Using MiSeq on DNA extracted from in-growth bags to observe ectomycorrhizal fungi with N, P and N+P additions in mixed hardwood forests at Bartlett Experimental Forest, New Hampshire

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INTRODUCTION

A number of studies have identified ectomycorrhizal fungi (EMF) that are important in N cycling, with some taking up inorganic N and others more important in taking up organic N (Lilleskov and Bruns 2001, Lilleskov et al. 2011). Far less is known about EMF and their uptake of phosphorus, which appears to be increasingly limited in many forest ecosystems.

In 2011 a large nutrient addition experiment began in Bartlett Experimental Forest in the White Mountains of New Hampshire (part of MELNHE - Multiple Element Limitation in Northern Hardwood Ecosystems; Fig. 1). Plots (10 x 10 meter) received N, P, N&P plus controls. While past studies have used sporocarp production or mycorrhizal roots to gauge the response of EMF, we sampled soil hyphae that grew into in-growth bags (Wallander et al. 2001) installed in plots. We extracted DNA from the in-growth bags and used the Illumina MiSeq approach to amplify fungal DNA from subsampled sand from in growth bag extracts.

METHODS

In growth bags were made by placing autoclaved sand into 10 x 10 cm bags made with 44µm nylon mesh (Plastok Co., UK) to allow hyphae, but not roots to grow into the bags. 132 bags were installed across three stands at Bartlett Experimental Forest (C1, C2, C4) that were harvested from 1978 to 1990 (Fig. 1). Stands contain a mix of American beech, yellow birch, white birch, balsam fir, red oak, red spruce, hemlock, sugar maple, red maple, striped maple, big tooth aspen, pin cherry and white. Each stand has four plots treated with N (30 kg N/ha/yr as NH_4NO_3), P (10 kg P/ha/yr as NaH_2PO_4), both N and P, and control, added annually since spring 2011. These relatively modest rates are designed to alter site fertility while minimizing artifacts associated with high doses of fertilizer.

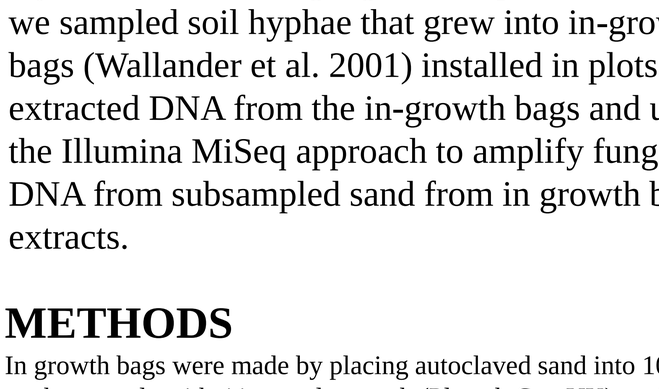

Figure 1: MELNHE site map at Bartlett Experimental Forest, in the White Mountains, New Hampshire, USA. We used plots treated with N, P, N+P, and Controls in stands C1, C2 and C4. Date of the last clearcut is shown with each stand.

Generation of DNA Sequences and Bioinformatics

DNA was extracted from in growth sand bags with with a Dneasy PowerSoil Kit (Qiagen, Inc.). One control sample was generated using the Powersoil kit on sand packaged in the sand bag but left in the lab. A second control sample was generated as a mock community using PCR amplified sporocarp DNA from *Rhizopogon*, *Paxillus*, *Laccaria*, *Hebeloma*, *Tuber*, *Tricholoma*, *Cortinarius*, *Thelephora*, *Boletus* and *Amanita* spp. (All genera from the mock community were recovered in the MiSeq run except *Tricholoma*.) We used modified primers to amplify fungal ITS 1 DNA using the Illumina MiSeq platform and following Smith and Peay 2016). The forward PCR primer contained an Illumina adapter, linker, and gene primer. The reverse PCR primer contained an Illumina adapter, barcode, linker, and gene primer. The same forward primer was used for all samples. Each reverse primer differed with a unique barcode. PCR reactions were made in 50µl volumes using NEBNext Ultra II Q5 Master Mix following the manufacturer's protocol. Following quantification with a Qubit 4 Fluorometer (ThermoFisher Scientific) on PCR products, 54 of 132 sand bag samples yielded enough DNA to process further. DNA was purified with Ampure magnetic beads following the manufacturer's protocols (Beckman Coulter, Brea, USA). Samples were run on the Illumina MiSeq platformusing a MiSeq Reagent Kit v3 (600 cycle).

MiSeq data were processed as follows: BBDuk was used to remove PhiX 174 and Illumina adapter sequences. Primers were trimmed with Cutadapt 1.13 (Martin 2011). BBMerg (from the BBMap package) was used to trim the 3' ends of reads to a phred quality score of ≥ 20, and then join paired reads with a required length of 50 bases (after quality trimming), a minimum overlap of 30 bases and a maximum error rate of 0.3. Cutadapt 1.13 was used to trim 27 and 15 bases from the 5' and 3' ends of merged reads, respectively, to remove the conserved 18S and 5.8S rRNA gene flanking regions. Sequences were quality filtered using VSearch 2.5.1 (Rognes et al. 2016). Qiime 1.9 (Caporaso et al. 2010) was used for demultiplexing, chimera filtering, and clustering. Chimeras were also identified in USEARCH61 (Edgar et al. 2011). All detected chimeras were removed. A pre-filter was applied to the dataset using a closed-reference OTU clustering at 60% sequence similarity against the UNITE Species Hypothesis dataset (sh_qiime_release_s_01.12.2017; Kõljalg et al. 2013) with USEARCH61 (Edgar 2010). The remaining sequences were then clustered denovo at 97% similarity with USEARCH61, and the centroids for each 97% cluster were clustered again at 95% similarity. This more conservative sequence clustering does a better job at recovering expected diversity in mock communities with Illumina MiSeq data (e.g., Nguyen et al 2016, Taylor et al. 2016).

Taxonomy was assigned to operational taxonomic units (OTUs) with the Ribosomal Database Project (RDP) Classifier (confidence = 0.5; Porras-Alfaro et al. 2014) and with BLAST ($E \leq 1x10^{-2}$; Altschul et al. 1990), in Qiime 1.9. Taxonomy was also assigned using the UNITE Species Hypothesis dataset (sh_qiime_release_s_01.12.2017), supplemented with additional ITS sequences from non-fungal eukaryotic lineages obtained from the NCBI nucleotide database (http://ncbi.nlm.nih.gov). OTUs matching non-fungal lineages, as well as any OTU with less than 10 sequences in the dataset were removed. OTUs were tentatively assigned to functional groups using FUNGuild (Nguyen *et al.* 2015). The final OTU matrix was rarefied to 1000 sequences per sample before further analysis.

Data Analyses

Data were analyzed on the full dataset after bioinformatics as given above.
-Bray Curtis distance was used for PERMANOVA and NMDS analyses.
-NMDS plots were produced for all OTUs for stands (C1, C2, C4) and for all OTUs by treatments (N, P, N+P, No addition Control).
-Histograms were produced to show the most abundant OTUs. Data are the mean (±SE) # of sequence reads per sample (DNA extracted from an in-growth sand bag), rarefied to 1000 by dividing each sample by 1000.
-We used PERMANOVA to test for differences between nutrient addition treatments and stands on relative abundances. We did two tests, one on non-transformed data and a second test on 4th root transformed data to down-weight the effect of abundant species.

RESULTS

- 3180 OTUs were recovered following clean up and filtering of the MiSeq data. All were assigned to fungal taxa, although some were only identified to Kingdom or Phlyum.
- The fungal communities separate by stand **(Figs. 2a and 2b)** and by treatment **(Figs. 3a and 3b)**. The NMDS results for separation of the communities by both stand and treatments (Figs. 2 & 3) were supported by PERMANOVA **(Table 1)**. PERMANOVA showed no N X P interaction at an $\alpha = 0.05$.
- DNA from a variety of fungi was recovered from the mesh bag sand extracts, including ectomycorrhizal, arbuscular mycorrhizal and saprotrophic taxa **(Table 2, Figs. 4 - 7)**.

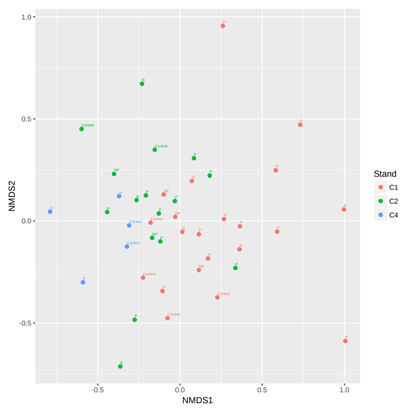

Fig. 2a: OTU ordination by stand – relative abundance data not transformed. Nutrient additions are color coded and stand is shown with the data points.

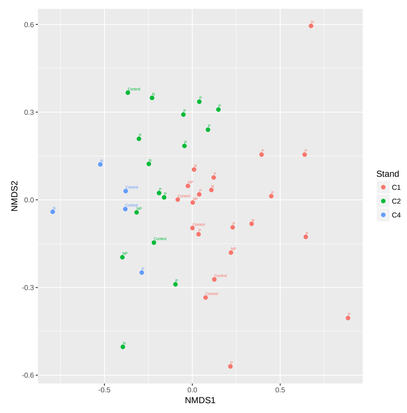

Fig. 2b: OTU ordination by stand – relative abundance data were 4th root transformed. Nutrient additions are color coded and stand is shown with the data points.

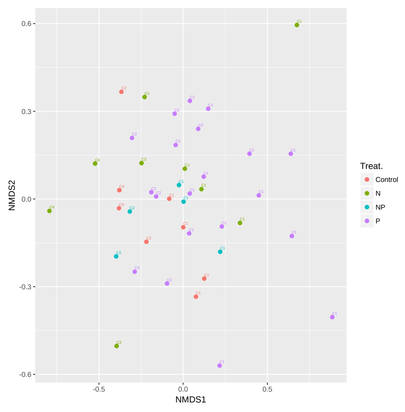

Fig. 3a: OTU Ordination by treatment – relative abundance data not transformed. Nutrient additions are color coded and stand is shown with the data points.

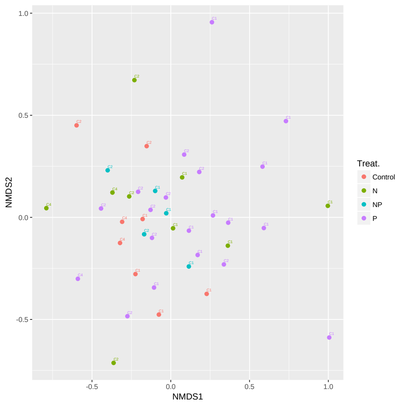

Fig. 3b: OTU Ordination by treatment – relative abundance data 4th root transformed. Nutrient additions are color coded and stand is shown with the data points.

Table 1a: PERMANOVA results on non-transformed data. Nitrogen, phosphorus and stand are signficant. There is no NxP interaction.

Factor in Model	P-values
Nitrogen	0.043
Phosphorus	0.047
N x P interaction	0.080
Stand	0.001

Table 1b: PERMANOVA results on 4th root transformed data. Phosphorus is not signficant, probably a function of down weighting abundant taxa (e.g., Genea).

Factor in Model.	P-values
Nitrogen	0.032
Phosphorus	0.234
N x P interaction	0.016
Stand	0.001

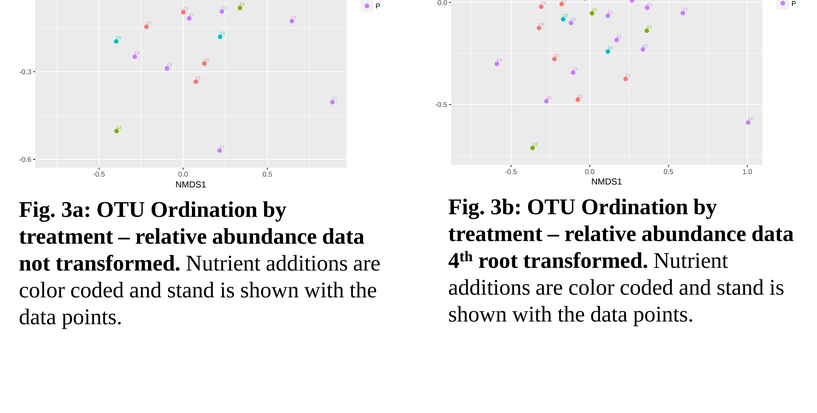

Fig. 4: Phosphorus addition plots. Data are mean (+SE) number of sequence reads per sample. The figure shows relative abundances because each sample was rarefied to 1000 sequences. Note that OTUs with same genus (e.g., the two *Boletus* OTUs) may or may not be different species because the identification was based less than 300 bp of the ITS1 rRNA gene. *Genea* appeared to be an indicator species of the P addition plots ($p < 0.05$).

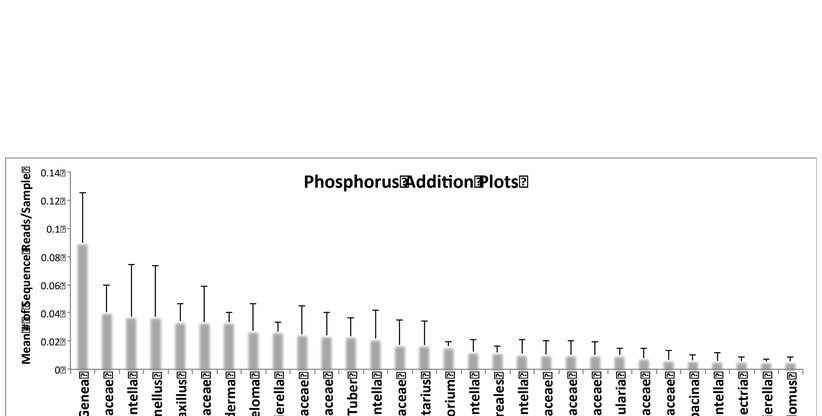

Fig. 5: Nitrogen addition plots. Mean (+SE) number of sequence reads per sample from nitrogen addition plots. The figure shows relative abundances because each sample was rarefied to 1000 sequences. Note that OTUs with same genus (e.g., the two *Tuber* OTUs) may or may not be different species because the identification was based less than 300 bp of the ITS1 rRNA gene. No indicator OTUs were found of the Nitrogen Addition plots.

TAKE-AWAYS

- We recovered many mycorrhizal and saprotrophic fungi.
- Fungal communities separated by stand and nutrient addition. This was expected considering the fine-scale patchiness of fungi belowground, and the fact that we use a small amount of sand as a sampling unit for the10 X 10 meter plots.
- Hypogeous and resupinate taxa were well represented, two groups of fungi that are underrepresented in sporocarp surveys.
- *Genea* was an indicator species of Phosphorous addition. This may be the first EM fungus identified with a positive response to elevated P.
- *Paxillus* and a Thelephoraceae were indicators of Nitrogen+Phosphorus addition. *Russula* was an indicator of Control stands. There were no indicators of Nitrogen addition.

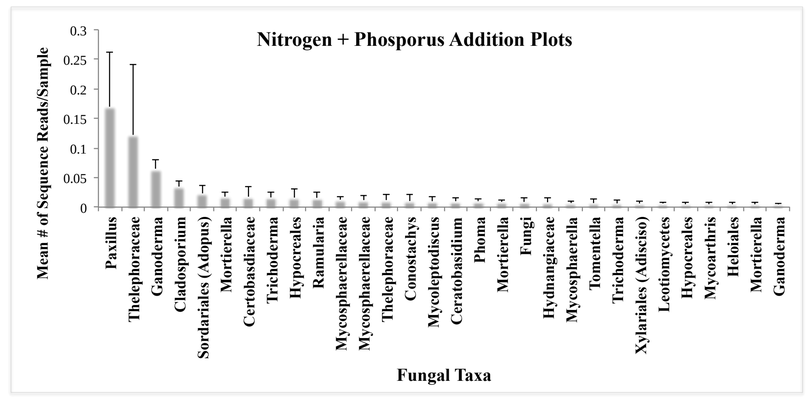

Fig. 6: Nitrogen + Phosphorus addition plots. Mean (+SE) number of sequence reads per sample. The figure shows relative abundances because each sample was rarefied to 1000 sequences. Note that OTUs with same taxon name (e.g., the two Mycospharellaceae OTUs) may or may not be different species because the identification was based on less than 300 bp of the ITS1 rRNA gene. The large error bar for the first Thelephoraceae is a function of one sample with a very high number of sequence reads. *Paxillus* and Thelephoraceae appeared to be indicator OTUs of the Nitrogen + Phosphorus addition plots ($p < 0.05$).

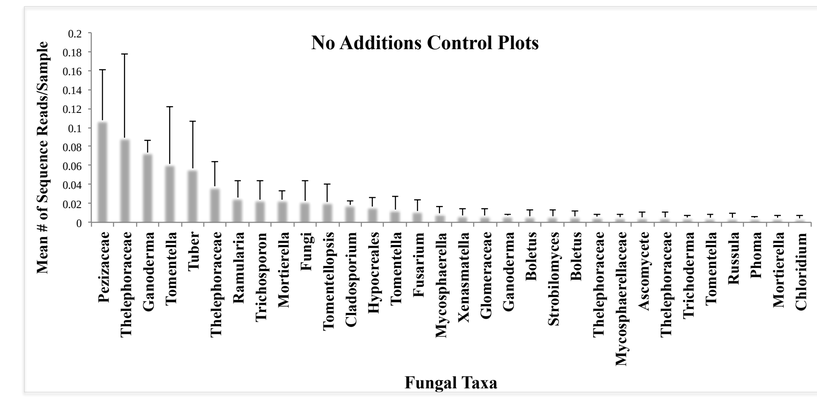

Fig. 7: No additions control plots. Mean (+SE) number of sequence reads per sample from nitrogen addition plots. The figure shows relative abundances because each sample was rarefied to 1000 sequences. Note that OTUs with same genus (e.g., the two Thelephoraceae OTUs) may or may not be different species because the identification was based less than 300 bp of the ITS1 rRNA gene. *Russula* appeared to be an indicator OTU of the control plots ($p < 0.05$).

Fig. 8: Genea harknessii. The genus Genea is a hypogeous fungus in Ascomycota. Genea was an indicator species of the phosphorus addition plots. Image courtesy of Matt Trappe from the North American Truffles web site.

Table 2: Fifteen most dominant OTUs from all plots in the study. The list is in order of greatest to least relative abundance. The finest taxonomic level possible from the data analyses is shown. When a species is shown, the specific epithet is given in parentheses given the lack of resolution from the <300bp of the nrITS. Known ectomycorrhizal taxa are well represented in the dominant OTUs.

1. family_Pezizaceae
2. genus_*Ganoderma*
3. species__*Genea_(hispidula)*
4. species__*Paxillus_(involutus)*
5. species__*Tuber_(separans)*
6. species__*Tomentella_(lilacinogrisea)*
7. species__*Mortierella_(humilis)*
8. genus__*Sebacina*
9. genus__*Cladosporium_(delicatulum)*
10. species__*Pseudotomentella_(tristis)*
11. family__Thelephoraceae
12. species__*Coprinellus_(micaceus)*
13. family__Pezizaceae
14. genus__*Pachyphloeus*
15. species__*Tuber_(separans)*

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Citations

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