition (Bidartondo et al. 2001). Nevertheless, my findings are a preliminary indicator of changes in EMF mycelial biomass in response to urbanization and nitrogen addition.

My data indicate that EMM biomass generally declines with the increasing soil nitrate levels and nitrogen mineralization rates determined by Maggie Anderson. This finding supports the hypothesis that the increase of accessible nutrient supply undermines the transactional relationship between EMF and their plant hosts. To what extent these relationships are conserved, however, is of interest to me.
A striking pattern emerged in the nitrogen addition plots regarding soil C:N (Maggie Anderson) and EMM biomass. As C:N decreased, EMM biomass declined as well. This relationship, however, did not hold true over the urbanization gradient, perhaps due to confounding factors that render nitrogen addition distinct from that accompanying urbanization. While the relationship in the nitrogen addition plots is founded on generally insufficient data, this trend warrants further research.

The relationship between ectomycorrhizal fungi and their hosts represents an essential facet of multiple element cycles in terrestrial systems. Investigating how these organisms react to increasing anthropogenic stressors like nitrogen deposition, therefore, is essential to understanding how our terrestrial systems will be molded by increasingly pervasive anthropogenic disturbance.

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Literature Cited


Figure 1: Proportions of sequences identified as non-EMF (Not EMF), EMF without species identification (EMF w/o ID), and EMF identified to species level (EMF ID).
Figure 2: Average relative abundance of EMF family

- Russulaceae: 82%
- Amanitaceae: 9%
- Elaphomycetaceae: 6%
- Other: 3%
Figure 3: Species richness across gradients wherein NDC, NDL, and NDH are the control, low, and high nitrogen addition plots, respectively.
Figure 4: Evenness across gradients wherein NDC, NDL, and NDH are the control, low, and high nitrogen addition plots, respectively.
Figure 5: Shannon Weiner Diversity across gradients wherein NDC, NDL, and NDH are the control, low, and high nitrogen addition plots, respectively.
Figure 6: Relative abundance of two species across nitrogen addition gradient, wherein NDC, NDL, and NDH are the control, low, and high nitrogen addition plots, respectively.
Figure 7: Total mantle length per square meter to 10 cm depth across gradients wherein NDC, NDL, and NDH are the control, low, and high nitrogen addition plots, respectively.

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Figure 8: Total EMM biomass (kg/ha) across gradients wherein NDC, NDL, and NDH are the control, low, and high nitrogen addition plots, respectively
Figure 9: Distribution of exploration type (contact, short, medium) across gradients wherein HW and SW are urban sites, HF1 and HF2 are rural sites, and NDC, NDL, and NDH are the control, low, and high nitrogen addition plots, respectively.
Figure 10: Relationship between N Mineralization (µg N/µg soil per year) and EMM biomass (kg/ha) across gradients wherein HW and SW are urban sites, HF1 and HF2 are rural, and NDC, NDL, and NDH are the control, low, and high nitrogen addition plots, respectively.
Figure 11: Relationship between soil nitrate (µg N/g soil) and EMM biomass (kg/ha) across gradients wherein HW and SW are urban sites, HF1 and HF2 are rural, and NDC, NDL, and NDH are the control, low, and high nitrogen addition plots, respectively
Figure 12: Relationship between soil molar C:N and EMM biomass across nitrogen addition gradient wherein NDC, NDL, and NDH are the control, low, and high nitrogen addition plots, respectively.
Figure 13: Relationship between soil molar C:N and EMM biomass across the urbanization gradient wherein HW and SW are urban sites, and HF1 and HF2 are rural