The effects of stem-girdling on ectomycorrhizal fungi growth and nitrogen cycling

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Abstract

Ectomycorrhizal (ECM) fungi have a symbiotic relationship with tree roots; the fungi make various nutrients in the soil, specifically nitrogen, accessible to trees in exchange for the carbohydrates produced by the tree via photosynthesis (Smith and Read 2008). This relationship makes hyphal growth of ECM fungi a relevant substrate for studying nitrogen cycling in forest ecosystems. Under the Forest Accelerated Succession ExperimenT (FASET), tree girdling was used to replicate disturbance and early forest succession. Labeled nitrogen was used to examine nutrient flux in hyphae varied by experimental plots. Although no significant relationship was found between stem-girdling and nitrogen levels in ECM fungi, nor stem-girdling and hyphae biomass, more data was added to the FASET study.

Introduction

Ectomycorrhizal (ECM) fungi have a symbiotic relationship with tree roots; the fungi make various nutrients in the soil, specifically nitrogen, accessible to trees in exchange for the carbohydrates produced by the tree via photosynthesis (Smith and Read 2008). Nitrogen is one of the fundamental elements of life, and is very often a limiting nutrient. Fungi are a very important nitrogen recycler, as they partake in ammonification, which leads to increased nitrogen availability for plants and other nitrate-utilizing organisms (Hobbie *et al.* 1999). The extent of N cycling within ECM after the death of related trees is not yet well understood and could give insight to the larger role that ECM play in forest succession.

The Forest Accelerated Succession Experiment (FASET) project is currently considering many ecological questions at the University of Michigan Biological Station (UMBS). Under the FASET study, all bigtooth aspen (*Populus grandidentata*), trembling aspen (*P. tremuloides*) and paper birch (*Betula papyrifera*) trees in a 39 ha. plot were stem-girdled. Stem-girdling a tree kills it by removing a ring of phloem around the circumference of the tree trunk. The death of these trees forces experimental forest succession by allowing more dominant, late-successional trees to grow and fill in gaps in the forest canopy (Nave *et al.* In Review). ECM fungi perform nutrient cycling in forests (Schweiger and Jakobsen 2006) and are thus one area of interest within FASET. In this specific experiment, labeled nitrogen was used to track nitrogen cycling in different successional stages of forests as well as the role ECM plays in this process.

Isotope labeling is a technique used to track the progression of an element through a system. Typically nitrogen in nature occurs as ^{14}N. The isotope ^{15}N occurs at very minute concentrations in nature, but is stable and acts the same as the more abundant ^{14}N isotope (Hobbie *et al.* 1999). By artificially applying labeled ^{15}N in high concentrations to a system,

some aspects of nitrogen cycling can be observed. Nitrogen provided to host trees by ECM is typically depleted of ^{15}N, while the nitrogen remaining in the ECM fungi is relatively rich in ^{15}N (Hobbie *et al.* 2009, Mayor *et al.* 2009). Using mass spectrometry, the isotope ratio of nitrogen can be determined to assess the progression of nitrogen throughout an ecosystem (Hobbie *et al.* 1999).

ECM continues to accumulate N after the death of the tree, at which point it no longer returns N to the tree. Also, dead trees are bleeding excess N out into surrounding soil (Nave *et al.* In Review). Over what is currently an uncertain time scale, we predict girdled plots to have higher nitrogen availability in their soil and we expect N levels of hyphae in girdled plots to be greater. This study also examined the relative hyphae biomass of girdled and ungirdled plots. Dead trees can no longer provide carbon to the mycorrhizae. Due to the mutualistic relationship between tree roots and ECM fungi, girdling and killing trees should decrease the hyphae biomass, leaving relatively more hyphae in the ungirdled plots. The experiment, though exploratory in nature, aided the FASET project in its goal of better understanding both how nitrogen cycles through forest ecosystems and how mass tree die-offs affect that cycle. It also provides insight to the role of ECM in forest ecosystems.

Methods

Sites

Data collection occurred within the University of Michigan Forest Accelerated Succession Experiment (FASET) plots and the AmeriFlux Tower research area at the University of Michigan Biological Station in Pellston, Michigan, USA. Three pairs of plots were used. Each pair contained one of FASET's girdled plots and one from the reference AmeriFlux Tower

plots (Figure 1). Plots were paired based on their tree species composition before

implementation of FASET stem girdling, as different forest stand types have their own species of

ECM fungus (Termorshuizen 1990). Plots B3 (girdled) and F8 (ungirdled) are high in maple

(*Acer spp.*), paper birch, and aspen abundance, typical of early succession. Plots C3 (girdled)

and I2 (ungirdled) are high in maple, oak (*Quercus spp.*), and pine (*Pinaceae*), but low aspen and

paper birch abundance. Plots D1 (girdled) and Q5 (ungirdled) are high in oak and pine

abundance, typical of late succession. All plots had a radius of 16m (0.1 hectare). Girdled plots

contain dying and dead bigtooth aspen, trembling aspen, and paper birch that were girdled in

May 2008 (three years prior to this experiment). In spring 2011, all paper birch were dead, 77%

of aspens were dead, and the remaining 23% of aspens dying (Curtis, unpublished).

Nitrogen labeling

Labeled nitrogen was used to better illuminate nitrogen flux in analysis. Isotopically

enriched ammonium chloride (NH_4Cl) was administered to plots using a backpack sprayer

during three applications: May, June, and July, 2010. 0.2kg of NH_4-N was sprayed per hectare,

which is not enough to affect the growth of trees (Nave *et al.* 2009). Spraying extended slightly

beyond the ranges of each plot to ensure that tree roots beyond the plot boundaries would be

drawing in labeled nitrogen.

Hyphal ingrowth bags: design and sampling

Within each plot, samples were collected from three clusters. Each cluster consisted of

four hyphal bags inserted vertically beneath the litter layer, 4-10 months previously. One bag in

each cluster was installed inside a PVC pipe (15 cm diameter, 30 cm depth) that had been driven

into the ground to exclude roots. By comparing biomass and isotope signatures of these hyphal

bags with those that did not have root ingrowth excluded, it may be possible to distinguish

between mycorrhizal and saprotrophic hyphae, the latter of which should predominate inside the

PVC root exclusion tubes. Hyphal ingrowth bags were made of SEFAR 03/30-18 NITEX mesh,

a nylon mesh with holes small enough (30 micron) to mostly exclude roots while permitting

hyphae to enter. The cores of the bags are glued together with a polyester resin (X-11 shower

pan sealer). Each bag is 10cm x 2.5cm and contain 50-60g of sieved sand (<355μ diameter) that

was ashed at 500°C for four hours. Ashing sterilizes the sand and removes organic matter. The

top of the bag is sealed with a staple. The design of these bags was modeled after Wallander *et

al.* 2001.

Three sets of samples were analyzed: one from before the labeled nitrogen was

administered, one from a year later, and one from two years later.

Processing

Hyphal ingrowth bags were kept refrigerated until processed. For processing, all non-

excluded hyphal growth bags from a single plot (9 bags) were brushed off to remove excess

material and then opened and emptied into two beakers. To extract the hyphae from the sand, a

series of soil sieves were used, water was added to the beaker and then stirred with a spoon.

While the sediment was suspended in the water, the water was then poured through a 125μm

sieve leaving sand behind in the beaker with a ridge of dark-colored hyphae clumped together.

The hyphae were scooped onto the sieve using the spoon. This was repeated at least three times

and until there were no visible clumps of hyphae remaining in the beaker. The sand was then

emptied out of the beaker, and the hyphae in the sieve were returned to the beaker and the

process was repeated to further decrease sand in the sample. The hyphae in the sieve were then sprayed with water using a wash bottle. This stirred up the hyphae and helped break it apart from the sediment particles. Careful rinsing of the sieve surface and forceps were used to extract clumps of hyphae and place them into a petri dish. Remaining water and sediment in the bottom of the sieve were disposed. In the petri dish, the hyphae were sprayed with the wash bottle, helping to further isolate the low-density hyphal mass from the denser sand grains. Forceps were used to extract hyphae of this dish into another one and this process was repeated until the hyphal mass was relatively free from sand grains. Finally, the cleanliness of the hyphal sample was inspected under a dissecting scope. The sample was then placed into a 20 mL scintillation vial and stored in the freezer. This process was done for the second beaker containing the same plot's remaining hyphal bags, which were then added to same scintillation vial. The three excluded bags from each plot were processed with the same method separately.

After the samples were freeze dried, additional cleaning was necessary to remove remaining sediment. Each hyphal mass was extracted from its vile with forceps and placed onto a white sheet of paper. Using forceps, the mass was then separated into increasingly smaller parts, allowing grains of sand to fall out of the mass and onto the paper. All pieces were then picked up with the forceps and placed back into the vial, while the remaining sand was discarded from the paper. The closed vial was shaken back and forth to release more sand from the hyphae; after shaking, the mass was again removed from the vial and placed onto the paper for further separation. The sand left behind in the vial was discarded. This process was repeated five times for each sample to maximize the amount of sand removed.

After this final cleaning was completed, the samples were individually weighed on a balance and the biomass was recorded. The samples were prepared for grinding by being placed

with forceps into labeled plastic grinding vials. Each vial was filled with hyphae, and a small plastic ball was placed on top of the sample. Grinding vials were placed into one of fourteen chambers on two metal disks. These disks were placed into clamps located inside of the grinding machine, and foam padding was used to ensure that the disks were secure within the clamps. The samples were ground for three minutes, after which the disks were removed from the machine, and the samples stored.

An isotope-ratio mass spectrometer was used to analyze the ratios of stable carbon and nitrogen isotopes of each sample.

Results

To account for excess sand and uneven levels of cleanliness in the samples, all sample biomasses was standardized using an expected 43% carbon composition. This 43% carbon composition was based on previous measurements of sporocarp carbon composition (Nave and Nadelhoffer, unpublished).

Biomass by Sample type

The mean corrected hyphae biomass of the root-included samples ($\bar{x}$ =.102 g) was much greater than that of the root-excluded samples ($\bar{x}$ =.013 g). Using a t-test, the difference was found to be very significant (p=.000) (Figure 2).

The mean $\delta^{13}C$ parts per thousand (‰) of the root-included samples ($\bar{x}$ =-26.6) was very similar to the mean $\delta^{13}C$ of the root-excluded samples ($\bar{x}$ =-26.8). A t-test showed no significant difference between the two means (p=.535) (Figure 3).

Biomass by Treatment

The mean hyphae biomass of the FASET samples ($\bar{x}$ =.053 g) was slightly lower than that of the samples from the reference plot ($\bar{x}$ =.064 g). Ignoring outliers, the range of hyphae biomass from the FASET samples was much smaller (.005 −.091 g) than that of the reference plot (.002–.210 g). Using a t-test, the difference between the mean corrected biomass of FASET plot samples and reference samples was found to be insignificant (p=.384) (Figure 4).

Nitrogen

The mean $\delta^{15}N$ of the 2009 ($\bar{x}$ =1.8), 2010 ($\bar{x}$ =130.0) and 2011 ($\bar{x}$ =60.1) were all very distinct. Using a one-way ANOVA test, the differences between 2009, the year prior to the application of labeled nitrogen, and 2010 and 2011, the years after the application, were very significant (p=.000) (Figure 5).

Of the samples collected after the application of radio-labeled nitrogen, the mean $\delta^{15}N$ of the FASET plot ($\bar{x}$ =88.7) was slightly lower than the mean $\delta^{15}N$ of the reference plot ($\bar{x}$ =98.0), but a t-test showed the difference was not significant (p=.087) (Figure 6)

Discussion

Our study sought to see the effects that stem-girdling has on the tree's associated ECM fungus. This was addressed in two ways: the effect of stem-girdling on hyphae growth and on nitrogen uptake.

Sample Type

The PVC pipe used to exclude roots from the samples worked very well. Ectomycorrhizal fungus grows predominately within tree roots, so by preventing root growth around the sample

bags, the hyphae biomass was significantly lower than the samples in which roots were not excluded.

Based on the analysis of $\delta^{13}C$ ‰ of each sample type, it appears that ECM was the dominant fungus type in the root-excluded bags and saprotrophic fungi were not a major component of either root-excluded or root-included samples. This aligns with the findings of Wallander *et al.* (2001) which concluded ECM fungus has $\delta^{13}C$ ‰ near -26.

Biomass

Ectomycorrhizal hyphae biomass was not significantly lower in the FASET plots than the reference plots as originally hypothesized. While the reference plot had a slightly higher mean hyphae biomass than the FASET plot, each plot had a very similar range of biomass measurements.

At a glance, it seems that stem-girdling of an ECM fungus' host tree has no effect on that fungus, but this is by no means conclusive. Biomass measurements were only collected from 2010 and 2011, two or three years after the stem-girdling occurred. Perhaps the stem-girdling affected the ECM fungus in a more immediate time frame and the ECM fungus has since then recovered.

Nitrogen

As expected, the $\delta^{15}N$ values for each sample year were very different. The ^{15}N that was measured in 2009 (i.e. the naturally occurring ^{15}N) was miniscule in comparison to the values measured after the application of labeled nitrogen. The $\delta^{15}N$ values for 2010 were also significantly higher than those of 2011, showing that the labeled nitrogen was being cycled through the ecosystem.

We originally hypothesized that, due to the increased deposition of nitrogen in the FASET plots (Nave *et al.* In Review), there would be higher ^{15}N concentrations in the FASET samples of ECM hyphae than in samples from the reference plots. However, the δ^{15}N analysis of samples showed that there was no significant difference in ^{15}N concentrations between the FASET plot samples and reference samples. It is clear that we do not yet fully understand the effects of nitrogen availability on ECM fungi, nor the effect that stem-girdling has on a tree's associated ECM fungi.

Future Studies

The technique for excluding tree roots from samples should be modified. Tree roots could still possibly grow under then up through the PVC pipe used to exclude them, so the pipes would need to be altered to better exclude tree roots.

The effects of forced forest succession on ECM fungi are still mostly unknown. The data collected during this study suggested that hyphae growth was slightly higher when the host trees were living, but it was not statistically significant. More samples from each year would help to determine if there is actually a correlation between the two. We attributed part of this insignificance to the lack of biomass measurements until two years following girdling. Biomass samples should be taken immediately after, and one year after stem-girdling occurs to give more insight into the effect tree death has on the growth of ECM hyphae. More samples might also help to smooth out the widely varying δ^{15}N measurements.
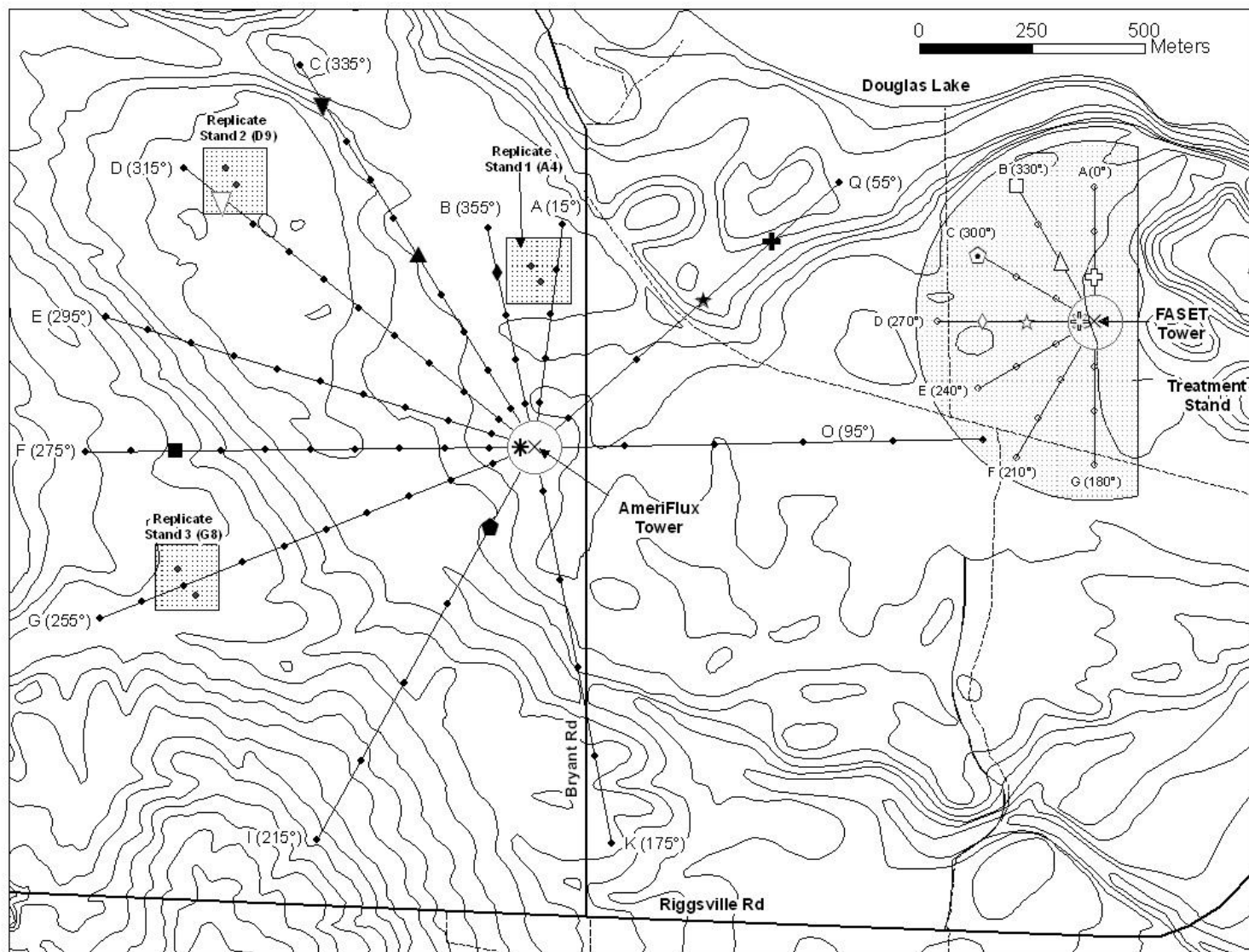
Fig. 1—Map of the studied area, showing both the FASET and reference areas. Paired plots B3 and F8 denoted by squares, C3 and I2 by pentagons, and D1 and Q5 by stars. FASET plots are marked in white and reference plots are marked in black.
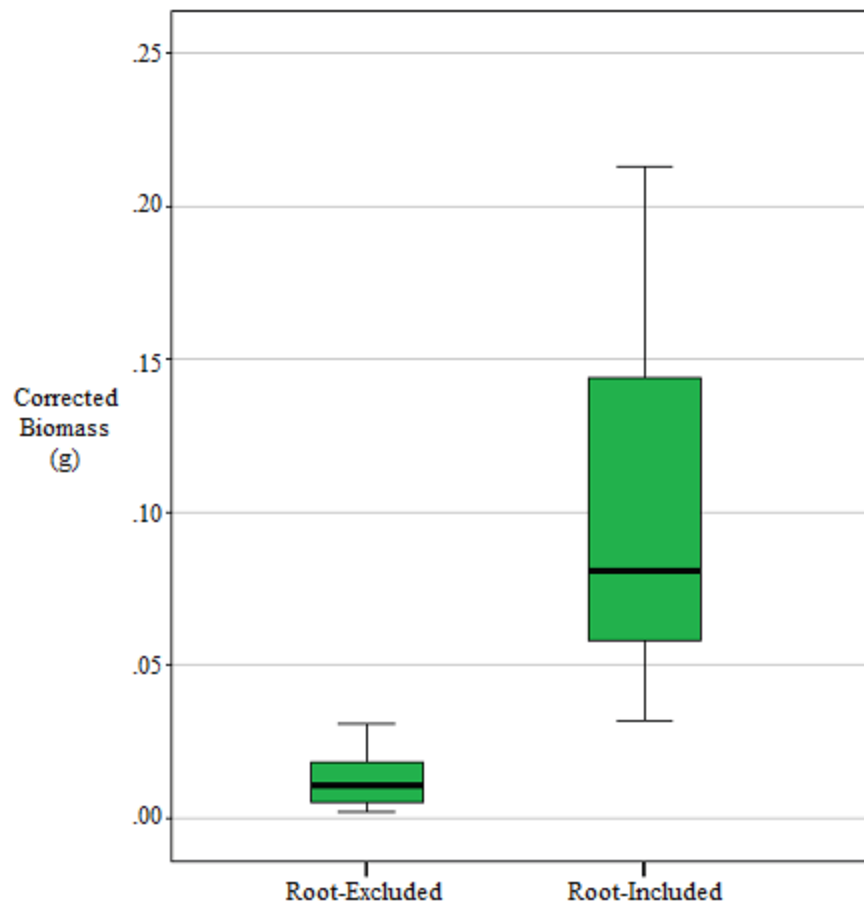
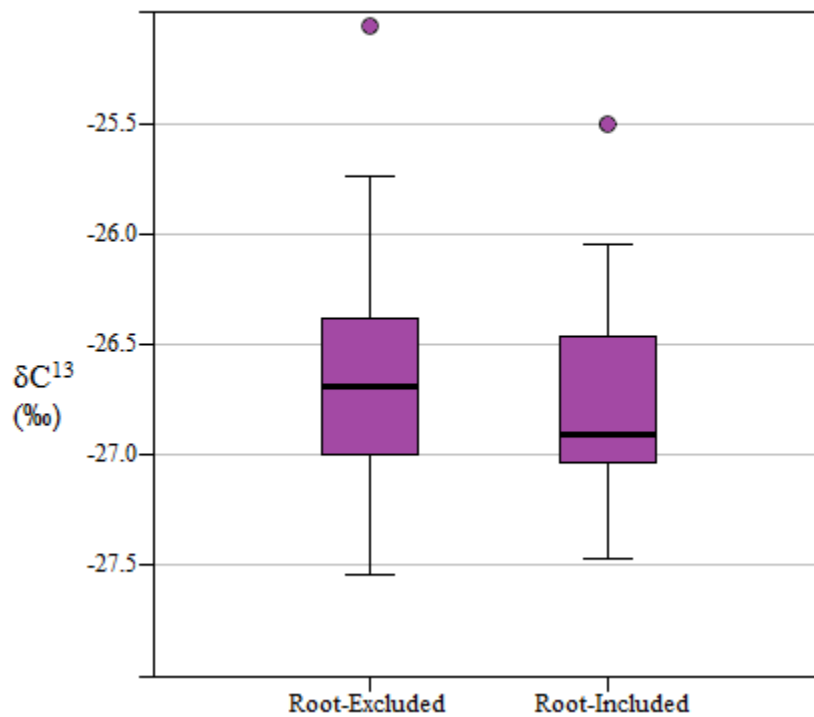**Fig. 2**—The corrected hyphae biomass distributions of all samples, separated by sample type.

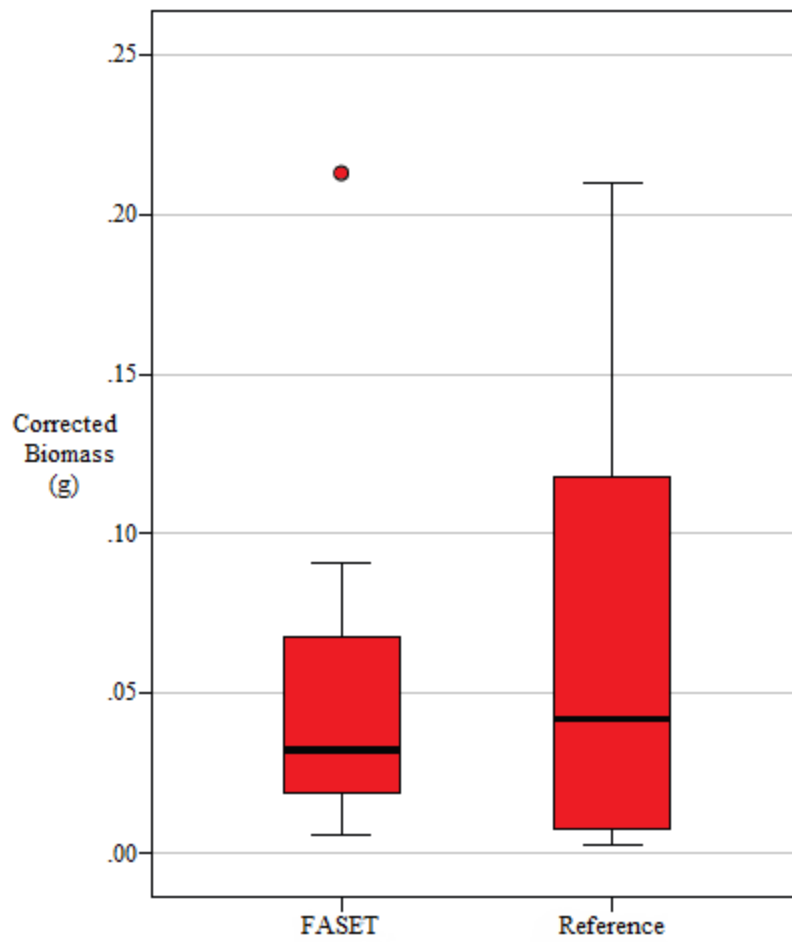**Fig. 3**—The $\delta^{13}C$ ‰ distributions of all samples, separated by sample type.

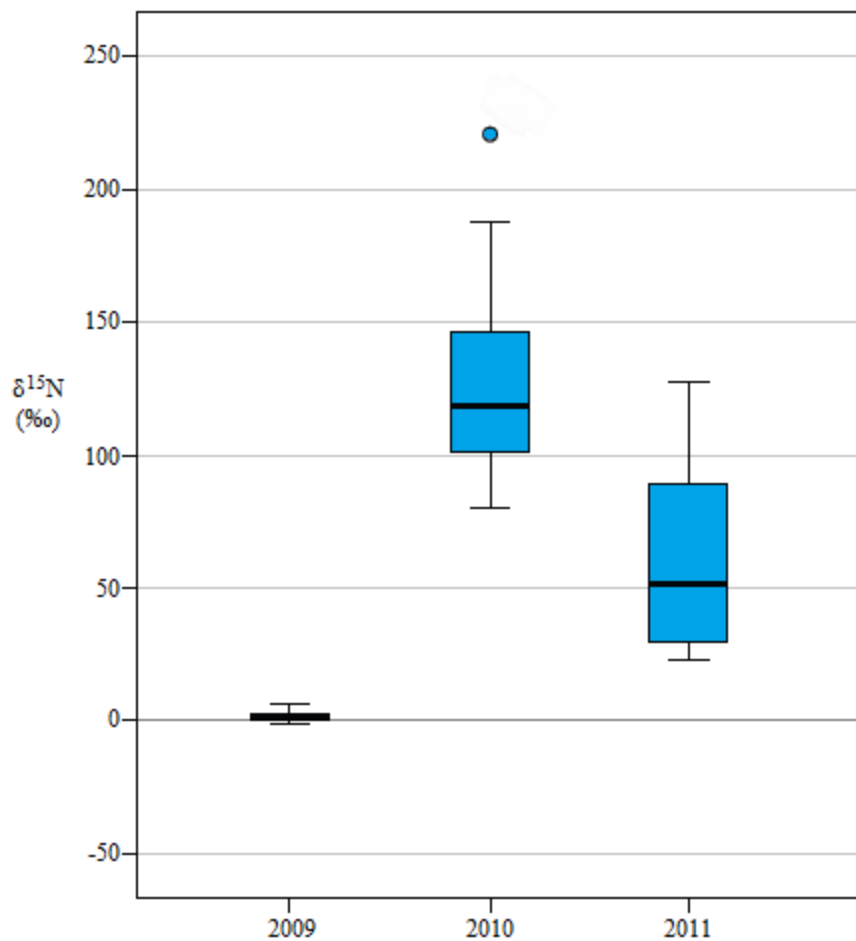**Fig. 4**—The corrected hyphae biomass distributions of all samples, separated by plot treatment.

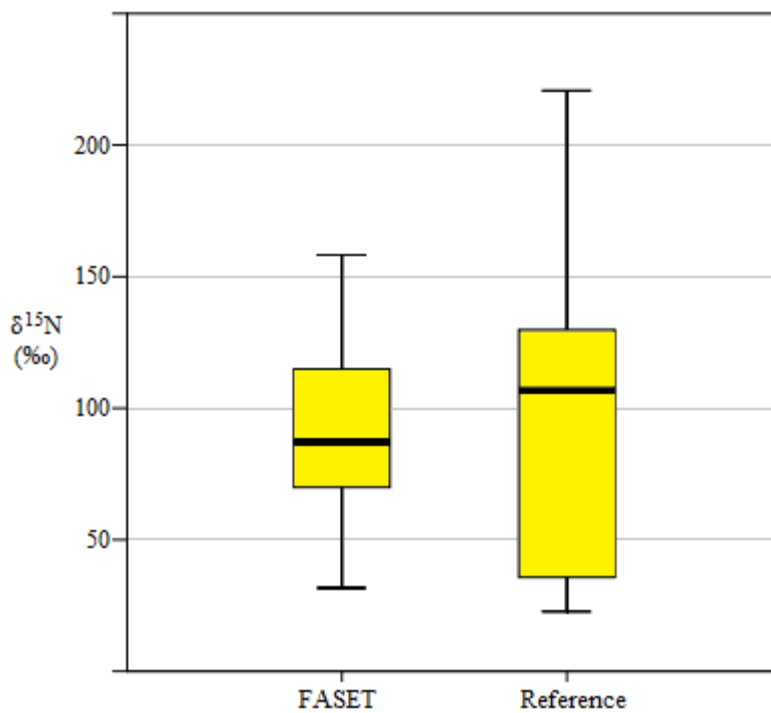**Fig. 5**—The δ^{15}N ‰ distributions of all samples, separated by sample year.

Fig. 6—The δ^{15}N ‰ distributions of samples from 2010 and 2011, separated by plot treatment.

Literature Cited

Curtis, P. 2011. Unpublished.

Hobbie, E.A., Agerer, R. 2010. Nitrogen isotopes in ectomycorrhizal sporocarps correspond to belowground exploration types. Plant and Soil 327:71-83.

Hobbie, E. A., Macko, S. A., and Shugart, H. H. 1999. Insights into nitrogen and carbon dynamics of ectomycorrhizal and saprotrophic fungi from isotopic evidence. Oecologia 118:353-360

Mayor, J.R., Schuur, E.A.G., Henkel, T.W. 2009. Elucidating the nutritional dynamics of fungi using stable isotopes. Ecology Letters 12:171-183.

Nave, L., Gough, C., Maurer, K., Bohere, G., Hardiman, B., Le Moine, J., Munoz, A., Nadelhoffer, K., Sparks, J., Strahm, B., Vogel, C., and Curtis, P. Disturbance, decoupling and resilience of carbon and nitrogen cycles in a northern temperate forest. In Review.

Nave, L. and Nadelhoffer, K. 2008. Unpublished.

Schweiger, P. and Jakobsen, I. 2006. Laboratory and field methods for measurement of hyphal uptake of nutrients in soil. Plant and Soil 226 (2):237-244

Smith, S. E. and Read, D. 2008. Mycorrhizal Symbiosis, third edition. Elsevier, Great Britain.

Termorshuizen, A. J. 1990. Succession of mycorrhizal fungi in stands of *Pinus sylvestris* in the Netherlands. Agriculture, Ecosystems, & Environment 28:503-507

Wallander *et al.* 2001. Estimation of the biomass and seasonal growth of external mycelium of ectomycorrhizal fungi in the field. New Phytologist 151(3):753-760.

Wallander, H., Göransson, H., Rosengren, U. 2004. Production, standing biomass and natural abundance of ^{15}N and ^{13}C in ectomycorrhizal mycelia collected at different soil depths in two forest types. Oecologia 139:89-97.