



The Effect of Parent Material on Ectomycorrhizal  
Community Composition and Diversity

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## **Abstract**

Nutrient availability is one of the most important components of soil chemistry, and has been shown to strongly impact ectomycorrhizal community composition and diversity. Phosphorous, magnesium, and potassium are important macronutrients for plant growth. Tree growth in the boreal and temperate forests is immensely dependent on their fungal mutualistic partners for their nutrition, particularly nitrogen and phosphorous. Studies have found that prolonged additions of nitrogen, sulfur and liming have altered the ectomycorrhizal community composition and diversity. In this thesis, I examined the effects of different nutrients' availability on the ectomycorrhizal community composition and diversity by comparing the communities on three different soil types which are derived from three contrasting parent materials. The granite bedrock is poor in magnesium and rich in potassium, the serpentinite is highly rich in magnesium and poor in potassium, and the amphibolite is fairly moderate in magnesium and rich in calcium. 798 sequences were successfully identified from 1392 ectomycorrhizal root-tips which were collected from soil cores. 37 OTU's were successfully identified from the 798 successful sequences. Non-parametric multi-dimensional scaling (NMDS) demonstrated that the ectomycorrhizal community composition were not appreciably different between the granite and amphibolite but was significantly different in the serpentinite. The ectomycorrhizal community composition was slightly different between the organic and mineral horizons in the granite and amphibolite, but was markedly different in the serpentinite. Rarefaction analysis demonstrated that the granite ectomycorrhizal diversity was significantly less than serpentinite and amphibolite ectomycorrhizal diversity. In this study the distinct difference in magnesium, calcium, and potassium availability between the granite and amphibolite was not associated with any significant difference in ectomycorrhizal community composition while the distinct difference in potassium, magnesium and phosphorous availability between the serpentinite and the other catchments was associated with a strong effect on the ectomycorrhizal community composition in both horizons. **Key words:** ectomycorrhizal fungi, granite, serpentinite, amphibolite, community composition, diversity, organic horizon, mineral horizon

# 1. Introduction

## 1.1 Ectomycorrhizal fungi and their role in the forest ecosystem

Ectomycorrhizal fungi (EMF) are a major functional group of soil organisms that form intimate mutualistic relationships with tree roots, supplying their partner-roots with nutrients and receiving carbohydrates from their plant-partners. It has been described as a plant adaptation to colonize soils where nutrients are bound in organic compounds (Read and Perez-Moreno, 2003). Plants roots cannot access many of the nutrients that are bound in organic forms whereas the mycelium of EMF fungus can get access to these organic nutrients by excretion of extracellular enzymes (Read and Perez-Moreno, 2003). EMF are primarily members of the two major divisions Basidiomycota and Ascomycota, and are a very species rich group, with an estimated 7-10000 fungal and 8000 plant species engaged in EMF symbiosis (Taylor and Alexander, 2005). Ectomycorrhizal fungi are the dominant mycorrhizal type of temperate and boreal forests where more than 95% of tree roots are colonized by EMF (Taylor et al., 2000). They predominate in the upper, organic-rich soil layer where organic matter accumulates (Meyer 1973; Harvey et al., 1976; Stober et al., 2001), though Rosling et al. (2003) found that two thirds of the collected EMF root-tips were located in the mineral horizon of a podzol soil. Ectomycorrhizal fungi play significant roles in a number of different ecological processes that govern the productivity and integrity of the forest ecosystem. Nitrogen (N) is the most commonly limiting nutrient in the boreal forest ecosystem for plants growth (Barbour et al., 1987); most N is sequestered in organic complexes (Leake and Read, 1997). Abuzinadah and Read (1986) showed that ectomycorrhizae were able to mobilize nitrogen from the organic N pool by producing potent acid-carboxypeptides. Hobbie and Colpart (2003) showed that up to 80% of the total N that was assimilated in the host plants came through their ectomycorrhizal partners. Additionally, other studies have demonstrated that EMF transferred significant amounts of P (Ekblad et al., 1996), Mg (Jentschke et al., 2000), and K (Van scholl et al., 2006) to their host plants. Abuzinadah et al. (1986) suggested that the ability of ectomycorrhiza to mobilize and utilize organic nitrogen would tighten the nitrogen cycle by increasing the effective competition with the decomposer populations. The intensive spread of ectomycorrhizal fungal mycelia increases the effective absorptive surface area of the root system and may link the roots of different trees

species together. Amaranthus and Perry (1994) showed that the hyphal networks of ectomycorrhizal fungi allow carbon and nutrients to pass between different plant species which may support the re-establishment of forests after disturbances. EMF are an essential component of the forest ecosystem; protecting host-roots from pathogens and environmental extremes, and maintaining soil structure (Harely and Smith 1983; Perry et al., 1989). The high production of oxalic acids by EMF under elevated levels of Al may be a response to decrease the toxicity of these metals (Ahonen-Jonnarh et al., 2000).

## **1.2 The role of ectomycorrhizal fungi in the uptake of mineral nutrients, especially P, K and Mg**

Plants nutrients, excepting N, C, O and H are largely derived from the weathering of primary minerals (Landeweet et al., 2001). Phosphorus is the second most common limiting mineral nutrient to the boreal forest trees (Dala, 1977). As much as 90% of soil phosphorus may be sequestered in organic compounds in the boreal forest (Cosegrove, 1967). Ectomycorrhizal fungi play a significant role in enhancing the uptake of P in forest trees. Brandes et al. (1998) showed that 76% of the total phosphorus of the norway spruce (*Picea abies*) seedlings were provided by EMF when inoculated with *Paxillus involutus*, and that the addition of phosphorus increased the EMF growth. Moreno and Read (1999) demonstrated the role of ectomycorrhizal phosphorous uptake by their ability to mobilize plant litter P; when they grew some mycorrhizal plants with litter and the others without, roots and shoots content of mycorrhizal plants growth with the litter exhibited 3 and 4 times, respectively, more phosphorous than the ones without litters. Plants deficient in phosphorus exhibit increased carbon allocation to the roots and associated mycorrhizal fungi (Wallander and Nylund, 1992). Placing 1% of apatite into sand-filled mesh bags stimulated the production of mycelium in a forest deficient in phosphorus (Hagerberg et al., 2003). Moreover, Potila and Wallander (2008) showed apatite and biotite addition increased the biomass of extrametrical mycorrhizal mycelia in a peatland forest which was deficient in both phosphorus and potassium. Jentschke et al. (2000) showed that the hyphae of *Paxillus involutus* presented significant amounts of Mg to its host Norway spruce seedlings. Mg deficiency has been shown to decrease the photosynthetic activity in the leaves of birch seedlings as well as decrease the root biomass (Ericsson and Kahr, 1995 and Ericsson, 1995). Wallander (1999) showed that *Pinus sylvestris* seedlings associated with EMF were able

to dissolve the provided biotite to get access to the potassium, however EMF did not show a positive effect in potassium uptake when k-containing biotite was added to a potassium poor forest (Hagerberg et al., 2003). There is ample evidence that EMF do play a significant role in releasing mineral nutrients through stimulation of weathering of insoluble minerals (see review by Hoffland et al., 2004). Lapeyrie et al. (1991) showed that different EMF species were able to solubilize insoluble phosphates and calcium when grown in vitro on agar plates. In another study, *Fagus sylvatica* seedlings that were inoculated with the ectomycorrhizal fungi *Laccaria laccata* increased uptake of potassium and magnesium when mica (phlogopite) rock was added and this was attributed to the excretion of organic acids (Leyval and Berthelin, 1989). EMF can actively solubilize insoluble minerals through their ability to produce low molecular weight organic acids (such as oxalic acid characterized by its high acid strength and complex formation with many different base cations), their proton exudation (either active or passive, a result of cation uptake), and, potentially, their ability to exude siderophores (which may increase weathering losses of Al or Fe, and concomitantly, losses of other mineral elements) or other ligands(see review, Landeweert et al., 2001).

### **1.3 Factors affecting the diversity and composition of ectomycorrhizal fungi**

Ectomycorrhizal fungi is a diverse group of soil organisms in the forest ecosystem. In one study, 172 ectomycorrhizal fungal species were observed in a study assessing the ectomycorrhizal diversity and structure in a wooded meadow (Tedersoo et al., 2006). Species richness in monodominant forests is often over 100 EMF species (Parrent and Vilgalys, 2007; Taylor et al, 2010). The high diversity of nutrient resources in soils, interspecific competition between EMF for roots, and soil heterogeneity may be some of the key factors maintaining the diversity of EMF communities (Bruns, 1995). Many studies have demonstrated the impact of many biotic and abiotic factors on the EMF fungal community composition and diversity (Arnolds, 1991; Avis et., al 2008; Kernaghan et., al 2003; Claridge et., al 2000). Nitrogen availability is one of the major factors that affects EMF community composition and diversity. Arnolds (1988) cited nitrogen deposition as the most important factor in the decline of ectomycorrhizal fungi in Europe. Ectomycorrhizal community composition has been dramatically changed and ectomycorrhizal species diversity strongly decreased when forests have been treated with high amounts of nitrogen (Kjoller et al, 2011, Cox et al, 2010, and Lilleskov, 2002).



Soil acidification and accumulation of organic matter are among the natural factors that affect the EMF communities which may also be accelerated by the deposition of pollutants (Arnolds, 1991). Stroo and Alexander (1985) showed that EMF colonization decreased at pH values less than 3.5 in a laboratory study. Air pollutants increase the acidity of soil since SO<sub>x</sub> and NO<sub>x</sub> react with moisture in the atmosphere forming acid rain (HNO<sub>3</sub> and H<sub>2</sub>SO<sub>4</sub>), while the uptake of aerielly deposited NH<sub>4</sub><sup>+</sup> also results in soil acidification. Host preference of EMF may also play a significant role in structuring the EMF community composition (Ding et al., 2011; Ishida et al., 2007), as may stand age (Visser, 1995; Smith et al., 2002) and atmospheric CO<sub>2</sub> concentration (Godbold et al., 1997). EMF species exhibit high differentiation in their preferences for several biotic and abiotic substrates of the forest soil which may be one of many reasons behind their high spatial distribution. Baier et al. (2006) suggested that EMF preference for the organic layer can be observed in their higher root-tip density in the organic horizon than the mineral layers. Soil horizon is one of the main factors that impact EMF communities composition and diversity since the soil physical and chemical properties differ with depth creating many different ectomycorrhizal habitats throughout the soil profile. Rosling et al. (2003) showed that there is a relationship between soil horizons and EMF community composition and found the majority of EMF species are restricted to the mineral horizon. Rosling and Rosenstock, (2008) suggested that different phosphorous release strategies of ectomycorrhizal fungi affected their spatial distribution in the podzolic stratified soil profile.

#### **1.4 Plants nutrient demands and their effect on ectomycorrhizal fungi**

Nitrogen and phosphorous are the most limiting nutrients for plant growth in the temperate and boreal forests and thus their availability controls ecosystem productivity, and serves as the dominant edaphic factor. Ectomycorrhizal fungi are an essential biotic component in the supply of these most limiting nutrients in the boreal and temperate forests. In temperate and boreal forests almost 80% of all the nitrogen and 75% of all the phosphorous in the plants are acquired by mycorrhizal fungi (Van der Heijden et al., 2008). Plant nutrient demands have a large influence on the carbon allocation rate between the above and belowground parts in the plants. Hermans et al. (2006) showed that plants allocate more carbon to the root system to improve their nutrients acquisition. Phosphorous and nitrogen deficiency of *Betula pendula* resulted in

increased carbon allocation to the roots, whereas magnesium and potassium deficiency did not show a similar response in a laboratory study (Ericsson, 1995). This suggests that plant productivity may be severely limited under the deficiency of magnesium and potassium. A review by Hobbie (2006) showed that 11 to 21% of the total net primary production was allocated by host plants to the ectomycorrhizal fungi, with a broad average around 15%. Plant carbon allocation to their ectomycorrhizal root partners has been observed to increase under phosphorous deficiency (Wallander and Nylund, 1992) and nitrogen deficiency (Correa et al., 2008), whereas carbon allocation was reduced to the roots and EMF symbionts under potassium deficiency (Ekbald et al., 1995). Correa et al. (2008) showed that plants' carbon investment in the mycorrhizal fungi did not affect the photosynthetic performance of the host plant, and that plant growth was related to the nutrient uptake (Corea et al., 2008; Correa et al., 2011). Hagerberg et al. (2003) examined the response of EMF mycelia to potassium or phosphorus sources by placing mesh bags containing either the mineral biotite (a K source) or the mineral apatite (a P source), in a nutrient poor spruce forest soil; EMF mycelia were not affected or stimulated by potassium addition while apatite addition increased the EMF biomass (Berner et al., 2012). This indicates the potential importance of plant carbon allocation to their EMF symbionts in responding to phosphorous deficiency by weathering the apatite mineral. For more information about the variable response of ectomycorrhizal weathering activity in response to plant nutrient demands in many field and pot studies see the review by Rosenstock (2009). The source and availability of plant nutrients may affect the plants response to ectomycorrhizal diversity. Baxter and Dighton (2005) showed that plants responded more greatly to ectomycorrhizal diversity in enhancing access to organic phosphorus nutrients than the inorganic phosphorous. EMF diversity increased the phosphorus uptake to the *B. populifolia* seedlings when they were inoculated with four different species of EMF (Baxter and Dighton, 2001). Kroehler et al. (1988) also found that ectomycorrhizal fungal species differed in their potential organic phosphorus mineralization; this may point to an important function of EMF diversity in meeting plants' nutrients demands.

## **1.5 The study questions**

In this study, we investigated how nutrient imbalances, soil type as a function of parent material, and soil horizon affect the ectomycorrhizal community composition and diversity by testing the following hypothesis:

- The different soils formed from different parent materials will have different ectomycorrhizal community composition and diversity.
- The mineral horizons and organic horizons within each catchment will also have different ectomycorrhizal fungal communities.
- The ectomycorrhizal fungal communities in the three different mineral soils will be more different from one another than those sampled from the organic horizons.

To address these questions we performed a study in the mountains of Northwest Czech Republic using three catchments closely situated to one another but occurring on very different bedrocks. The high variation in the parent materials of each catchment corresponds to high variation in the mineral element concentrations in the different soils, and, as we have observed, different limiting nutrients in the forests on the different catchments (potassium, magnesium, and phosphorous). This site provides a unique opportunity to study the effect of soil type and, by correlation, nutrient availability on the ectomycorrhizal community composition and diversity in the organic and mineral horizons.

## **2. Materials and Methods**

### **2.1 Study Site**

The study was conducted in three small forested catchments in the Slavkov forest in northwestern Czech Republic, about 120 km west of Prague (figure 1). The forest is a protected mountainous region with an area of 610 km<sup>2</sup> (Bencokova et al., 2011). The catchments (Lysina, Pluhuv Bor, and Na Zelenem) are situated in close proximity to each other (<5-7 km<sup>2</sup> apart) and have similar topography, altitude, tree species, atmospheric deposition fluxes, mean annual air temperature (5-6°C) and stand history, but are underlain by clearly different parent materials which is considered to be the major difference between these three catchments. Needle nutrient analysis previously done suggested that the serpentinite has below optimum levels of potassium and

phosphorus, and the granite site has below optimum levels of magnesium, while the amphibolite site is likely nitrogen limited (Håkan Wallander, pers. comm.) (table1).

Element (µg/g)	Deficiency Range	Granite	Serpentinite	Amphibolite
K	3500	4774	2883	4829
Mg	400-700	481	1808	919
P	1200-1300	1153	826	1314
N	12000-13000	12870	11700	14330

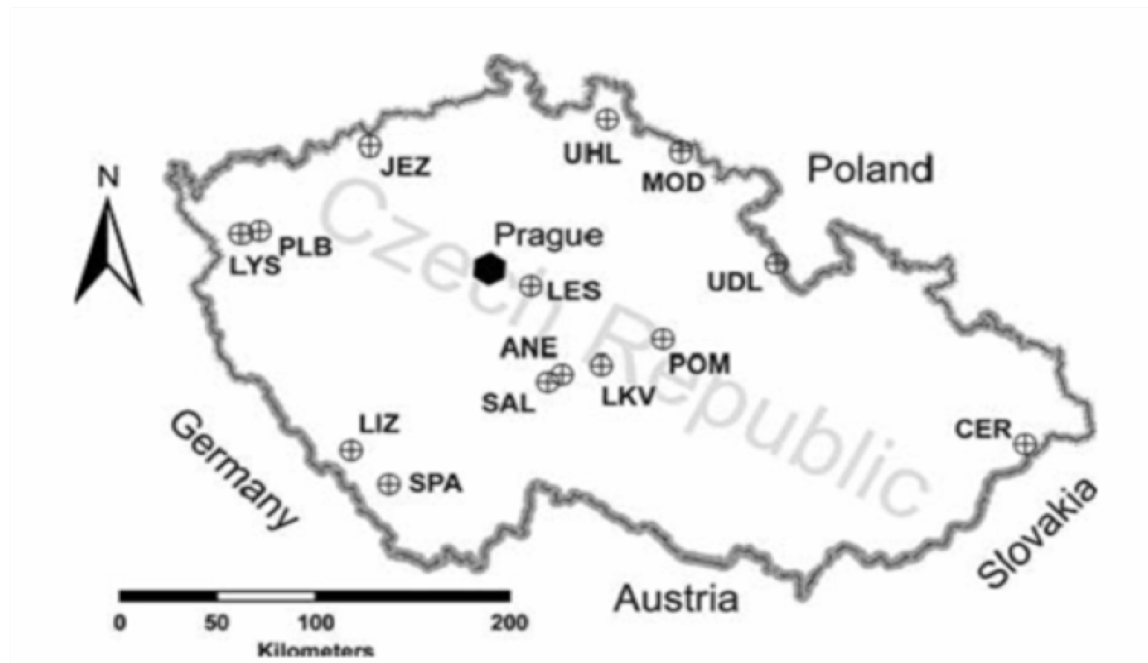
**Table 1:** Needle nutrient contents from needles taken from the Slavkov forest (unpublished; Håkan Wallander, pers. comm.)

a. The Lysina catchment has an area of 0.273 km<sup>2</sup> with an altitude of 829-949m a.s.l. (Bencokova et al., 2011). Mature Norway spruce (*Picea abies*) trees cover the most part of the catchment area while some small areas are covered by the young spruce trees and grasses (e.g. *Calamagrostis villosa*). The soil type that dominates the catchment is podzolic soil which is underlain by coarse-grained leucogranite bedrock. Cation exchange capacity and base saturation are low in the podzolic soil because it derived from base-poor rocks. Leucogranite is poor in magnesium and rich in potassium being comprised of 0.1% MgO and 4.4% K<sub>2</sub>O by mass (Table1). Average annual precipitation and temperature are 945 mm and 5°C.

b. The Pluhuv Bor catchment has an area of 0.216 km<sup>2</sup> with an altitude of 690-804 m a.s.l. (Bencokova et al., 2011). Approximately, 92% of the vegetation in the catchment is Norway spruce (*Picea abies*) mixed with 8% of scots pine (*Pinus sylvestris*) (Bencokova et al., 2010). The soil is classified as a eutrophic brown soil. It developed on magnesium-rich serpentine rocks. It is a highly base-cation rich soil. Serpentinite is rich in magnesium and poor in potassium being comprised of 36% MgO and 0.02% K<sub>2</sub>O by mass (table1). Average annual precipitation and temperature are 844 mm and 5.7 °C respectively.

c. The Na Zelenem has an area of 0.55 km<sup>2</sup> with an altitude 736-802 ma.s.l (Kram et al., 2012). Norway spruce (*Picea abies*) trees cover the vast majority of the catchment area.

Cambisol is the dominant soil type covering the amphibolite bedrock. It is a base-cation rich soil. Amphibolite has moderate amounts of magnesium and high amounts of calcium being made up of 8% MgO and 9% CaO by mass (table2). Average annual precipitation and temperature are not known at this time, but are assumed to be within the range of the other two sites (Pavel Kram, pers. comm.).



**Figure 1:** Location of lysina and pluhuv por catchments on the Czech Republic map. Study sites are named “LYS” and “PLB” and are located in the upper left portion (Northwest) of the map.

Chemical composition	Leucogranite Mass %	Serpentinite Mass %	Amphibolite Mass %
MgO	0.1	36	8
K <sub>2</sub> O	4.4	0.02	0.23
CaO	0.5	0.3	9

**Table 2:** Percentage of the magnesium, potassium, and calcium element in the bedrock, by mass Leucogranite data are from Kram et al. (1997), amphibolite and serpentinite data are from Kram et al. (2009).

## 2.2 Sample Collection and Processing

Ectomycorrhizal fungal sampling was carried out in September 2010. Within each of the three study catchments three subplots (200-400 meters apart) were chosen such that all 9 subplots had similar slope and aspect. Ten soil samples were collected from each subplot; each sample was a composite sample of two cores, the top portion (organic horizon) or the bottom portion (mineral. Hence, from each site 5 organic

horizon and 5 mineral horizon samples were collected. Each core was partitioned into organic and mineral fraction visually. The samples were immediately stored in 4°C and then processed over the course of the next two months. Sample collection was equally distributed in each subplot of each plot. [3 plots X 3 subplots X 5 composite cores X 2 horizon = 90 samples]. Each sample was washed and cleaned over a 2 mm sieve with distilled water. Cleaned roots were then collected and put into Petri dishes filled with distilled water for ectomycorrhizal root tip sampling. We examined the roots that were under the dissecting microscope to remove the roots that were not turgid and that appeared senescent. After we examined and sorted the roots, we randomly selected 24 fine ectomycorrhizal root tips from each sample using a gridline intersect method, and immediately placed them in Sigma (Sigma-Aldrich; St. Louis, MO, USA) XNAP™ extraction solution for DNA extraction. In all, 2160 ectomycorrhizal root tips [90 samples X 24 root tips] were collected for ectomycorrhizal fungal community composition and diversity characterization.

### **2.3 Molecular Analysis**

To assess the diversity and community composition of ectomycorrhizal fungi in each parent material, we extracted the DNA from 1392 of the 2160 ectomycorrhizal root-tips collected. This amounted to 2-3 samples for each horizon in each subplot. DNA was extracted from the root tips using the Sigma XNAP™ extraction protocol. We selected the ITS region for PCR amplification and sequencing; it is the most common region for fungal identification due to its high copy number and high variability among the fungal species (Horton and Bruns, 2001). In order to amplify this region of DNA we used the following PCR protocol (2µl DNA extract, 2.5µl of 10x PCR buffer, 1µl of 10µM ITS1F, 1µl of 10µM ITS4, 0.5 µl of 10µM dNTPs, 0.125µl of 20mM MgCl<sub>2</sub>, 0.25µl of DreamTaq polymerase, 15.5 of dH<sub>2</sub>O, and 2µl of 10mg/µl BSA). 25 µl of the PCR reaction were loaded in each well of the plate for each sample then we ran them on the PCR thermocycler to amplify the targeted ITS DNA region. We used the following thermocycling parameters: 94°C denature, 51°C annealing, and 72°C extension for 35 cycles. 8µl of PCR products were run on 1.5% agarose gel in SB buffer at 150 milliamps for 30-40 minutes to check positive amplification. After then we ran 15µl of the same PCR product on 2% agarose gel in TAE buffer at 110 milliamps for 2-3 hours to separate double bands. Double bands were extracted using the band-stab technique (Bjourson

and Cooper, 1992) and individually re-amplified using the same PCR protocol. PCR products were cleaned to remove the excess of primers and salts using isopropanol/ ethanol precipitation. Sequencing of PCR amplicons was performed using Applied Biosystems Big Dye™ (v3.1) sequencing chemistry (Applied Biosystems, CA, USA). The sequencing reaction includes the following reagents (1µl of cleaned PCR product, 1µl of BD mix, 1µl of BD buffer, 1µl of 1.25Mm ITS4, and 1µl of dH<sub>2</sub>O). We chose the reverse primer (ITS4) in the sequencing reaction because it has exhibited better performance in the sequencing of the DNA target region. 5µl of the sequencing reaction were loaded in each well of the plate for each sample to run them on the thermocycler. The thermocycler were programmed on the following sequencing cyler program (96°C denature, 50°C annealing, and 60 °C extension) for 25 cycles. We removed then the excess primers and unincorporated dNTPs after sequencing reaction using the ABI recommended ethanol/EDTA precipitation. Single pass sequencing was performed on an ABI 3700 Sanger sequencing machine (Applied Biosystems, CA, USA). Sequences were identified by comparison with the sequences database at the national center for bioscience informatics (NCBI) using the basic local alignment search tool (BLAST) Ectomycorrhizal sequences that matched BLAST at hit 90% similarity were positively identified grouped according to their genera names. The sequences within each genus were then clustered separately to form OTU group using sequencher 4.7 (Gene Codes Corp., Michigan, USA) with a minimum sequence overlap of 30% and minimum sequence identity of 97%. The sequences that were clustered together in one contig were categorized according to their species name when one member of the contig matched an NCBI accession of a vouchered specimen at >98% sequence similarity, otherwise they were simply termed by genus (e.g. Piloderma sp.1).

## 2.4 Statistical Analysis

Species richness and species evenness are the two main components of biodiversity and we measured both aspects of the ectomycorrhizal fungal communities. Species richness was measured by observing the number of different species. We used the chao1 estimator and rarefaction curves to estimate the species number of the communities sampled. Species diversity was assessed by using the Shannon and Simpson diversity indices. The Shannon diversity index (H) was calculated as

$$H = -\sum p_i \ln(p_i)$$

The Simpson diversity index was calculated as

$$1 / (\sum p_i)$$

Species evenness was calculated as

$$H / \log(S)$$

$p_i$ : the proportion number of the total number of species made up of the  $i$ th species,

$H$ : the Shannon index, and

$S$ : the total number of observed species.

Chao1 estimator was calculated as

$$S = S_{obs} + (a^2/2b)$$

$S_{obs}$ : the number of species observed

$a$ : the number of species observed just once

$b$ : the number of species observed just twice

Eighteen different ectomycorrhizal communities were used in the analysis (3 parent materials X 2 depths X 3 subplots). Each community represents the total number of ectomycorrhizal sequences that were positively identified in all the replicate samples from one horizon/subplot/parent\_material. We used the ordination method non parametric multi dimensional scaling (NMDS) to visually compare the community similarity between the eighteen different ectomycorrhizal communities. Using NMDS the communities were compared between the parent materials ( $n=6$ /parent material) or between soil horizons ( $n=9$  /horizon). NMDS ordination is a suitable method to examine the relationship between microbial communities' composition patterns, the underlying environmental factors that influence these patterns and the similarity between the communities because it does not assume a normalized distribution of species or equivalent variance between communities (Clarke, 1993). The effect of parent material on community composition was compared on the NMDS plot using the factfit function (factfit attempts to find the optimum fit between the points in a 2 dimensional ordination and a vector representing one chosen factor, in this case parent material) and using partial mantel test in the R statistical package VEGAN stat (Oksanen et al., 2012). In both cases, parent material was defined as independent character states. The effect of parent materials or horizons on the abundance of the 11 most common species was examined using a two way ANOVA, with the student's-t-test for species abundance comparison between the horizons and Tukey's HSD test for species



abundance comparisons between the parent materials. All ANOVAs were done with JMP v5.0.1 (SAS institute, NC, USA).

### **3. Results**

#### **3.1 Sequencing Details**

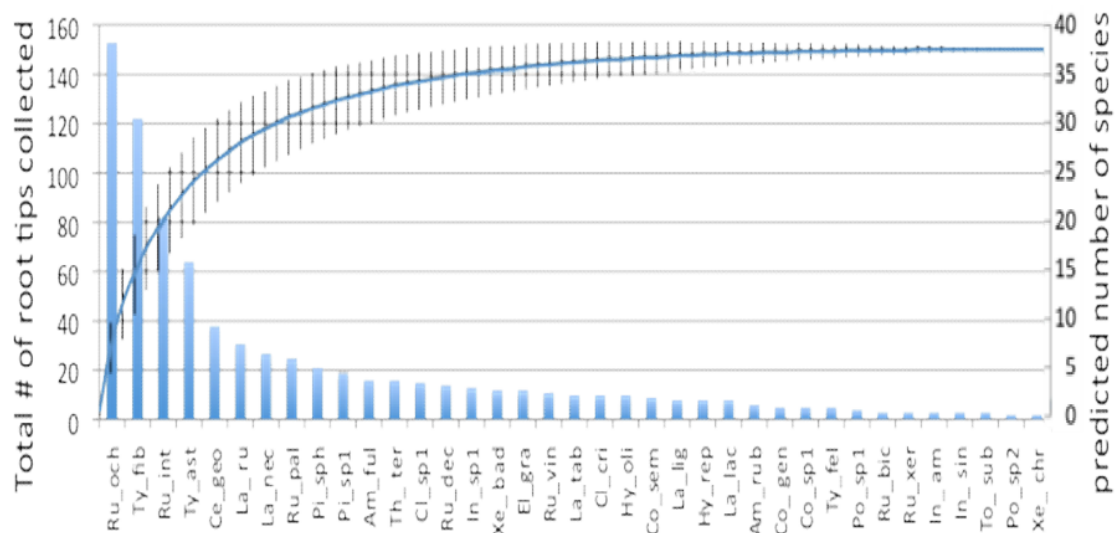
While we were able to successfully amplify DNA from most of the root tip DNA extracts, the vast majority of the PCR products yielded double bands when viewed with agarose gel electrophoresis due to the presence of other co-colonizing fungi. After performing band-stab and re-amplification, 2400 bands were sequenced. The majority of these sequences were either known saprotrophs or unidentified members of the Heliotiales, and were excluded from further analysis. 798 ectomycorrhizal root-tips were successfully sequenced and identified as ectomycorrhizal fungi out of the 1392 root tips dedicated for the DNA extraction. Of these 798 ectomycorrhizal sequences, 361 were from the organic and 437 from the mineral horizons, and 240 from granite, 222 from serpentinite, and 336 from amphibolite sites, respectively. Rarefaction analysis (figure 2) for all the parent materials combined yielded a species accumulation curve which appeared to plateau, suggesting that we adequately sampled the community to capture most of the ectomycorrhizal diversity.

#### **3.2 Ectomycorrhizal community composition**

The 798 ectomycorrhizal fungal sequences were sorted into 37 OTU's. Ectomycorrhizal fungal species were highly variable in their abundance, from the most abundant species *Russula ochroleuca* (153 root-tips) and *Tylospora fibrilosa* (122 root-tips) to the most rare species *Polyozellus sp.2* and *Xerocomus chrysenteron* (2 root-tips each) across all the parent materials and horizons. The overall species abundance shows that a few species exhibited high abundance whereas many species exhibited low species abundance across all the parent materials (figure 2).

*Russula ochroleuca*, *Tylospora fibrillose*, *Tylospora asterophora*, and *Cenococcum geophilum* were the most abundant species in all parent materials, making up 47.1% of all sampled root tips. *Russula*, *Tylospora*, *Lactarius*, *Piloderma*, and *Cenococcum* were the most abundant genera in all parent materials, making up 36.4%, 23.3%, 9.5%, 5.0%, and 4.6% respectively of all sampled root tips. Five of the ten most

abundant species showed a preference for one parent material. *Russula integra* was significantly more abundant in the serpentinite site ( $p < 0.04$ ), *Piloderma spharosporum* more abundant in the granite ( $p < 0.01$ ), and *Piloderma sp.1* more abundant in the amphibolite catchment ( $p < 0.02$ ). Additionally, *Tylospora asterophora* ( $p < 0.08$ ) and *Cenococum geophilum* ( $p < 0.06$ ) were marginally more abundant in the amphibolite and granite catchments, respectively. No genera or family for which we found more than one species was significantly more abundant on a particular parent material. *Russula paludosa* and *Russula integra* were the only two species that significantly preferred the organic or mineral horizons, respectively (table 3).



**Figure 2:** Bars show the number of root tips collected for each species and the line curve is the species accumulation curve, generated by analytical rarefaction, for the total ectomycorrhizal community across all three parent materials. Thin vertical lines on the curve represent the 95% confidence interval for each rarefaction estimate. 37 species were observed out of 798 successful sequences.

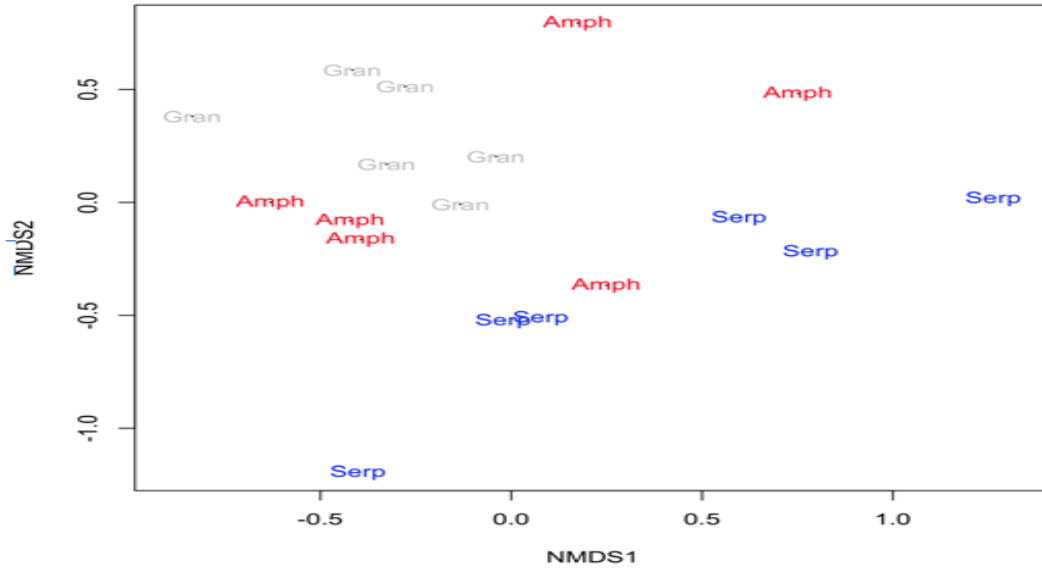
Taxa name	#of tips	# of subplots observed (out of 9)	Response to parent material	P value	Horizon preference	P value
<b>1. <i>Russula ochroleuca</i></b>	153	9	No effect		No preference	
<b>2. <i>Tylospora fibrillosa</i></b>	122	9	No effect		No preference	
<b>3. <i>Russula integra</i></b>	82	4	S High	0.04	Min*	0.01
<b>4. <i>Tylospora asterophora</i></b>	64	7	A High	0.08	No preference	
<b>5. <i>Cenococcum geophilum</i></b>	37	7	G high	0.06	No preference	
<b>6. <i>Lactarius rufus</i></b>	31	4	No effect		No preference	
<b>7. <i>Lactarius necator</i></b>	27	5	No effect		No preference	
<b>8. <i>Russula paludosa</i></b>	25	3	No effect		Org *	0.1
<b>9. <i>Piloderma sphaerosporum</i></b>	21	5	G high	0.01	No preference	
<b>10. <i>Piloderma sp.1</i></b>	19	2	A high	0.02	No preference	
<b>11. <i>Amanita fulva</i></b>	16	4	No effect		No preference	
12. <i>Thelephora terrestris</i>	16	2				
13. <i>Clavulina sp.1</i>	15	2				
14. <i>Russula decolorans</i>	14	3				
15. <i>Inocybe sp.1</i>	13	1				
16. <i>Inocybe sindonia</i>	13	1				
17. <i>Xerocomus badius</i>	12	5				
18. <i>Elaphomyces granulatus</i>	12	3				
19. <i>Russula vinosa</i>	11	3				
20. <i>Clavulina cristata</i>	10	3				
21. <i>Hygrophorus olivaceoalbus</i>	10	3				
22. <i>Lactarius tabidus</i>	10	1				
23. <i>Cort. semisanguineus</i>	9	6				
24. <i>Hydnum repandum</i>	8	1				
25. <i>Lactarius lignyotus</i>	8	3				
26. <i>Laccaria laccata</i>	8	4				
27. <i>Amanita rubescens</i>	6	1				
28. <i>Cortinarius gentiles</i>	5	1				
29. <i>Cortinarius sp.1</i>	5	2				
30. <i>Polyozellus sp.1</i>	4	2				
31. <i>Tilopilus felleus</i>	4	3				

**Table 3:** Summary of the 31 most common (out of 36) ectomycorrhizal fungal taxa observed. Number of tips is the total number of tips that were successfully identified for one ectomycorrhizal taxa. Number of subplots indicates the number of subplots that had at least one successful identified sequence for each taxa. Response to parent material: “G high” refers to the species that were significantly more abundant in granite, “S high” significantly more in serpentinite, “A high” significantly more in amphibolite. “Min\*” refers to the species that was significantly more abundant in the mineral horizon and “Org\*” significantly more in the organic horizon. Not shown were *Russula bicolor*, *Russula xerampelina*, *Inocybe amicta*, and *Tomentella sublilacina* (each found on three root tips) and *Polyozellus sp.2* and *Xerocomus chrysenteron* (found on two root tips each).

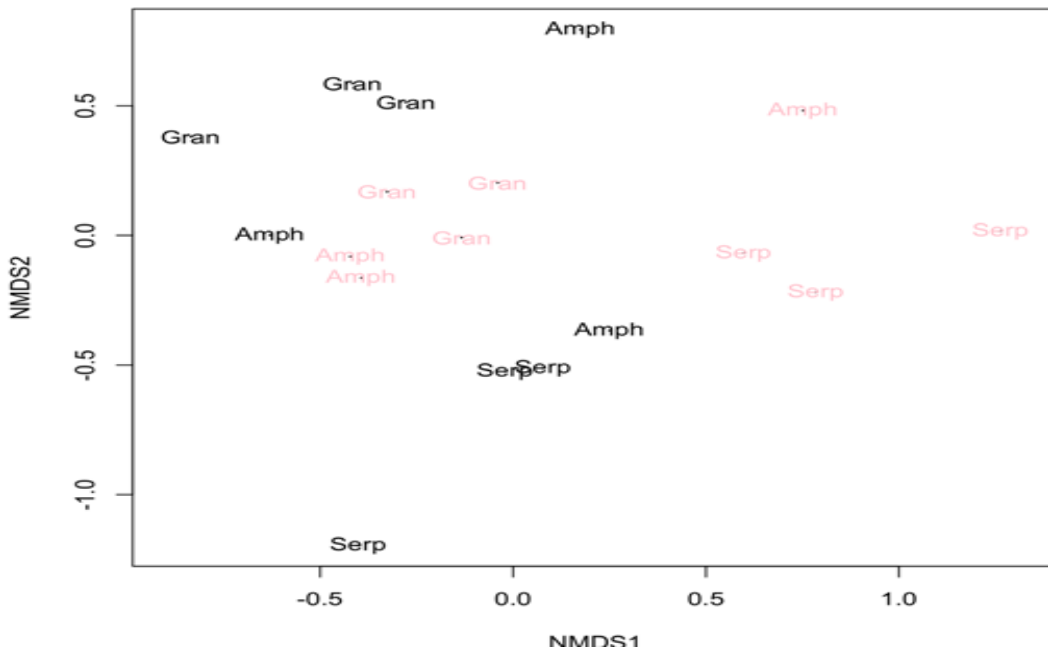
While the factorfit function found a significant segregation of communities in the NMDS plot according to parent material ( $r^2 = 0.3564$ ,  $p < 0.007$ ) partial mantel tests found that the effect was mostly due to the serpentinite communities' difference from the remainder of the community. When the effect of each parent material was examined independently, the amphibolite community mantel's r statistic and corresponding p value were -0.091 and 0.752, respectively, the corresponding values for granite were Mantel's  $r = 0.084$  ,  $p < 0.246$  , while for serpentinite they were Mantel's  $r = 0.28$ ,  $p < 0.008$ . Neither the partial mantel test nor the factorfit function showed any study-wide effect of horizon on the community.

When we visualized the 18 different ectomycorrhizal fungal communities (3 parent material X 3 subplot X 2 horizon ) using the two dimensional non-parametric multi-dimensional scaling (NMDS) plot (figure 4), the following are visually apparent:

- All six granite communities (3 organic and 3 mineral) are similar to each other (figure 3).
- The amphibolite communities vary more than the granite communities and are not distinct from the granite communities (figure 3).
- The serpentinite communities are distinct from the granite and amphibolite communities (figure 3).
- There is some distinct segregation in community composition between the organic and mineral communities in the granite catchment (figure 4), but not in the amphibolite catchment.
- The organic and mineral communities in the serpentinite catchment vary distinctly from one another, more than the distinction within the granitic communities (figure 4).



**Figure 3:** NMDS plot shows the 18 different ectomycorrhizal community compositions in the parent materials. Gran refers to granite, Serp refers to serpentinite, and Amph refers to amphibolite.



**Figure 4:** NMDS plot shows the organic and mineral communities composition in the parent materials. Black colored labels represent the organic horizon communities for a specific parent material. Grey colored labels represent the mineral horizon communities in each for a specific parent material

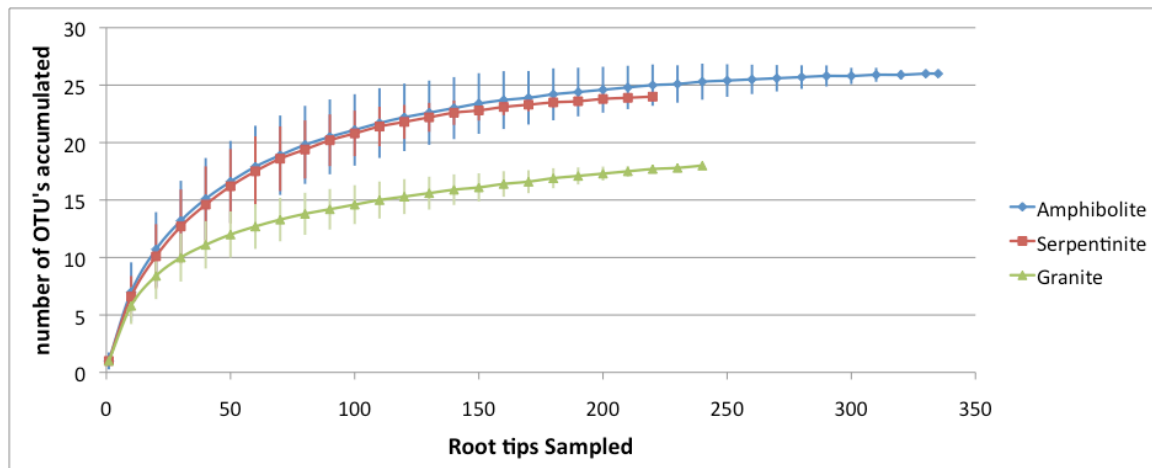
### 3.3 Ectomycorrhizal diversity

The rarefaction analysis (figure 5) shows that the amphibolite (26 OTU's observed) and serpentinite (24 OTU's observed) communities have similar diversity, while the granite (18 OTU's observed) is significantly less diverse. The total number of observed OTU's for each parent material/subplot/horizon combination (n=18) varied between 3 and 13, was not significantly affected by either the horizons or the parent materials.

As measured by the chao1 estimator, diversity was not significantly different between the parent materials (table 3). Shannon's and Simpson's index indicated that the EMF diversity increased as granite < serpentinite < amphibolite, but we were not able to test if this was statistically significant. The overall chao1 estimated for the organic and mineral horizons were 32.7 +/- 15.5 (S.D.) and 38.4 +/- 19.2 (S.D.), respectively.

Parent material Horizon	Granite organic	Granite mineral	Serpentinite organic	Serpentinite mineral	Amphipolite organic	Amphipolite mineral
<i>Number of species</i>	12	15	14	16	17	20
Shannon index	1.87	2.32	2.24	2.13	2.44	2.51
Simpson index	4.09	7.61	6.91	5.03	8.45	8.51
Evenness	1.19	1.48	1.43	1.35	1.56	1.60
Parent material	Granite		Serpentinite		Amphipolite	
Total species /parent	18		24		26	
Chao1 estimator (+/-S.D.)	38.2 (+/- 97.7)		38.0 (+/-60.9)		32.7 (+/-16.5)	
Shannon index	2.20		2.55		2.78	
Simpson index	6.25		7.99		12.05	
Evenness	1.40		1.62		1.77	

**Table 4:** Ectomycorrhizal species diversity and richness across all parent materials



**Figure 5:** species accumulation curves for each parent material generated by analytical rarefaction. Vertical bars represent the 95% confidence interval for each rarefaction estimate.

## 4. Discussion

Granite, serpentinite, and amphibolite are very different bedrocks in terms of their chemical composition: granite is poor in magnesium and rich in potassium whereas serpentinite is highly rich in magnesium and poor in phosphorous (and, comparatively, potassium), and amphibolite is fairly moderate in magnesium content and highly rich in calcium (see table1). Soil pH also varies between the different soils originated from the three chemically contrasting bedrocks. The average pH in the top 30 cm of mineral soil was 3.7 in the granite-derived podzolic soil, 5.9 in the serpentinite-derived cambisol, and 4.6 in the amphibolite derived stagnosol soil (unpublished data). The other components of the catchments such as tree species, stand age, topography, annual air temperature, precipitation, and altitude are similar across all the parent materials. We cannot separate the effects of pH and other soil factors, such as texture, from the effects of nutrient availability, but the needle nutrient analysis does suggest the differences in nutrient availability correspond to meaningful differences in tree functioning.

### 4.1 Ectomycorrhizal diversity

Rarefaction analysis (figure 5) showed that serpentinite and amphibolite communities have similar ectomycorrhizal (EMF) diversity while that on granite is significantly lower. Eighteen, 24, and 26 OTU's were observed in granite, serpentinite, and amphibolite, respectively. While the total numbers of observed species between

horizons/subplots/parent material were not significantly different (table 4), our sampling of the different subplots was uneven so we believe that the species accumulation curves generated by analytical rarefaction are the best metrics of diversity in our system, and they were generally consistent with the Shannon and Simpson's indices. These results were somewhat consistent with Gehring et al. (1998), which found no difference between the number of ectomycorrhizal species in two different soils with different nutrient availability while also consistent with Branco (2010) and Branco and Ree (2010) who found that serpentine soils exhibited more ectomycorrhizal diversity than the non serpentine soils. While serpentine soils often have high levels of heavy metals, it is possible that the high concentrations of Al in the granite could be a reason behind the low observed diversity. Edwards and Bell (1989) showed that high concentrations of toxic metals such as Al and the low availability of base cations affected the development of the mycorrhizal fungi. Nitrogen fertilization or deposition has been commonly observed to strongly reduce ectomycorrhizal diversity (Rosenstock, 2010; Lilleskov et al., 2002; Cox et al., 2010), and these forests were subjected to high levels of sulfur and nitrogen deposition in the twentieth century, and continue to experience high levels of nitrogen deposition. Sulfur and nitrogen deposition was the highest in 1993 when they were recorded as 34 and 14 Kg/ha/year, respectively, and while sulfur deposition has decreased greatly since then, nitrogen deposition levels remain above 10 Kg/ha/year.

## **4.2 Ectomycorrhizal community composition**

The community composition of granite and amphibolite were slightly, but not significantly, distinct from each other but both were significantly different from the serpentine ectomycorrhizal communities. There was no consistent difference between the organic and mineral communities in the amphibolite soils. In contrast, there was a consistent though small difference between the EMF communities in the different horizons in the granite soils, and a larger difference between horizons in the serpentine plots (figure 4).

Nitrogen deposition has many impacts on the soil chemistry such as leaching base cations and soil acidification and has been repeatedly associated with significant changes in ectomycorrhizal communities (Lilleskov et al., 2001; Cox et al., 2010).



Calcium and magnesium (liming) additions, used to fertilize forests and increase soil pH, have also been observed to markedly change the ectomycorrhizal community composition (Kjoller et al., 2009; Rineau and Garbaye, 2009). Other studies have also reported that the ectomycorrhizal community composition can be correlated with natural variations in soil chemical properties such as nitrate, pH, and soil texture (Newbouns et al., 2012; Toljander et al., 2006). Gehring et al. (1998) compared the ectomycorrhizal community between two soil types which were different in their soil moisture and nutrient availability such as phosphate and nitrogen, and found different ectomycorrhizal community composition between the two soils. In contrast, we did not find as large an effect of different nutrient concentrations on the ectomycorrhizal community composition across all the parent materials (figure 3). The granite and amphibolite community composition were quite similar to each other, despite the differences in pH, magnesium availability, phosphorous availability, potassium availability, and forest productivity, while the serpentinite communities were distinct from the other two. We think that decades of high acid deposition and continued high levels of nitrogen may have been a perturbation that led to a convergence of species composition, though we have no evidence to support this potential explanation. Acid deposition, and nitrogen deposition in particular have repeatedly been shown to alter community composition and species diversity (Rosenstock, 2010; Kjoller et al., 2011; Avis et al., 2008; Lucas and Casper, 2008), and some have suggested that it favors more generalist species and more aggressive fungal colonizers (Lilleskov et al., 2002, Wallenda and Kottke, 1998). Agganga et al. (1996) showed that different ectomycorrhizal fungi were not significantly affected by pH, while Letho (1994) showed that the ectomycorrhiza formation decreased as the pH was increased from 3.6 to pH 7. The ectomycorrhizal community on serpentinite-derived soil exhibited markedly different community composition, both from the communities on other parent materials and between the different subplots. The cambisol soil which has formed on the serpentinite is very different from the podzolic and stagnosol soils formed on the granitic and amphibolite parent materials owing, in part, to its high pH (5.9) and magnesium content, and low phosphorous content as well as marked textural differences. Branco (2010) and Branco and Ree (2010) compared EMF species diversity and community composition between serpentine and non-serpentine soils that had the same climate, aspect, and host species (*Quercus ilex*). They found somewhat elevated

ectomycorrhizal diversity in the serpentinite sites, although the ectomycorrhizal communities were not significantly different. Serpentine soil is generally considered a stressful soil in regards to plant establishment and growth, because it has unbalanced calcium:magnesium ratios, is poor in nutrients such as phosphorous and potassium, and has high concentration of heavy metals particularly nickel. Brady et al. (2005) stated that plants growing on serpentine soil are characterized by their low productivity and diversity.

We found that only 5 of the 11 most common species exhibited a significant preference for a particular parent material. *Tylospora asterophora* and *Cenococcum geophilum* were significantly more abundant in amphibolite and granite, respectively, among the most abundant species. *Russula integra* was the only species that exhibited significant preference for the serpentinite. The *C. geophilum* was more abundant in the organic horizons across all the parent materials. Baier et al., (2006) and Göbl and Thurner (1996) found *C. geophilum* to be the most predominant species in soils derived from Raibler dolomite and also found that it exhibited a strong preference for the organic horizon. *Russula ochroleuca* and *Tylospora fibrillosa* were the most abundant species in all the parent materials in our study. Rineau et al., (2009) observed that *R. ochroleuca* was significantly repressed in limed plots. In our study it does not appear that soil pH alone has an effect on the ectomycorrhizal composition. Most of the ectomycorrhizal species were common in the soils of parent materials that varied greatly in pH. Rineau et al. (2009) found that *Cenococcum geophilum* and *Russula ochroleuca* were repressed under elevated calcium and magnesium availability, but we did not observe this pattern in our study. The high variation in nutrient availability between amphibolite and granite, especially the magnesium, calcium, and potassium, did not seem to strongly impact the ectomycorrhizal community. A number of studies have been conducted to investigate the efficiency of EMF in solubilizing recalcitrant sources of mineral nutrients such as potassium and magnesium and their ability to supply their plant partners with these nutrients, but not on the effects of the availability of these mineral nutrients on the ectomycorrhizal community composition.

### **4.3 The soil horizons and their effect on the ectomycorrhizal community composition**

The ectomycorrhizal communities were moderately segregated between the organic and mineral horizons in granite plots and clearly segregated between the organic and mineral horizons in the serpentinite (figure 5). The segregation in the ectomycorrhizal community between the organic and mineral horizons was to some extent consistent with our hypothesis. Serpentinite communities were significantly affected by horizon. Rosling et al. (2003) showed that the ectomycorrhizal community was different between the organic and mineral horizons, and two thirds of the collected root-tips were found in the mineral horizon. Dickie et al. (2002) found that the ectomycorrhizal communities in four soil layers were significantly different. We think that the phosphorous deficiency in the serpentinite-derived soils may be a reason behind the distinct difference between the organic and mineral soil ectomycorrhizal communities. Rosling and Rosenstock (2008) suggested that the spatial distribution of ectomycorrhizal fungi in a podzolic stratified soil profile may be because of the different phosphorous release strategies in the ectomycorrhizal species. Dickie et al. (2002) also suggested that the substrate-resource gradient was behind the highly different ectomycorrhizal species composition in the soil layers. In the organic horizon where phosphorus is found in organic forms, ectomycorrhizae that have the capacity to solubilize this P (through phytase and phosphatase production may be selected for via higher carbon allocation by the plant to the fungus. The majority of phosphorous in the mineral horizon is bound to the aluminum, calcium, or iron and the ectomycorrhizae that are able to solubilize these compounds and release the bound P by producing oxalic acids or siderophores may be favored in the mineral horizon. Courty et al, (2010) showed that ectomycorrhizal species were highly varied in their phosphatase enzymatic activity even within the same species in different soil horizons. It is also possible that nutrient acquisition of magnesium or potassium may be selected for by the host plants. Jentschke et al. (2000) showed that the ectomycorrhizal fungi in a laboratory study were able to transfer significant amounts of magnesium to their seedlings partners, and van Scholl et al. (2006) also showed that ectomycorrhizae transferred significant amounts of potassium to their plants partner.

*Russula intergra* and *Russula paludosa* were the only two species that exhibited significant preference for either the mineral or organic horizons, respectively, across all the parent material. Rosenstock (2010) found that, overall, the Russulacea exhibited a preference for the organic horizon. The ectomycorrhizal communities of the granite and amphibolite in the organic and mineral horizon were only slightly different from each other, a similarity that potentially could be explained by the historic nitrogen and sulfuric deposition levels. Rosenstock (2010) found that the differences between the ectomycorrhizal communities in the organic and mineral horizons were lower under high levels of nitrogen fertilization. Simultaneously, the high historic nitrogen and sulfur deposition may be a driving factor behind the differences in the organic and mineral horizons observed in the serpentinite communities, if these differences are a function of phosphorus acquisition capabilities.

## **5. Conclusions**

We were able to characterize the community composition and diversity in the ectomycorrhizal communities in three catchments formed on different parent materials. Granite and amphibolite-derived soils had similar ectomycorrhizal community composition whereas, serpentinite-derived soils have a significantly different ectomycorrhizal community composition. Serpentinite and amphibolite-derived soils have similar ectomycorrhizal community diversity, whereas granite has significantly lower diversity. Amphibolite-derived soils have similar ectomycorrhizal community composition in the organic and mineral horizons whereas serpentinite and, to a lesser extent, granite have different ectomycorrhizal community composition between the organic and mineral horizons. Large differences in the availability of magnesium, potassium, and calcium did not result in any significant effects on the ectomycorrhizal community composition, at least in the granite and amphibolite catchments. The soil formed on serpentinite has distinctly different ectomycorrhizal community composition than the other parent materials, and show differences between the organic and mineral horizon, possibly due to the lower phosphorus or potassium availability in the serpentine soils.

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