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Quantifying carbon allocation to mycorrhizal fungi by temperate forest tree species across a nitrogen availability gradient

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Environmental Conservation and Sustainability Honors Thesis

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Abstract

Terrestrial ecosystems make up the largest carbon pool with a major portion of that being forests. With carbon being a major concern due to global climate change, being able to make accurate models is increasingly important. Studies have shown that trees may allocate up to 50% of their photosynthetically fixed carbon underground; however these values haven't been accurately quantified and underground carbon allocation has been historically overlooked. Mycorrhizal fungi may be a large portion of underground carbon allocation, as they have a symbiotic relationship with trees where they provide the plant with water and nutrients in return for sugars (carbon). New methods and knowledge will allow us to quantify carbon allocation and fungal biomass. Ergosterol is a biomarker that is the human equivalent of cholesterol for fungi which can be used to measure fungal biomass. Since both free-living and mycorrhizal fungi have ergosterol, a series of open and closed cores located at Bartlett Experimental Forest will separate the amount of ergosterol due to free-living versus mycorrhizal fungi. This is one of the first studies that will quantify fungal biomass and carbon allocation under a variation of natural settings and compare two different methods to estimate these values.

Introduction

Significance

Carbon dioxide is a greenhouse gas. Greenhouse gases trap radiation in the earth's atmosphere, and can lead to a warming of the earth's surface, which in turn can affect a number of processes that affect climate patterns. Terrestrial ecosystems contain 3 times more carbon than the atmosphere and forests annually release more than 10 times the amount of CO2 to the atmosphere through soil respiration than fossil fuel emissions (Trumper et al., 2009; Heinemeyer et al., 2007). However, natural soil respiration fluxes are balanced by fixation of atmospheric CO2 through photosynthesis (Aber & Melillo, 1991). Terrestrial ecosystems store 2100 gigatonnes of carbon globally, with 699.1 gigatonnes stored in temperate and boreal forests forests alone (Trumper et al., 2009). For this reason, forests are a very important part of the global carbon budget. Still, our understanding of their role in the carbon balance would be improved with a more thorough understanding of whole-plant carbon allocation. Knowing how much carbon is allocated to and stored in different components of forest ecosystems will help scientists better model the effects of future climate change (Heinemeyer et al., 2007).

Through photosynthesis plants take in carbon dioxide and convert it to energy, which is used to create biomass. Trees can allocate carbon to leaves to photosynthesize more, to wood in order to better reach the sunlight, to roots and mycorrhizae to gather water and nutrients (Aber & Melillo, 1991). The ability to grow and store carbon in temperate forests is largely limited by the availability of soil nitrogen (Hasselquist et al., 2012; Trumper et al., 2009). Through a symbiotic relationship with mycorrhizal fungi, forests can better access nitrogen, even in soils with low N-availability (Read & Smith, 2008). Although many studies measure the carbon allocated to

foliage, roots, and wood, few if any measure the fraction of photosynthetically fixed carbon allocated to mycorrhizal fungi (Meyer, Grote & Butterbach-Bahl, 2012). For this reason, many ecosystem models do not include the flux of carbon supplied to mycorrhizal fungi by plants, which can be as high as 30% of all photosynthetically fixed carbon (Hobbie, 2006). Knowing the amount of carbon allocated to mycorrhizal fungi will improve both modeled predictions of forest growth and allow for better estimates of whole plant and ecosystem carbon budgets (Meyer, Grote & Butterbach-Bahl, 2012). If mycorrhizal fungi aren't included, then these models are missing at least 30-40% of soil microbial biomass (Hasselquist et al., 2012).

Nitrogen limitation restricts the net primary productivity (NPP) of terrestrial ecosystems, both in the Northeast and globally (Vadeboncoeur, 2010). Although we know the importance of the relationship between nitrogen and ecosystem carbon storage, there is a lack in the knowledge of belowground carbon allocation by trees for the acquisition of nitrogen (Fisher et al., 2010). Measuring belowground carbon allocation, especially to mycorrhizal fungi, will enhance our understanding of whole plant carbon allocation patterns and our estimates of the ability of ecosystems to store carbon. Most studies only account for carbon allocated to wood, foliage and less often roots (Grote, 1998). This is largely due to the difficulty in making belowground measurements (Grote, Kiese, Gruenwald, Ourcival, & Granier, 2011).

Measuring carbon allocation belowground is particularly important in environments that are nitrogen limited. Symbiotic mycorrhizal fungi facilitate plant access to inorganic and organic nutrients, and are the main pathway of nutrient and water uptake for plants in natural systems (Clemmensen et al., 2006; Read & Smith, 2008). As much as 10-40% of all photosynthetically fixed carbon by the plant may be allocated to ectomycorrhizal (ECM) fungi (Simard, Jones, & Durall, 2002; Wallander, 2006). It is suggested that ECM fungi can also reduce decomposition

rates in the soil by suppressing other microbial groups; therefore allowing forests to store carbon longer (Langley, Chapman, & Hungate, 2006; Wallander, 2006).

Background

Mycorrhizal stems from the words *myco* meaning fungus and *rhizo* meaning root (Aber & Melillo, 1991). Mycorrhizal fungi have a symbiotic relationship with most terrestrial plants in which they provide the plant with greater access to growth-limiting soil. The plants in turn allocate carbon to the fungi, supplying fungi with carbon (sugars) necessary for fungal growth (Read & Smith, 2008). Allocating carbon to the fungi can be more effective for the plant than increasing root length, because fungi (with a very small diameter of only a few microns) require a lower carbon cost per unit length compared to thicker roots (Aber & Melillo, 1991; Hasselquist et al., 2012). There are thousands of different species of mycorrhizal fungi, but in temperate forests there are two main types: ectomycorrhizal (ECM), which creates a sheath around root tips and extends out into the soil; and arbuscular-mycorrhizal, which penetrates the cells of the roots, directly providing the nutrients to the cells (Read & Smith, 2008; Brundett, 2004). The type of mycorrhizal fungi present at a site depends on the nutrient (mainly nitrogen) richness of the soil, and the species of the plants (Read & Smith, 2008; Correa et al., 2008; Lang & Polle, 2011). Generally ECM have stronger enzymatic capabilities for accessing soil nitrogen, have much larger biomass, and require much higher carbon fluxes from plant hosts. In low nitrogen habitats, the dominant trees tend to be species that associate with ECM fungi.

The amount of carbon allocated to ECM fungi is thought to correlate with the degree of soil nitrogen limitation. Generally, this is because if there is high soil nitrogen availability, then trees can assimilate nitrogen using their roots, with little dependence on mycorrhizae. If there is

low soil nitrogen availability, then plants should depend more on the fungi (Hobbie & Hobbie, 2008).

Field and lab studies show that allocation to ECM ranges from 0-40% (Hobbie, 2006), however estimates in the field under natural conditions are very hard to make. Using multiple methods to quantify carbon allocation to ECM fungi will provide more confidence in our estimates. Specifically, both fungal ingrowth techniques and new isotopic techniques could refine these estimates and provide a better view of carbon allocation belowground and the importance of fungal biomass in forest ecosystems (Hobbie, 2006).

Ergosterol is a biomarker found in living tissues of fungi (more in methods) and can be used to estimate the amount of living fungal biomass in forests.. For example, in a culture study in a controlled lab setting with only a single species, (Correa, Strasser, & Martins-Loucao, 2008) measured the amounts of ergosterol to quantify ECM fungal biomass under conditions of varying nitrogen limitation. They found that there was a negative relationship between fungal biomass and N availability (more fungal biomass when N availability was low). Culture studies under controlled conditions are important; however Wallander (2006) stressed the importance of quantifying ECM in natural systems. One difficulty in using ergosterol to quantify ECM biomass in natural settings is that both symbiotic ECM fungi and free-living (non-symbiotic) saprotrophic fungi contain ergosterol, making it difficult to quantify just ECM fungi. Some studies have dealt with this by measuring ergosterol content in small mesh bags filled with sand that are buried in the soil (Clemmensen et al., 2006; Wallander, Ekblad, & Bergh, 2011). This technique makes the assumption that only ECM fungi will grow into the sand bags (and not saprotrophic fungi). One issue with using mesh bags is that they provide a very unnatural substrate containing no carbon

or nutrients, and can greatly underestimate fungal growth (Wallander, 2006). There is a need for studies that measure fungal production in natural systems (Courty et al., 2010).

Objectives

In this study I (1) used established methods to quantify the biomass of ectomycorrhizal (ECM) fungi in grams per meter^2, (2) created an ingrowth study to understand ectomycorrhizal productivity, and (3) created a better understanding for the use of ergosterol as a biomarker of fungal growth. This will be done in plots with different amounts of available nitrogen and different tree species composition in a northern temperate forest in New Hampshire.

Methods

Overview

Because it is difficult to directly measure carbon allocation to we used two independent methods to estimate carbon allocation, and compare their results. The first method uses a paired in-growth soil core approach to quantify the production of ectomycorrhizal fungal biomass in soils, by measuring ergosterol concentrations in in-growth cores. Ergosterol is a fungal specific biomarker that makes up about 0.3% of living fungal biomass and is the fungal equivalent of cholesterol in humans (Read & Smith 2008). The second method uses bulk soil cores to understand the standing fungal biomass. These two methods each make different assumptions and will provide two independent estimates of carbon allocation to mycorrhizal fungi. The ingrowth cores give us a production value, while the bulk soil cores allows us to understand the amount of standing biomass. By comparing results from both methods I will have more confidence in my estimates of carbon allocation and mycorrhizal biomass.

Site Description

The study site for my project is the Bartlett Experimental Forest in northern New Hampshire, which has a series of established forest research plots that span a range of species and soil N availability (Appendix A). I focused my work on six plots—three at N-rich sites (labeled C2, 14Z and 9D) and three at N-poor sites (6N, 10T and 32P). The N-rich sites are mostly hardwood dominated, while the N-poor sites are dominated by conifers. All of these sites are heavily researched, allowing us to use previously collected data in order to know the N-richness of all the sites.

Study Design

10 gallons of organic soil (shallow and including greater than 40% soil organic matter (SOM)) and of deeper mineral soil from each site was sieved to 6mm at each site in the beginning of June. The soil was then brought back to Durham and baked using heat and light from the sun for 48 hours in order to kill all the living fungal biomass in the soil. The light exposure turns the ergosterol to vitamin D; therefore theoretically clearing the soil of all ergosterol. The soil was then sieved to 2mm in order to remove roots and coarse particles. Before inserting the newly processed soil into the cores, an inoculum of active soil consisting of roughly 10% of total soil volume was added to the cleaned soil in order to spark growth; however samples of the cleaned soil and mixed soils were collected in order to analyze how well we removed the ergosterol, and how much was present at time zero. A week after the soil had been processed, I returned to Bartlett and removed two (paired) cores of soil from twelve different points from within each of the six plots, for a total of 144 cores. The cores were made using a sharpened 2-inch diameter PVC pipe, to 30 cm depth, from which the soil was discarded. The

depth of the mineral and organic horizons was measured, and the empty cores were filled with the processed (root and fungal-free) soil to the appropriate depths of mineral and organic soils. At each of the 12 points within each plot, we installed one closed core where a 1.5inch PVC pipe remained in the ground surrounding the added soil, and an adjacent core which was open and marked by three metal rods. Only free-living, saprotrophic fungi would be able to colonize the closed core since the underground network of ECM fungi which rely on root carbon will not be able to penetrate the walls of the core. In the open core both ECM fungi and free living fungi would be able to grow. At the first six of the twelve points per plot, a core of bulk soil was also removed in order to analyze the amount of ergosterol that is in the soil profile at any given time.

The cores were left alone for two months, from July to September, in order to allow for root, ECM fungal, and free-living fungal ingrowth. The soil in the cores were collected at the end of the two months, and were separated by organic and mineral soil.

In the lab, each collected sample underwent a process of ergosterol extraction. All together that included 60 cleaned soil samples (5 per horizon for each site), 60 samples of soil mixed with the inoculum, 72 bulk soil samples, 144 open core samples, 144 closed core samples, and some duplicates and blanks to supplement the data. For this process, 0.5 grams of soil from organic horizons and 1.0 grams of soil from mineral horizons were extracted in a solution of methanolic sodium hydroxide. After centrifugation to separate the solid and liquid phases, pentane was added. Ergosterolreadily dissolves in pentane, separately from other contaminants in the methanol phase (liquid-liquid separation). (Detailed information on the ergosterol extraction method is Appendix B.) Ergosterol extracts were put into glass vials and sent to Kevin Kuehn at the University of Southern Mississippi for quantification of ergosterol concentrations using high performance liquid chromatography (HPLC).

ECM fungal biomass and fungal production for the ingrowth cores is calculated using the following equations:

$$E_{soil} = E_{methanol} / Mass_{soil} \quad (1)$$

$$E_{corehorizon} = Mass_{corehorizon} \times E_{soil} \quad (2)$$

$$C_{fungal/corehorizon} = E_{corehorizon} \times E/C \times 0.40 \quad (3)$$

$$C_{AllocationFungi} = C_{fungal/corehorizon} / \text{incubation time} / \text{core area} \quad (4)$$

where, ($E_{pentane}$) is the concentration of ergosterol per milliliter of Methanol, ($Mass_{soil}$) is the mass of soil used in the extraction, (E_{soil}) is the concentration of ergosterol per the initial mass of soil used for the analysis, ($E_{corehorizon}$) is the total amount of ergosterol in either the mineral or organic horizons of the soil, ($Mass_{corehorizon}$) is the mass of the total soil of the core horizon, ($C_{fungal/corehorizon}$) is the total amount of fungi carbon in either the mineral or organic horizons of the soil, (E/C) is the ratio of erogosterol to fungal biomass which is 3 milligrams of ergosterol per gram of fungal biomass (Clemmensen, Michelsen, Jonasson, & Shaver, 2006; Salmanowicz & Nylund, 1988), 0.40 is the fraction of fungal biomass that is carbon (Henn et al. 2002), ($C_{AllocationFungi}$) is the carbon allocation to fungi for that core soil horizon per area per unit time, (incubation time) will be 60 days, and (core area) will be the inner diameter of the PVC pipe used to collect soils. This analysis will be done for the organic and mineral soil horizons for every core, resulting in twelve values for carbon allocation to fungi in the organic layer and twelve values for the mineral layer at each plot. Equations 1-4 estimate total fungal C production. For closed cores, which exclude ECM fungi, this method will measure only saprotrophic fungal C production. In the open cores where both ECM and saprotrophic growth

occur, ECM production will be estimated by subtracting the amount of saprotrophic growth measured in the closed cores.

Data Analysis

All collected soils were analyzed in the lab to find moisture content and organic matter content. The ergosterol data from the HPLC gave total ergosterol in micrograms per sample. Knowing the weight of the original soil sample, combined with moisture and organic matter content, gave us the amount of micrograms of ergosterol per gram of dry soil and per gram of organic matter. Using the conversion factor of 3 milligrams of ergosterol per gram of fungal biomass we can furthermore find grams of fungal biomass per gram of dry soil or organic matter. Nilsson et al. (2010) show a mycorrhizal and saprotrophic ratio of 0.49 which was used to isolate mycorrhizal fungi. A factor of 0.4 can be used to determine how much of the mycorrhizal biomass is carbon (Hobbie & Hobbie, 2006). Tukey HSD tests were run on all figures in order to measure statistical significance.

Results

Bulk Soil

Figure 1 shows the amount of fungal carbon in grams present at each site. Sites are color-coded by type, where: 10T (mean=608.4) and 6N (mean=574.7) are high elevation low-N conifer dominated sites; 32P (mean=295.76) is a low elevation low-N conifer dominated site; 9D (mean=278.58) is a high elevation mixed hardwood high-N site; 14Z (mean=169.04) and C2 (mean=194.59) are high-N low elevation mixed sites. Figure 2 shows the same sites, but gives an average fungal biomass per gram of organic matter instead of per unit area. The values portray a different story with not much deviation between sites. They all have much higher concentrations

of fungal biomass in the organic horizon. The descending order the sites are as follows: 6N (mean=40), 9D (mean=39), C2 (mean=35), 10T (mean=31), 32P (mean=35), and 14Z (mean=23). The mineral soil values in descending order are: 9D (mean=21), 10T (mean=19), C2 (mean=11), 6N and 14Z (means=10), and 32p (mean=8)

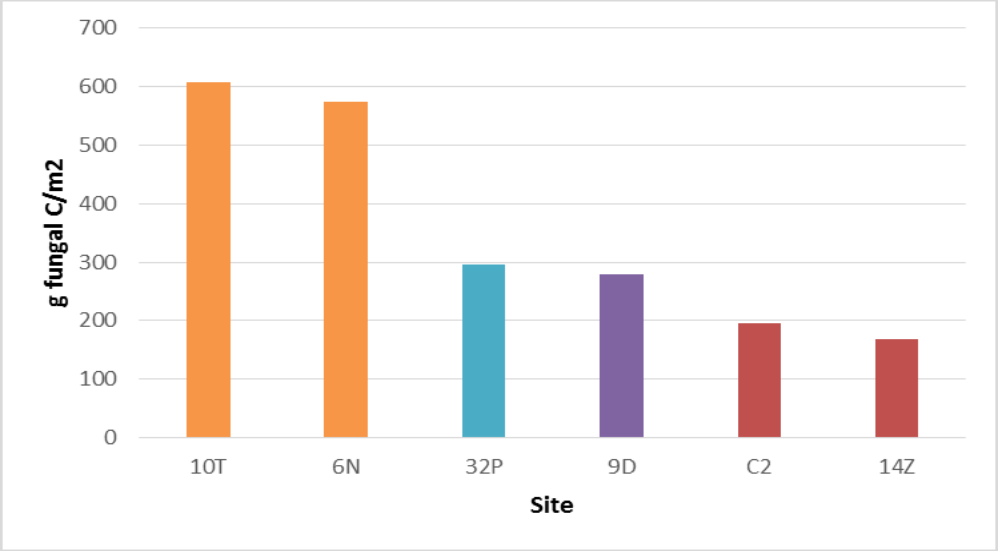

Figure 1. Average amounts of fungal carbon per meter squared for each site

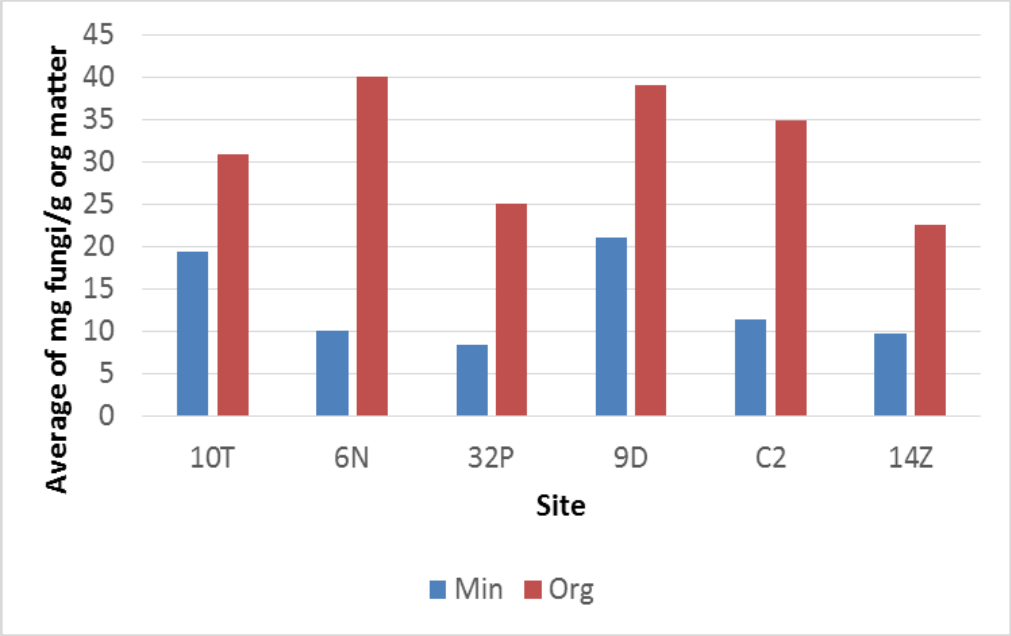

Figure 2. Average milligrams of fungal biomass per gram of organic matter by site

Soil Type Analysis

All the different types of soils used in the study were sampled for their ergosterol concentrations. The t-tests show that, in the mineral soil, the open core type is statistically different than the mixed and pre-inoculum (Figure 3 and Table 1). However, in the organic horizon the bulk soil is statistically different than all the other soil types (Figure 4 and Table 2). Figure 5 (A and B) shows the same analysis as four, but separates the samples by low and high-N sites.

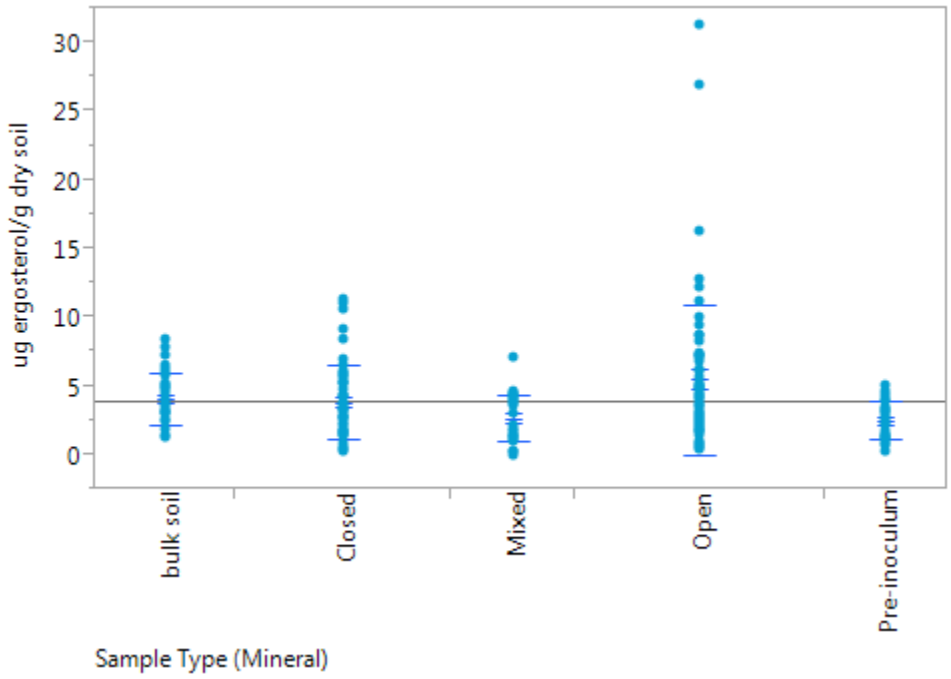

Figure 3. Analysis across soil types of micrograms of ergosterol per gram of dry soil for the mineral horizon

Table 1. Means and std deviations for mineral soil across soil types

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
bulk soil	34	4.05882	1.85425	0.31800	3.4118	4.7058
Closed	53	3.82264	2.67673	0.36768	3.0848	4.5604
Mixed	30	2.67333	1.74513	0.31862	2.0217	3.3250
Open	61	5.47377	5.49339	0.70336	4.0668	6.8807
Pre-inoculum	30	2.49000	1.39565	0.25481	1.9689	3.0111

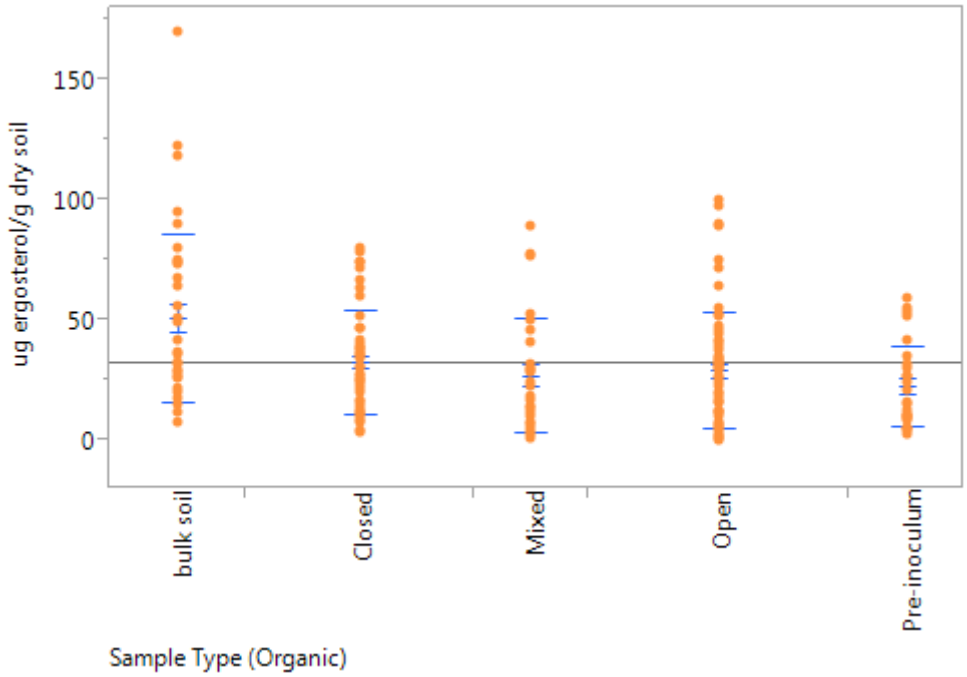

Figure 4. Analysis across soil types of micrograms of ergosterol per gram of dry soil for the organic horizon

Table 2. Means and std deviations for organic soil across soil types

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
bulk soil	36	50.6361	35.2166	5.8694	38.721	62.552
Closed	60	32.4533	21.6420	2.7940	26.863	38.044
Mixed	30	27.0100	23.4713	4.2852	18.246	35.774
Open	69	28.9435	24.2463	2.9189	23.119	34.768
Pre-inoculum	30	22.4733	16.6385	3.0378	16.260	28.686

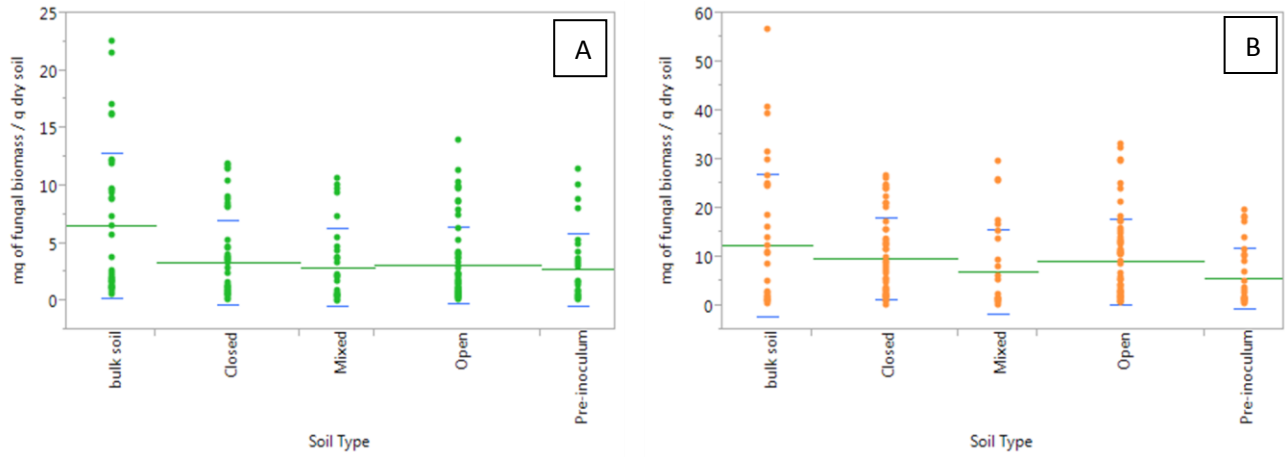

Figure 5.mg of fungal biomass per gram of dry soil, by soil type with (A) showing samples from Low N Sites (B) are samples from High N sites

Production

The ingrowth core ergosterol values give a production estimate versus the standing biomass derived from bulk soil cores. In terms of fugal biomass per gram of dry soil and per gram of organic matter, 10T showed to be the highest in all four scenarios and 14Z the lowest (Figure 6). In order to estimate mycorrhizal abundance using the paired cores we minused the closed core value from the open core value (Table 3). Some of the values are negative and many are low, however, 10T is once again the most abundant site, with 14Z being the lowest.

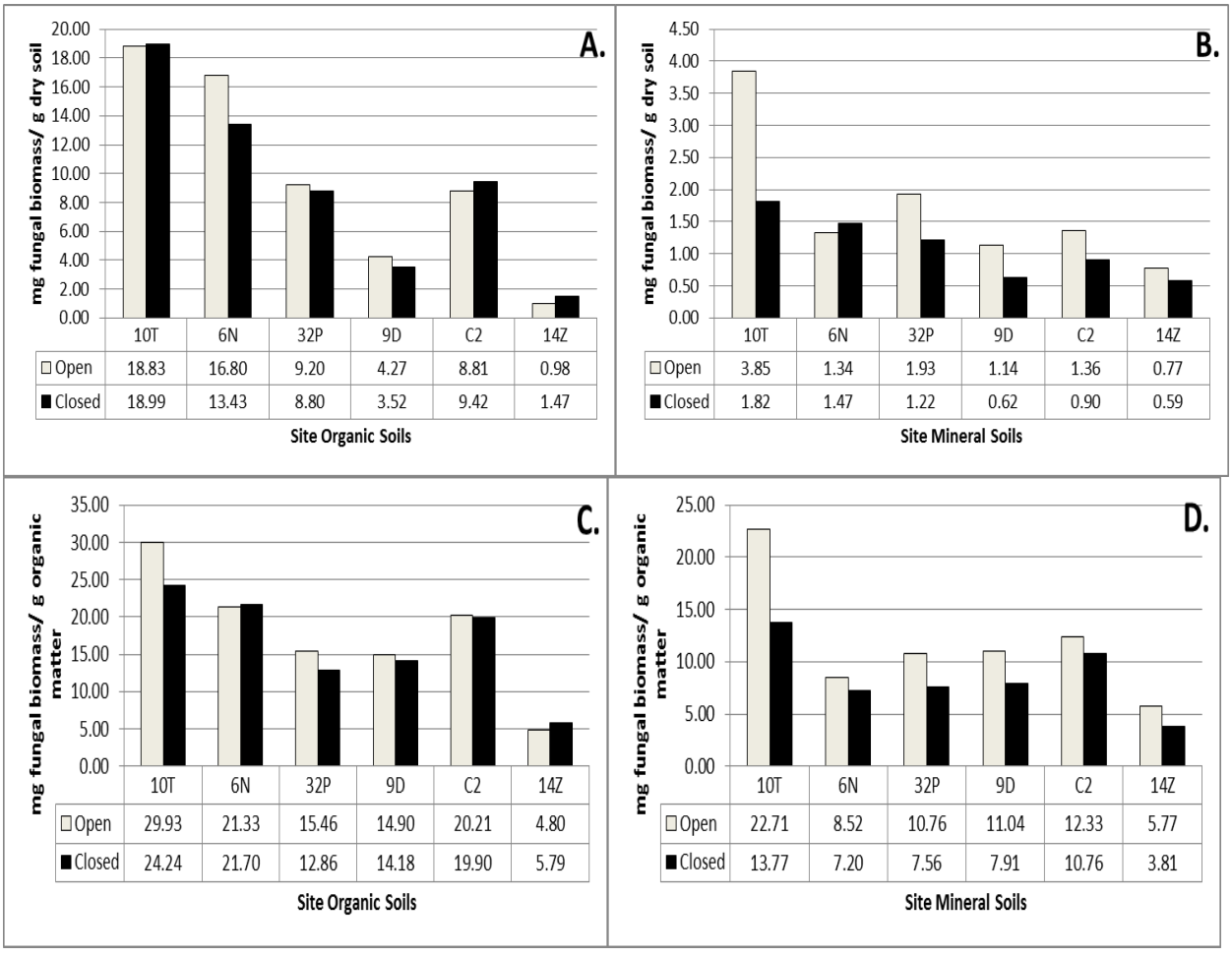

Figure 6. A&B shows the milligrams of fungal biomass per gram of dry soil for each site and C&D shows the milligrams of fungal biomass per gram of organic matter for each site

Figure 7 and Table 3 shows that 10T has the highest amount of fungal ingrowth for the organic horizon, followed by C2, 9D, 6N, 32P, and 14Z. 10T, C2 and 9D show up to be statistically different than 32P, 14Z and 6N. Figure 6 and Table 5 portray a different story where 10T is again the highest, however it is followed by 32P, 6N, C2, 9D, and then 14Z. In Figure 8 and Table 4, 10T is significantly different than 6N, C2, 14Z and 9D; 32P is significantly different than 9D and 14Z; 6N and C2 are significantly different than 10T; 14Z and 9D are statistically different than32P ad 10T.

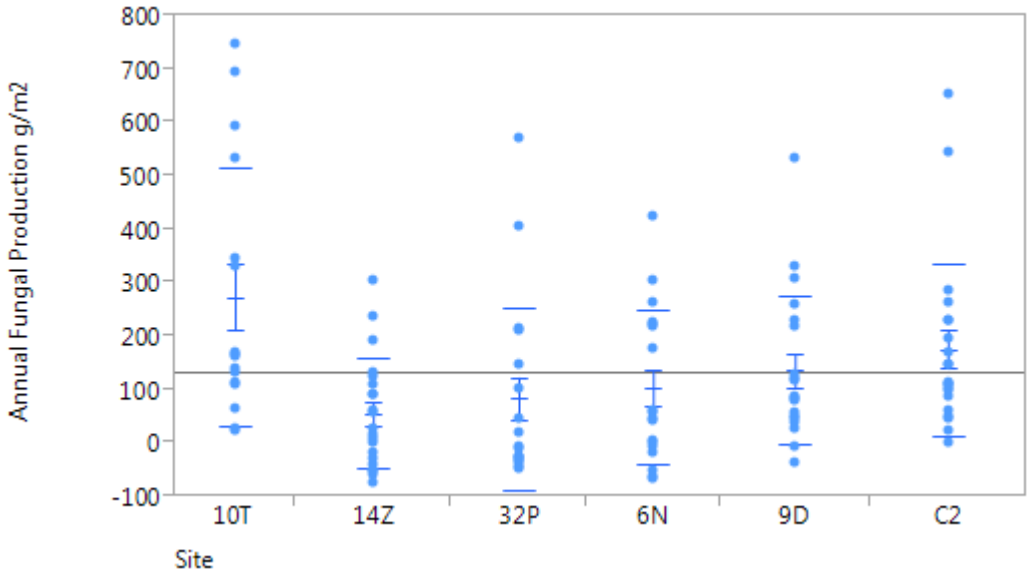

Figure 7. Annual fungal production estimates per meter squared for the mineral horizon and by site

Table 3. Means and standard deviation for the annual fungal production by site (mineral horizon)

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
10T	16	272.868	241.146	60.287	144.4	401.37
14Z	21	54.012	102.989	22.474	7.1	100.89
32P	18	82.337	171.833	40.501	-3.1	167.79
6N	18	102.915	144.736	34.114	30.9	174.89
9D	20	135.345	139.571	31.209	70.0	200.67
C2	21	174.856	161.878	35.325	101.2	248.54

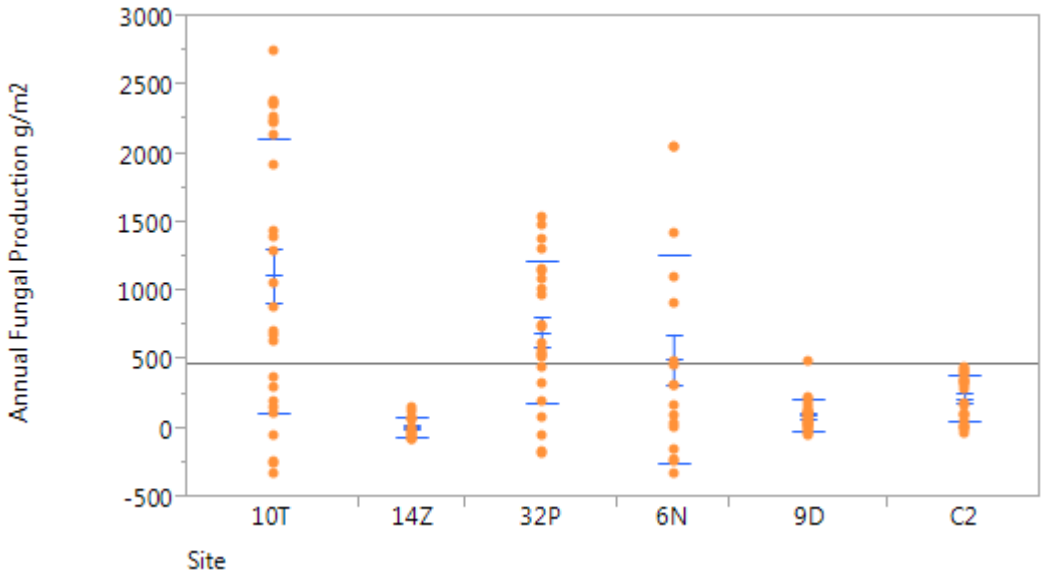

Figure 8. Annual fungal production estimates per meter squared for the organic horizon and by site

Table 4. Means and standard deviation for the annual fungal production by site (organic horizon)

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
10T	26	1117.30	998.777	195.88	713.9	1520.7
14Z	16	11.10	69.491	17.37	-25.9	48.1
32P	23	698.92	518.351	108.08	474.8	923.1
6N	17	502.22	758.000	183.84	112.5	892.0
9D	24	95.66	112.225	22.91	48.3	143.1
C2	23	220.35	164.509	34.30	149.2	291.5

Using the productions values in the ingrowth cores, we were able to estimate yearly production. First, the statistical significance of different data comparisons were tested including: grams of fungal carbon per gram of dry soil, gram of fungal carbon per gram of organic matter, and grams of fungal carbon compared to moisture content. The strongest relationship, found with doing stepwise modeling was found when comparing fungal carbon to grams of organic matter. Using this information we scaled up the grams of fungal carbon per gram of organic matter by multiplying it by the total amount of organic matter in a meter squared. We then separated what was ECM, and what was saprotrophic. The results of this analysis are shown in table 5.

Table 5. Estimated grams of fungal carbon per meter squared using ingrowth values.

	Both	Saprotrophic	ECM	Percent ECM
High-N	137.59	28.99	108.61	0.79
Low-N	675.84	367.61	308.23	0.46

Fungal and SOM relationship

Compared to all other parameters, there was a strongest relationship between fungal biomass and organic matter (Figure 9). The relationship we found in our data is compared to a literature value from Hobbie et al. (2009) where the same type of comparison was made.

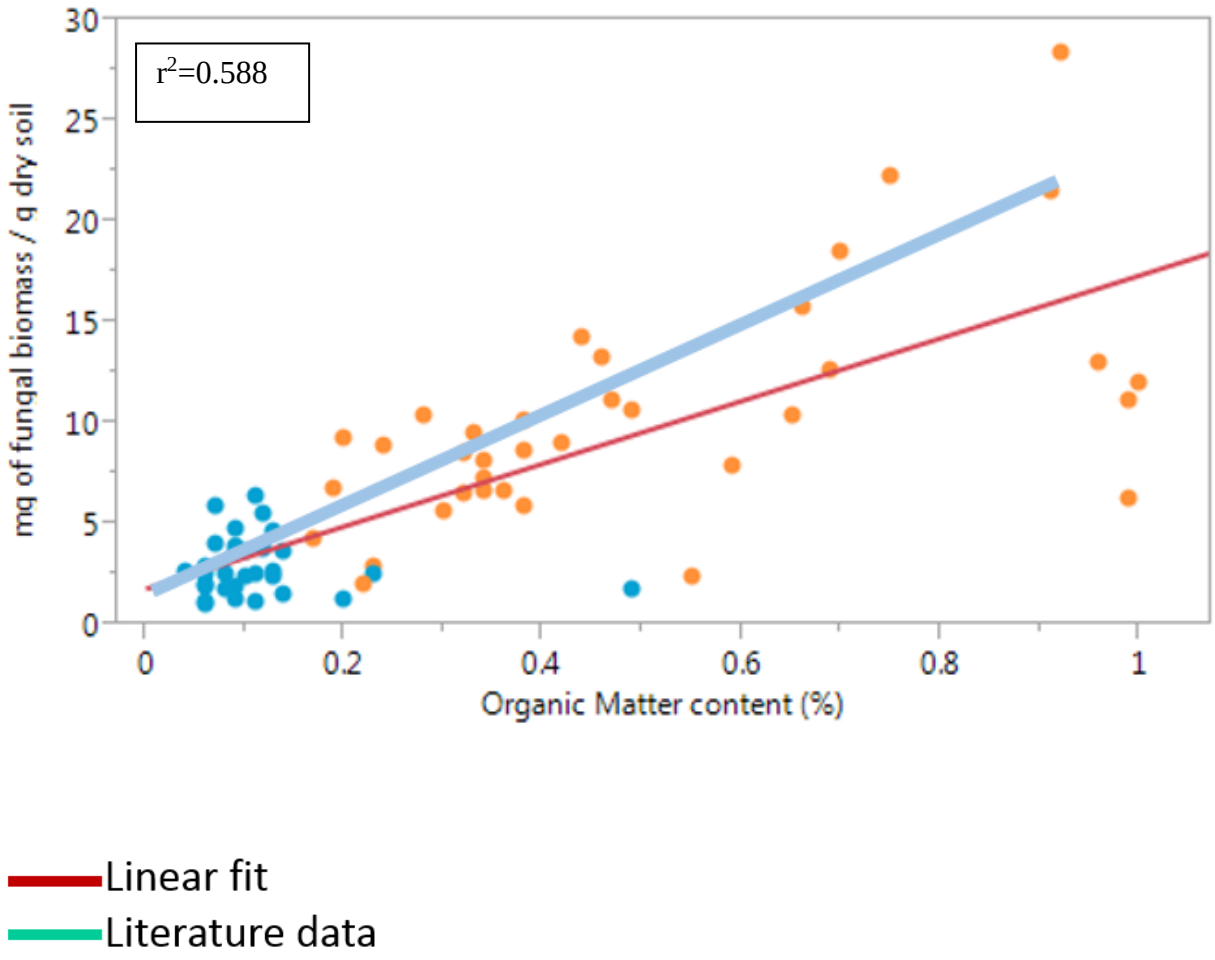

Figure 9. Regression of soil fungal abundance versus soil organic matter (SOM). Literature data adapted from Hobbie et al. (2009)All of the bulk soil samples from the six sites were used in Figure 9 to show the tight relationship between soil fungal biomass and soil organic matter. The mineral samples are shown in blue and the organic in orange.

Discussion

Fungal Biomass

The low nitrogen sites such as 10T, 32P, and 6N had overall higher values of fungal biomass in the bulk cores when compared to the high nitrogen sites such as 9D, C2, and 14Z. The low-N sites had 1123 grams of fungal biomass in the organic layer and 87.5 grams in the mineral layer per m^2 (Appendix data) . The high-N sites had 380 grams in the organic and 154 in the mineral. Figure 1 shows the gradient of sites from low-N conifer and high elevation sites to high-N hardwood and low elevation sites, and it is simple to see that the amount of fungal biomass decreases across the gradient. Figure 2 offers an explanation for this that ties the amount of fungal biomass to organic matter, where all sites are relatively similar in the amount of fungal biomass per gram of organic matter. In order to turn this data to a ECM estimate we use the saprotrophic and mycorrhizal ration found in Nilssonn et al. (2012) of 0.49. This reduces the low-N biomass to 550 for organic and 43 for mineral, and the high-N biomass to 186 for organic and 76 for mineral.

Mycorrhizal Production

In order to find mycorrhizal biomass we used a system of paired cores that would theoretically deduce the amount of mycorrhizal ingrowth while accounting for saprotrophic ingrowth. We know that the open minus closed values did not completely agree, as we had an entimated 50 samples give negative results when using the fungal biomass per dry soil values. However, using fungal biomass per gram of organic matter agreed with the values shown in Hobbie et al. (2009), Wallander et al. (2004), and Clemensen et al. (2006). The mycorrhizal biomass in mg per gram of organic matter ranges from 29.9 at 10T and 4.8 at 14Z for the organic horizon and 19 to 5.7

for the mineral horizon (Appendix data). Wallander et al. (2013) shows us how mycorrhizal ingrowth works on a periodic and yearly scale. From this we can estimate that the July to September ingrowth period is about 1/3 of the mycorrhizal growing capacity. 10T once again has the highest annual mycorrhizal productivity with 1117 grams per m^2 in the organic and 272.7 in the mineral soil (Tables 3&4). The low N site of 14Z has the lowest with 11 grams in the organic and 54 in the mineral (Tables 3&4). Table five estimated the production for low-N and high-N sites showing an interesting trend. The most surprising of which is that the high-N sites had an ECM percentage of 78%, compared to the 45% of the low-N sites. We expected the high-N sites to have a lower percentage, because they have some non ECM dependent trees such as maples (*Acer spp*); however this anomally may be minimal as the sites as mostly American beech (*Fagus grandifolia*) dominated which is still an ECM reliant tree species. The low–N sites did still have an overwhelmingly higher amount of fungal carbon compared to the High-N (Table 5).

Fungal Carbon and SOM

As seen in Figure 9, there is a very close relationship between fungal biomass and SOM (r^2 of 0.588) Most of the data agrees with the fact that the more organic matter a horizon has, the higher the amount of fungal biomass. The relationship per gram of organic matter is much better than that of per gram of dry soil. Hobbie et al. (2009) shows the same relationship with their ergosterol analysis (r^2=0.76). The Hobbie et al. (2009) data shows a range of 3 to 303 micrograms of ergosterol per gram of orgaic matter, which overlaps with the 50-160 micrograms of ergosterol to gram of organic matter seen by Clemmensen et al. (2006). Our data also overlaps with both sets with a range of 12-185 micrograms of ergosterol per gram of organic matter (Appendix data).

Ergosterol Difficulties

The Soil type analysis including figures 3 and 4 show us a fundamental problem with our current understanding of ergosterol. The literature stresses that one must be very careful when dealing with ergosterol samples as it degrades quickly when in the presence of heat and light (Wallander et al. 2013). Clemmensen et al., (2006) urges the importance of storing ergosterol samples at -20 degrees celcius. Figures 3 and 4 and Tables 1 and 2 show ergosterol values for the processed soils which we put into the ground (labeled mixed and pre-inoculum). The average amount of ergosterol in micrograms per gram of dry soil is 22.4 for organic soil and 30 for mineral soil (tables 1 and 2). This means that the soil processing meant to relieve the soil of any ergosterol did not work. It is believed that the only truly effective way of relieving the soil from its ergosterol is by auto claving the soil with a certain humidity (personal communication with Kevin Kuehn). There has been a debate in the literature as to whether ergosterol measure only living biomass(Mille-Lindblom et al.,2004) , and I believe this study confirms that ergosterol can still be present after the fungi is no longer living.

Another fundamental issue was that when we minused this value from the ingrowth, we received several negative numbers (as seen in the last column of Appendix K). It is implausible to have negative values unless you lost ergosterol instead of having any ingrowth.A loss of ergosterol can be explained by competition by incoming microbes, since the soil was void of all living organisms.

Conclusions

Fungal biomass is strongly positively correlated with soil organic matter. We can see a correlation between mycorrhizal biomass and production, and the nitrogen and tree species gradient; where, low-N high elevation coniferous sites have more fungal carbon than low elevation high-N hardwood sites. Our study shows that ergosterol is not as fragile as the literature shows; after drying the soil and giving it ample amounts of sunlight, ergosterol was still present in relatively high concentrations. A better understanding of the mycorrhizal and saprotrophic ratio can allow us to better quanitfy production and biomass.

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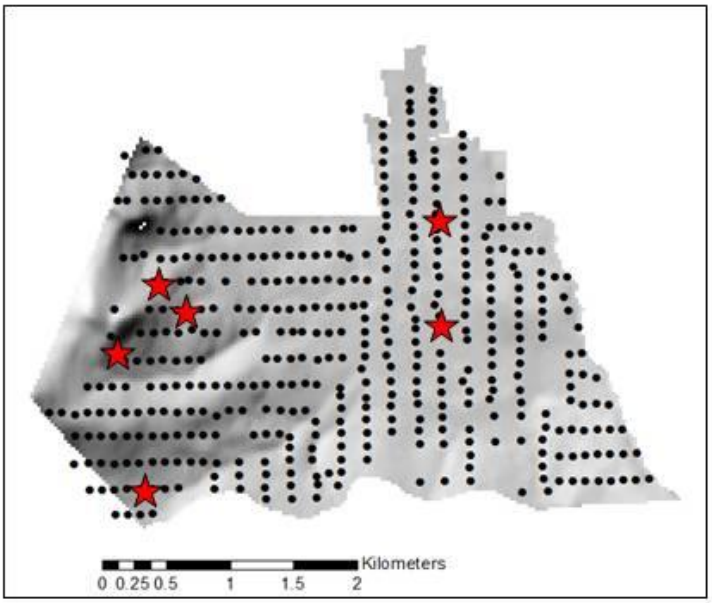
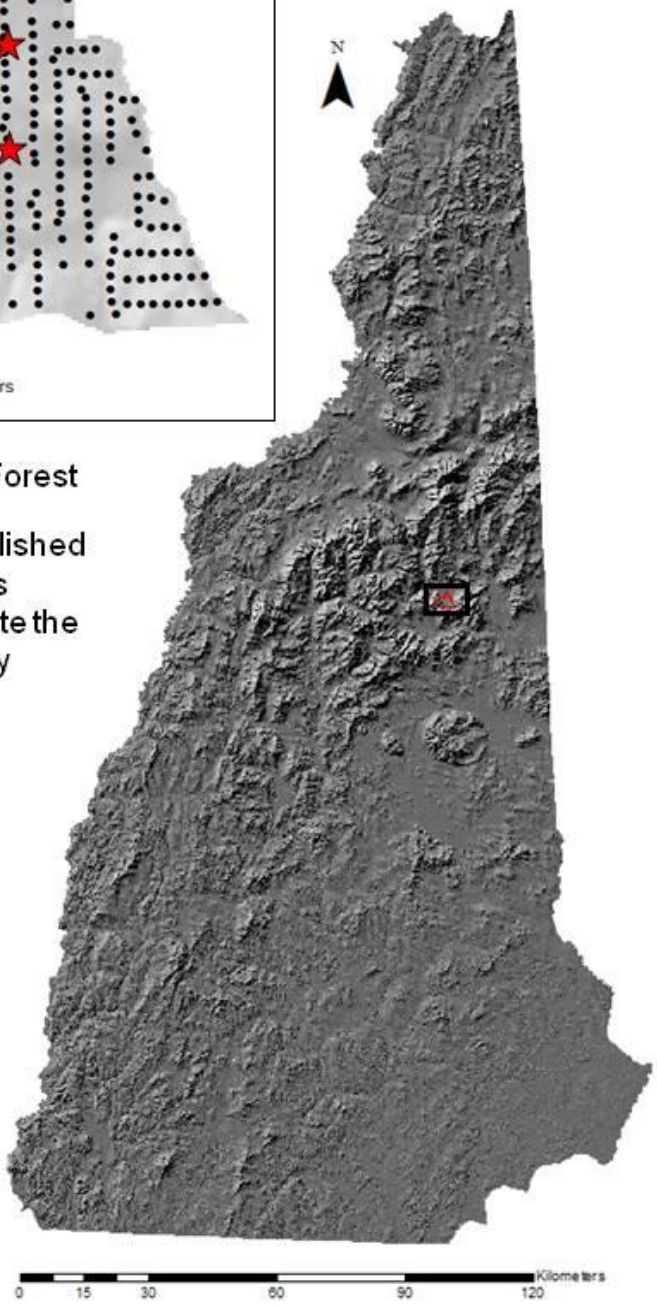
Appendix A



Inset: Bartlett Experimental Forest

Black dots indicate the established network of experimental plots within the forest. Stars indicate the six plots where I will focus my work.



Appendix B

Ergosterol Procedure

1. Weigh out sample (sieved) 0.5g soil *(organic)*, 1.0g sand *(mineral)*, 5.0 g rocks usual masses
    a. *Set up an excel spreadsheet with the original sample ID, how much was weighed out for ergosterol extraction and the ergosterol label*
    b. Use the screw cap test tubes that have a gap between the neck and the screw part, set up in a test tube rack by rows of ten
    c. *Ergosterol is typically labeled by letter and number1-9, with a control every tenth sample. The first nine samples would be A1-A9 with AC as the control. The next nine samples would be B1-B9 and BC as the control. 18 samples (plus 2 controls) is usually a good number to work with, especially if new to the process*
2. Add 2 mL MeOH and 0.5 mL 2M NaOH
    a. *Use the 1mL pipet, change tips between chemicals*
3. Cap and place in the hotbed for 90 mins at 70C
    a. The hot bed is the hot water bath, only use under the hood, raise the gas injection plate (not needed for this part). 70C is the heat setting 7 on the dial
    b. This is a good place to stop for the day, the next part can be lengthy (at least 2 hours) if stopping, wrap the test tube rack in aluminum foil, label and put in the freezer
4. Add 1 mL of MeOH
5. Add 3 mL pentane, vortex for 20 seconds, centrifuge, collect top layer
    a. *Use different tips for pentane and MeOH*

   b. *Centrifuge settings are speed 2000 time 2 temp 24*
   c. *Collect top layer using glass pipettes*
6. Repeat step 5 twice more each with 2mL washed of pentane.
7. Place all collections in marked vials/test tubes
   a. *Use non screw top test tubes, mark with label from test tube in previous step*
8. Blow down with N2 or He
   a. *Make sure the gas plate is fully lowered*
   b. *Once the three extractions are done and the extracted parts are being evaporated in the new test tubes, dump excess dirt, sand, ect from the old tube into a hazardous waste container*
9. Bring back up with 1mL of MeOH by weight (~0.792g~101 μL)
10. Vortex 10 seconds
11. Pipette to 90-95% into small vials, cover tray with foil and freeze

For cleaning:

Ergosterol glassware gets muffeled.

-Soak glassware in soapy water for 30 minutes, scrub out and rinse, make sure all debris is removed

-Soak in DI for 30 minutes and rinse in DI, wrap in foil

-Muffle at 500C for 5 hours once the muffler gets to temp, then allow to cool

Test tube caps get sonicated:

- put caps in beaker with water and dish soap, sonicate for 30 min with heat

Appendix C

Ergosterol and Isotope Overview:

Significance of Measuring Mycorrhizal Biomass and Abundance

With the numerous benefits that mycorrhizae provide to its host plants and communities, such as providing access to nutrient pools and being a main pathway for uptake (Read and Smith, 2008) it is important to understand how much mycorrhizal fungi there truly is. Mycorrhizae also play a major role in the carbon (C) cycle of forests (Read and Smith, 2008) Plants with mycorrhizal symbiosis allocate an estimated 10-50% of their net primary production (NPP) to the mychorrhizae (Simard et al.,2002), and although 75% may contribute to soil respiration (Hogberg et al., 2003), some may become long lived in the soil and sequestered away from the atmosphere (Aber et al., 1998). Mycorrhizae are great contributors to the C cycle and C sequestration of forests, but only a few studies have focused on the environmental effects on mycorrhizal mycelia, due to a lack of methods to accurately distinguish mycorrhizal myelia from other fungi (Nilsonn et al., 2012).Correct measurements of ECM are needed to be accurate in describing the C cycle of terrestrial ecosystems (Nilsonn et al.,2012), since there is a major lack in the understanding the below ground C flux (Litton and Giardina, 2008)

Overview of Ergosterol

Ergosterol is the dominant sterol in most fungi and Olsen (1973) found that it is mainly in the plasmalemma of the fungi and that there is a 1-7mg sterol/g mycelium conversion factor when in pure culture. Basically ergosterol is the fungal equivalent of cholesterol in humans. Ergosterol is found in ectomycorrhizal (ECM) fungi which is the dominant mycorrhiza in temperate and boreal forests when compared to arbuscular mycorrhizal (AM) fungi which is predominantly found in the tropics and sub-tropics (Read and Smith, 2008 ; Nilsson et al., 2012).

Study Designs

Most common way to measure mycorrhizal production is to use root free ingrowth bags (Wallander et al., 2001; Hendricks et al., 2006; Korkama et al., 2007; Hedh et al., 2008). Not all ingrowth bag studies are the same or measure for the same variables, but all assume that ingrowth is primarily mycorrhizal, and this has been verified through trenching and DNA analysis (Nilssonn et al., 2012; Hedh et al., 2008). Ingrowth bags are primarily made of 25-50 micron vinyl mesh, which have pores large enough for mychorrhizae, but not roots (Wallander et al. 2001). Most studies use mineral sand to ensure no previous fingerprints of mycorrhizal are present, however it has been shown that different mycorrhizal species do not colonize the bags and they overall prefer soil as a medium instead of the sand (Nilssonn et al., 2012; Hendricks, 2006). Sandbags are usually put in the soil profile at a suggested 45 degree angle at different depths, however optimal mycorrhizal production occurs at the innerface of organic and mineral soil (Wallander, 2001; Nilssomm et al., 2012; Lindahl et al., 2007) These sandbags studies can measure the ingrowth of carbon, ergosterol, chitin, observable mycelia/hyphae, phospholipid fatty acids (PLFAs), and any other biomarkers (Nilssonn et al.,2012). The size of the ingrowth period also changes throughout he studies depending on the focus of their question (Nilsonn et al., 2012)

Ergosterol Assay

Most ergosterol studies have designs as that described above and explained in (Wallander, 2001; Wallander, 2004; Salmanowics and Nylund, 1998; Clemmensen and Michelsen,2006) Ergosterol is extracted from the sand or soil substrate into a highly volatile media, and this stage is repeated numerous times. The suspended ergosterol (free ergosterol) is then blown down by gas, and brought back by alcohol. The samples are ran through a High Performance Liquid Chromatography (HPLC) and the amount of chromatograms is measured under UV light. The

results are a raw amount of free ergosterol. This is converted to fungal biomass through a conversion factor of 3 micro grams of ergosterol per mg of fungal biomass (Salamanowics and Nylund, 1998). This can be extrapolated from mg of fungal per gram of sand/soil to fungal biomass per cm^3, and further to grams of fungal biomass per section of soil profile (Wallander et al., 2004).

Other Methods

The simplest way to quantify fungal biomass in mesh bags is to burn the sand at high temperatures and the loss in weight is used to estimate biomass (Korkama et al., 2007; Hagerberg et al.,2003). The C concentration of fungal material is almost always around 43% (Taylor et al., 2003), this can be used to extrapolate fungal biomass. One problem with this method is that bacterial growth and any other forms of carbon could skew the C amounts. More conventional methods also include isolating the mycelia from the sand or soil substrate and simply quantifying it (Nilssonn et al., 2012).

PLFAs are a major component of cell membranes and they decompose quickly after death (Nilssonn et al., 2012). They have been used as a biomarker for soil fungi, but have been recently been questioned since, some different bacteria and organism groups also contain the same PLFAs, therefore positively skewing the data and making PLFAs less accurate (Frostegård et al., 2011). There are different PLFAs present in different organism groups, but there is too much overlap to confidently say that PLFA measurements from ingrowth bags come from mycorrhizal (Cavigelli et al., 1995). Whole cell fatty acids have also become a biomarker for microbial biomass (Nilssonn et al. 2012).

Chitin is another biomarker popularly used in many studies, but measurements can be flawed because some soil organisms such as microarthropods have chitin in their exoskeletons, although roughly only 0.5% of biomass (Simpson et al., 2004). Chitin does however represent a significant portion of fungal biomass, roughly 5% (Appuhn and Joergensen, 2006). Chitin, like most of the biomarkers, is flawed in the fact that it can not distinguish between saprotrophic fungi (SAP) or ECM fungi (Nilssonn et al. 2012). Chitin also cannot distinguish between alive and dead fungi, meaning that soil does not make for a good substrate in Chitin studies (Nilssonn et al., 2012). Chitin has been used in numerous studies by isolating glucosamine with the use of strong acids and measuring the free glucosamine through chromatographic techniques (Appuhn et al., 2004; Zamani et al., 2008).

Benefits of Ergosterol Analysis

Using ergosterol has by far been the most accepted and used method of quantifying ECM biomass as seen in the amount of studies done (Nilssonn et al., 2012). Ergosterol is very sensitive to light which could lead to underestimations of fungal biomass, especially when combined with the expected underestimations in sandbag studies (Nilssonn et al., 2012). However, the sensitivity to light could allow for soil to be cleaned of ergosterol and used accurately in ingrowth core studies. Using ergosterol is more time consuming than conventional studies, but is comparable to the resources needed for chitin and PLFA analysis and cheaper than DNA (Salmanowics and Nylund, 1998). Ergosterol can also be used to better understand changes in biomass under different conditions through continuous studies, and when combined with other methods may give a very accurate representation of the mycorrhizal community. The largest downfalls seen are its sensitivities and the fact that it can’t identify a difference in SAP or ECM fungi.

Isotope Review

Another promising alternate technique to measure carbon allocation to ECM fungi under natural conditions is the use of stable isotopes. For example (Craine et al., 2009) used natural nitrogen isotopic ratios (15N:14N, referred to as $\delta^{15}N$) to clarify the nitrogen cycle and reveal the symbiotic relationship between ECM and plants involving nitrogen. (Craine et al., 2009) found that isotopic techniques may be able to quantify the degree of reliance by plants on ECM fungi, because ECM fungi changed the $\delta^{15}N$ from the soil to the plant by an average of 3.2‰ Meaning that the more the plant relies on mycorrhizal fungi the more the nitrogen ratio ($\delta^{15}N$) changed from the soil to the plant.

Background on Isotopes

Isotopes are a variant of an element, where although all atoms of an element have the same number of protons, different isotopes will have a different number of neutrons. Measuring the relative abundance of stable isotopes has become a popular research tool, and has lead way to the field of stable isotope geochemistry. These isotopes are analyzed using a mass spectrometry instrument, which separates different isotopes by measuring their mass and charge, and calculates the abundance of these isotopes. The most common elements used in stable isotope analysis to understand natural systems are nitrogen, carbon and oxygen.

Isotope Method

The process that uses stable isotopes to measure mycorrhizal carbon allocation is described in Hobbie & Hobbie (2008). Specifically, the ^{15}N:^{14}N ratio ($\delta^{15}N$) of root and soil material will first be used to estimate the nitrogen transfer ratio (Tr), which is the fraction of N assimilated by ECM fungi that is transferred to the tree. (1-Tr) is the fraction that remains in ECM fungal biomass and is calculated following Hobbie et al. (2008) as:

$$(1\text{-}Tr) = (\delta^{15}N_{Soil} - \delta^{15}N_{plant})/\Delta f \quad (5)$$

where, $(\delta^{15}N_{Soil})$ is the $\delta^{15}N$ measured in the soil, $(\delta^{15}N_{plant})$ is the $\delta^{15}N$ measured in tree roots, and (Δf) is a fractionation constant, typically with values between 8-10‰. Once Tr is calculated we will use the following equation (from Hobbie & Hobbie, 2008) to estimate carbon allocation to ECM fungi:

$$C_{fungal} = (1/T_r\text{-}1) \times N_p \times C/N \times (1/e) \quad (6)$$

where, (C_{fungal}) refers to the carbon allocated to ECM fungi, (N_p) is the total amount of nitrogen uptake by the plant, (C/N) refers to the ratio of carbon to nitrogen of fungi (typically between 10-20), and (e) is the microbial efficiency (typically around 0.50 or 50%).

Using natural nitrogen isotope ratios, the carbon allocation to mycorrhizal fungi by trees could be derived by using a series of equations proposed by Hobbie & Hobbie (2008).

Conclusion

The best way to accurately measure abundance, biomass and carbon allocation to mycorrhizal fungi is by employing several different methods (Nilssonn et al., 2012). Most of the methods have limitations that must be accounted for, and can be minimized through the employment of the different methods with different strengths and weaknesses, including but not limited to chitin, ergosterol, DNA, stable isotopes and PLFA analysis. The most apparent issue in all methods is the lack of ability to separate and determine a difference between SAP and ECM fungi (Nilssonn et al., 2012). Understanding belowground carbon dynamics is important for scientists to model our environment under current and future conditions; with ergosterol being such a major portion of the overall flux, its carbon values cannot be ignored.

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