# Plant Productivity, Ectomycorrhizae, and Metal Contamination in Urban Brownfield Soils

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# Plant Productivity, Ectomycorrhiza and Metal Contamination in Urban Brownfield Soils

Running Title: Ectomycorrhiza and Plant Productivity in Brownfield Soils

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**Abstract**

The soil contamination legacy of post-industrial sites has become an issue of increasing ecological and public health concern. This study examines the ectomycorrhizal and above ground plant relationships in the metaliferous soil of an urban brownfield. Ectomycorrhizal fungi (EMF) were microscopically identified by physical morphotyping followed by sequencing of ribosomal DNA. Plant productivity was assessed through Leaf Area Index (LAI) measurements taken from May through July of 2012 and 2013. Results indicate that there were significant changes in EMF community composition and plant productivity based on their position along a total soil metal load gradient. *Cenococcum geophilum* was the dominant species in the soils where total soil metal load was below previously established threshold values - and *Russula sp.* was the dominant genera in soils where the total soil metal load was above the threshold value. Higher LAI values are seen in environments with higher soil metal levels. However, higher LAI could be due to multiple factors such as increased moisture and the dominance of metal-tolerant tree species. This study demonstrates that soil metal contamination does have an effect on plant productivity and EMF community composition, and supports the idea that EMF species have varying levels of tolerance for metals.

Keywords: heavy metals, mycorrhizae, plant productivity, restoration

## Introduction

As global demographic transition tends to favor the development of cities, the role of urban ecology is becoming increasingly significant. Many post-industrial sites include significant areas of abandoned or vacant plots that have been colonized by novel vegetative assemblages that offer a variety of ecological services. However, soil metal contamination in such areas is cause for both environmental and public health concerns (Albering et al. 1999, Luo et al. 2012, Qian et al. 2012). Plant species that occur in brownfield sites often exhibit a metal tolerance threshold, beyond which they exhibit signs of stress (Gallagher et al., 2011). However, plant responses to stress and their productivity are likely ameliorated by ectomycorrhizal fungi (EMF) (Smith and Read 1997). To fully understand the mechanisms of this, EMF community composition and plant productivity must be measured in the field, and contaminated brown fields make excellent and highly relevant case studies.

Soil metal induced stress in vascular plants is well documented. The specific symptoms of stress vary according to the metal and its concentration. For example, *Convolvulus arvensis* (field bindweed) seedlings grown in an agar-based medium exhibited metabolic stress at equivalent Cd soil concentrations above 20 mg/l; whereas, in the same experiment, the effective levels for soil Cu(II) and Cr(VI) were not reached at 80 mg/l (Gardea-Torresdey et al. 2004). In the field, vegetative assemblage composition and trajectory can also be impacted, if not driven by soil metal concentrations (Gallagher et. al. 2011). In most of these cases the metal load dose-response curve generally indicates a reduction in metabolic efficiency after a specific threshold concentration has been exceeded. This paper examines the possibility that such responses in the above

ground plant assemblage are linked to changes in the below ground ectomycorrhizal fungi (EMF) community structure (Wardle et al. 2004).

Soil metal contamination, acid rain (Schützendübel and Polle 2002, Ochimaru and Fukuda 2007, Bojarczuk and Kieliszewska-Rokicka 2010), habitat fragmentation (Peay et al. 2010) and enhanced nitrogen levels (Lilleskov et al. 2002, Krumins et al. 2009) can all contribute to the stress urban environments place on the associated vegetative assemblages. EMF grow in association with plant roots and form a mutualistic relationship that assists in nutrient acquisition and tolerance to stressful environments (Courty et al. 2010, Jones et al. 2012, Karliński et al. 2013). The role that above/below ground feedbacks are playing under these conditions is of increasing interest to ecologists. For example, there appears to be a strong positive effect of mycorrhiza in nutrient poor environments where they facilitate nutrient uptake, resistance against disease, and drought tolerance (Van Der Heijden et al. 2008). However, there are few studies clarifying the extent to which the diversity of mycorrhizal communities contributes to increases in plant health and productivity. Baxter and Dighton (2001) found that mycorrhizal plants were able to take up higher amounts of P when infected with enhanced mycorrhizal diversity and less when infected with a single mycorrhizal species. Additional studies however, determined that the apparent effect of diversity was actually a sampling effect and that the inclusion of specific species better explained the increase in productivity (Van Der Heijden et al. 2006, Vogelsang et al. 2006). In order to further understand this relationship we must look at the shifts that take place in a field setting, particularly a metal contaminated one where community composition will be subject to environmental selection.

89 Urban brownfields provide a unique place to study EMF and plant relationships
90 because they frequently contain soil contaminants (*e.g*. metals) that negatively impact
91 both above ground and below ground communities (Hrynkiewicz et al. 2008, Krpata et al.
92 2008, Gallagher et al. 2008b, Regvar et al. 2010). Ectomycorrhizal species vary in their
93 tolerance to soil metal contamination, and shifts in community composition will favor
94 species that are more tolerant to contamination (Hartley et al. 1997, Blaudez et al. 2000,
95 Regvar et al. 2010, Hui et al. 2011), often resulting in a loss of species richness
96 (Chappelka et al. 1991, Baxter et al. 1999, Krpata et al. 2008). Since many mycorrhizal
97 species inhibit metal uptake by host plants, it has been proposed that a loss in EMF
98 species richness has the potential to reduce this function (Bojarczuk and Kieliszewska-
99 Rokicka 2010). In addition, it has been demonstrated that many EMF are host-specific
100 and if those mycorrhizal species are not able to tolerate high metal loads, their associated
101 plant host may not be able to persist. The loss of this facilitative function will have an
102 effect on the plant assemblage (Ledin 2000, Fomina et al. 2005, Melo et al. 2011).
103 Further studies that explore EMF diversity levels and identify metal tolerant species, as
104 well as their tolerance thresholds, would greatly enhance remediation efforts (Leski et al.
105 1995, Hrynkiewicz et al. 2008, Urban et al. 2008, Bojarczuk and Kieliszewska-Rokicka
106 2010, Luo et al. 2014). The research presented here addresses one aspect of the
107 relationship between plant productivity and mycorrhizal community structure with
108 respect to soil metal load. We characterized the EMF community in association with
109 plant productivity (as measured by Leaf Area Index, LAI) across a known soil metal
110 contamination gradient (Figure 1) (Gallagher et al. 2008a). Microscopic and molecular
111 techniques were used to identify differences between sites and ectomycorrhizal

community trends along the gradient. The initial hypothesis was that below ground diversity would correlate negatively with increased soil metal loads and positively with increased LAI values.

## Materials and Methods

### *Study Site*

Liberty State Park (LSP) is located on the west coast of the Upper New York Bay in Jersey City, NJ, USA. The area surrounding the park is one of the most densely populated urban environments within the United States. Much of the soil in the park is fill material composed of debris brought from New York City during its development in the 19$^{th}$ century. From 1889 to 1967 the area was used as a railroad terminal connecting the New York harbor area to the rest of the country and allowing commuters access to ferries to and from New York City (Gallagher et al. 2008b). The rail yard was abandoned from 1967 to 1970, and the area was gradually acquired by the State of New Jersey for use as a park. However, there is approximately 102 hectares within the interior section of the park that remains un-remediated and has very limited human access. Since abandonment, a naturally occurring deciduous forest consisting of early succession species such as: *Populus tremuloides, Betula populifolia, Rhus Copilinum* and *Rhus glabra* has assembled. The specific sites chosen for this study were dominated by *B. populifolia,* a pioneer species that competes well in soils of little nutritional value (Elias 1980).

134 *Soil Contamination*

135 While the USDA has given the soils of LSP their own designation, the Lady
136 Liberty Series (National Cooperative Soil Survey 2012), the edaphic conditions vary
137 significantly throughout the site (Gallagher et al. 2008a, 2008b) (Table1). To assess the
138 cumulative impact of the soil metals, a total soil metal load (TML) index (Juang et al.
139 2001) has been developed by performing a rank order transformation on the soil metal
140 concentration of As, Cr, Cu, Pb and Zn. These metals were chosen as they regularly
141 exceeded both ecological (USEPA 2003) and residential soil screening criteria (NJDEP
142 2004). The summation of the rank order values, at each of 41 sampling sites across the
143 contaminated portion of LSP, produced a total TML ranking which scaled between 0 and
144 5. The results were back-transformed using the reverse function of the linear regression
145 (Wu et al. 2006), and a TML map was then developed by Kriging these data. (Gallagher
146 et al. 2008a). A TML index of 3 has been identified as a critical threshold beyond which
147 plant function and seed viability in *Betula populifolia* (Gallagher et al. 2008a, 2008b)
148 were significantly impacted.

149 *Sampling Design*

150 Four sites within the interior section of LSP were examined in 2012 and 2013
151 based on their position along a metal contamination gradient. Two sites were below the
152 TML threshold of three (L1 TML = 1.56 and L2 TML = 1.64) and two sites were above
153 the TML threshold concentration (H1, TML = 3.56 and H2, TML= 3.08). In addition, as
154 differences in EMF community composition and LAI varied so greatly between the two
155 high soil metal load sites in 2012 (Figures 2a and 3a), we added a fifth site in 2013 (site
156 H3 (TML = 4.31) to better resolve trends at sites with high soil metal contamination.

*Mycorrhizae Sampling and Morphotyping*

Five bulk soil cores (5 cm through approx. 20-25 cm depth) were removed at four meter intervals along a 20 meter transect within each site. The cores were not separated into soil horizons as soils at LSP are unique and horizons are not always relevant or easily separated. Samples were placed in separate Ziploc bags and immediately transferred to a 4°C refrigerator where they were stored prior to analysis. Sampling was conducted once in early June 2012 and once in early July 2013. Roots were manually separated from each soil sample and gently rinsed with warm water to remove large pieces of soil and debris. They were then placed on a gridded Petri dish for examination. Ectomycorrhiza were characterized following the standards established by Agerer (1997). Ramification pattern, shape of the unramified end, mantle texture, color, luster and presence of emanating hyphae and/or rhizomorphs were all used to identify tips. Tips that shared the same characteristics were assumed to be the same morphotype. Tips were separated into their respective morphotypes in order to calculate the relative abundance of each tip and overall diversity of the mycorrhizal community post sequencing (Agerer 1997, Krpata et al. 2008).

*Molecular Identification*

To increase the rigor of our morphotyping effort and match identity to the tips present at our site, we microscopically collected mycorrhizal tips and sequenced them for identification. Following morphotyping, ectomycorrhizal tips of the same type were clipped from the root and grouped together into one 200 µl tube and stored at -20°C for future DNA extraction. DNA was extracted from the root tips using the Mo Bio

PowerSoil DNA Isolation Kit (Mo Bio, Carlsbad, CA) following manufacturer's procedures. At the last stage of the extraction, in samples with a small number of mycorrhizal tips (less than 5), 30µl (rather than 100 µl) ultra-pure water was used to further concentrate DNA. Extracted DNA was stored at -20°C prior to amplification by PCR using the fungal primers ITS-1F and ITS4; these primers target ascomycete and basidiomycete DNA (Landeweert et al. 2003, Murat et al. 2005, Krumins et al. 2009). The reactions were run in an Eppendorf Mastercycler Pro thermocycler (Eppendorf, Hauppauge, NY) under the following conditions: 94°C initial denaturization for 5 minutes followed by 34 cycles of 30 seconds at 94°C, 2 minutes at 50°C, 3 minutes at 72°C and a final elongation at 72°C for 5 minutes. The PCR product was then stored at -20°C.

To obtain pure mycorrhizal clones for sequencing, PCR products were first ligated and then transformed using the Promega pGEM®-T Vector System (Promega, Madison, WI) following the manufacturer's recommended protocol (Landeweert et al. 2003). Two successfully cloned colonies were chosen from each sample at random to be sequenced. The cloned and amplified DNA was prepared for sequencing by the removal of excess nucleotides, salts and amino acids by running through a PERFORMA® Spin Column (Edge BioSystems, Gaithersburg, MD). The samples were then sequenced using an Applied Biosystems Sequencer model 3130 Genetic Analyzer (Thermo Fisher Scientific Inc. Waltham, MA). The Applied Biosystems kit Big Dye version 3.1 was used to prepare the samples according to the manufacturer's recommended protocol. DNA sequences were entered into the National Center for Biotechnology Information (NCBI) BLAST website (National Center for Biotechnology Information 2009) to

compare the sequence to voucher specimens and generate probable species identity. Most of our morphotypes were matched to known sequences, however, those that were not successfully sequenced were labeled as Unknown and numbered 1-9 to differentiate them. Sequencing analysis allowed us to rigorously identify and quantify the arbitrary morphotyped EMF tips. The identities and sequences collected in this analysis will allow for future work that quantifies individual taxa through methods like quantitative PCR.

*Above Ground Plant Productivity Measurements*

To characterize above ground plant productivity, the Leaf Area Index (LAI) was calculated using a LI-COR 2200 (LI-COR, Inc, Lincoln, NE) once weekly from May of 2012 and June of 2013 until the ectomycorrhizae sampling dates (early June 2012 and early July 2013). This plant canopy analyzer uses the gap fraction technique at five zenith angles to assess sunlight penetration. All data is presented as the mean and standard error of the five sampling points along the established transect at each site (n=5). All measurements were collected between 6:30am and 8:30am to preclude the potential for interference from direct sunlight. The results from 2012 and 2013 are presented separately.

*Data Analysis*

The relative abundances of each sequenced and unknown morphotype were used in a Principal Components Analysis (PCA) to determine variation in ectomycorrhizal community composition among the sites. PCA was followed with a multivariate analysis of variance (MANOVA) testing for differences among the sites and considering the first three component scores as response variables. The MANOVA was followed with the

Bonnferroni test for means separation. To test for a relationship between the EMF community composition and LAI of each site, we ran a Spearman Rank Correlation between the first two component scores and the LAI values of each site. This analysis parallels that of the site comparison, but it also demonstrates the possible relationship between primary productivity and the mycorrhizal community composition of the soil. The Shannon Index was calculated as a means of representing the ectomycorrhizal diversity level of each site. The Shannon Index was calculated separately for each pin and then the values were averaged together for each site, resulting in one Shannon Index value for each site (n=5). All of the statistical tests were carried out in SAS Version 9.1 (SAS Institute, Inc. Cary, NC) or Minitab (Version 12.3).

## Results

### *Sequencing Results*

Nineteen taxa from 13 families and 12 orders were identified by cloning and sequencing the ITS region of ribosomal DNA (Table 2). *Cenococcum, Inocybe* and *Leptodontidium* were the only genera found in all five sites. The genus *Russula* was seen only at the highly contaminated sites, while the genus *Sebacina* was seen only at the low contaminated sites.

### *EMF Community Analysis*

In 2012, 21 different EMF morphotypes were identified to order or higher after sequencing. The MANOVA revealed a significant effect of site on EMF community composition as determined by morphotyping followed by sequence identification (Figure 2a, Wilks' Lambda $F = 2.43$, $P < 0.05$). The Bonnferoni test revealed that the EMF

communities of the low metal sites were most significantly similar to each other and site H2 while the high metal sites were most significantly similar to each other and site L1. The factor scores generated by the PCA were based on the relative abundances of each sequenced morphotype. Factor 1 was positively and significantly correlated to the relative abundance of *Tomentella sublilacina* and *Inocybe lacera*, and negatively correlated with *Cenococcum geophilum*. Factor 2 was positively and significantly correlated to *Russula illota* or *R. laurocerasi* (could not be distinguished to the species level by sequencing), and negatively and significantly correlated with Unknown 5 and *Tomentella sublilacina*.

In 2013 an additional high metal load site was added to the sampling regime. Twenty two EMF taxa were identified to the level of order or higher. The MANOVA revealed a significant effect of site on EMF community composition as determined by morphotyping followed by sequence identification (Figure 2b, Wilks' Lambda $F = 4.13$, $P < 0.001$). The Bonferroni test revealed that the EMF communities of the low metal sites were most significantly similar to each other and those of the high metal sites were most significantly similar to each other. Factor 1 was positively and significantly correlated to the relative abundance of *Tomentella*, and *Inocybe lacera*, and negatively and significantly correlated to *Cenococcum geophilum*. Unknown Species 6 and *Scleroderma bovista* are correlated with Factor 1, but not at a significant level ($p = 0.07$). Factor 2 was positively and significantly correlated to *Scleroderma bovista*, *Fusarium oxysporum* and Unknown 9, and negatively correlated to *Tomentella*, *Inocybe lacera* and Unknown 5.

Interestingly an ANOVA of the Shannon Index showed no significant difference among sites for 2012 (Figure 4a, $F = 0.73$, $P > 0.5$) or 2013 (Figure 4b, $F = 0.88$, $P > 0.4$).

*Leaf Area Index*

We were interested in the relationship between primary productivity of the plant assemblage and the mycorrhizal community composition of the soil. In 2012 a significant correlations between the LAI values and PC2 (Figure 3a. $r=0.4484$ and $P<0.05$), and in 2013 between LAI and PC1 (Figure 3b. $r=-0.5138$ and $P<0.001$) and PC2 (Figure 3b. $r=-0.4079$ and $P<0.05$) was found. Interestingly the scores correlated positively in the first year and negatively in the next, which may be an artifact of the arbitrary values assigned in the PCA. Although most taxa are distributed across all of LSP (Table 3), they are not evenly distributed and this also varies between years. When we consider which taxa correlate with the component scores across the two years, *Rusulla sp*. and *Schleroderma sp.* correlate with sites of high metals and high LAI. This is especially the case for site H1 which has the highest LAI values. *Russula sp*. was seen in both 2012 and 2013, while *Scleroderma sp*. was seen only in 2013. *Inocybe sp.* was seen in both years and correlates with low metal sites that have lower LAI than site H1, but higher than site H2 (Table 3).

**Discussion**

The inherent difficulty in identifying ectomycorrhizal communities has restricted the number of studies which address community structure and functioning. While studies of EMF communities under conditions of soil metal stress are limited, one study found pronounced differences in microbial community composition and functioning associated with a copper mine (Wang et al. 2007), and another study found differences in microbial community diversity (Chappelka et al. (1991). In addition, studies of copper mine waste

(He et al. 2010) have found metal resistant microbes within the rhizosphere of the associated plant assemblage. Since the site in this study has had a relatively long development history, approaching 50 years, perhaps a metal tolerant microbe community has also developed. In this study, a significant difference among EMF community composition at sites of differing contamination levels (Figure 2 a, b) was found. Specifically, the low metal contamination sites were most similar to one another and, likewise, the high metal contamination sites were most similar to one another. In addition, there was a much greater variation between the high metal sites than there was between the low metal sites, possibly due to differences in site concentrations of the different metals or variation in other abiotic factors such as organic content or nutrient availability. However, since differences in pH and moisture levels did not correlate either individually or collectively with the observed changes in EMF community composition in a separate analysis (*data not shown*), we believe the difference in EMF community structure to be metal driven.

Interestingly, although EMF composition was shown to vary along the metal concentration gradient, there was no significant difference in the Shannon indices of species diversity at each site. Although the diversity levels were slightly lower in 2013 than they were in 2012, the trends between the sites remained consistent. There was no correlation with metal concentration, as proposed in our original hypothesis that there would be decreased diversity with increasing metal load. This demonstrates that, at least in this setting, composition of the EMF community, rather than diversity, is more strongly related to metal contamination.

Most of the sites had relatively few dominant species and many rare species (Table 3) similar to studies by Baxter et al. (1999) and Regvar et al. (2010). This is in contrast to findings which observed a much higher species richness and evenness across metal contaminated sites in Austria (Krpata et al. 2008). The lack of similarity in diversity between field studies in metal contaminated environments supports the notion that there is a highly dynamic relationship between soil conditions and biotic organisms. The difference between studies also suggests that these relationships are contextual since there is a high degree of variability. Factors such as nutrient availability, competition with other soil organisms and disturbance can have large effects on EMF diversity (Bruns 1995). Studies which examine a combination of variables such as nutrient levels, competition, plant species composition and contamination both singularly and in the same treatment may help to resolve which factors have the most influence on EMF diversity and composition.

The species composition between sites showed a large degree of variation. Only three genera, *Cenococcum, Inocybe* and *Leptodontidium,* were found across all of the study sites. Additionally, there was a large variation in the relative abundance of these species (Table 3). Such factors may include interspecific competition, metal contamination, interactions with other soil microbes, plant species composition, soil characteristics or a combination of these factors. In a study on global patterns of fungal diversity, researchers found that the phylogenetic family of the host plant was the largest driver of EMF composition (Tedersoo et al. 2012). Additional studies characterizing the plant assemblage composition at each of the sites would determine if that pattern is also

seen at LSP. However, this study demonstrates that even in sites with the same dominant plant species, considerable variation in EMF community composition can occur.

As in other studies (Cripps 2003, Krpata et al. 2008), *Cenococcum geophilum* was the dominant species across several sites. This species is known to be highly adapted to metalliferous soils (Chappelka et al. 1991). Interestingly, in contrast to the aforementioned studies, the LSP study shows that *C. geophilum* was dominant at the two sites with relatively low metal concentrations and only one of the sites, H2 (Table 3) above the established TML threshold. Site H3 was dominated by *Scleroderma bovista* and *Inocybe lacera* while site H1 was mainly dominated by *Russula illota/laurocerasi* and *Inocybe lacera* (Table 3). It should be remembered however, that the concentration of metals at the LM sites of this study are low only compared to the other sites within the study area. All sites had soil metals that exceeded ecological screening standards. In addition, even at the high metal load sites the concentration of the individual species of metal varied significantly. Perhaps *C. geophilum*, while metal tolerant, has a threshold limit for a particular species of metal. Of particular interest is the extremely high concentrations of Cu and Pb at the high metal load site (H3) not dominated by *C. geophilum* While other edaphic conditions also vary between the higher metal load sites, the absence of *C. geophilum* may indicate a specific relationship with these two metals and deserves further study.

In general, variation in EMF community composition was observed based on their position on the metal contamination gradient. While EMF composition changed depending on the level of metal contamination, diversity levels remained relatively constant. *Cenococcum geophilum* was the dominant species in the low contaminated

environments. The variation in dominant EMF species at high soil metal concentrations supports the idea that EMF species have different tolerance to concentrations and/or types of metals.

The relationship between the EMF community and the plant community Normalized Difference Vegetation Index (NDVI) was also interesting. Contrary to expectations, two of the high metal load sites exhibited significantly higher NDVI readings than those of the low soil metal load sites. The difference in the corresponding EMF communities support the notion that the above and below ground feedbacks are context specific (Reynolds et al. 2003) changing in a relatively small geographic area. In addition, the temporal (between years) change in the EMF community composition at the high metal load sites, indicates that the feedback relationship is also dynamic (Bardgett et al. 2005). Furthermore, it is known that while sites H1 and H3 exhibited higher NDVI values, the rate of tree growth (DBH/time) is slower than at site L1 and L2 (Dahle et al. 2014). This indicates that maintenance and/or reproduction is requiring more energy than growth in comparison to the norm for the area.

While restoration initiatives that include inoculation of the soil with metal tolerant ectomycorrhiza have been recommended (Quoreshi 2008) and may be beneficial to the above ground plant communities, the relatively high LAI at two of the high soil metal load sites with differing EMF community composition, may indicate that the ecological legacy of the community is also significant.. The effectiveness of a locally specific EMF community legacy that enhances the stability of the above ground plant community may eventually change paradigms associated with the long term ecological risk of brownfield

sites. Specifically, stable plant communities result in stronger water balance models and decreased erosion potential, which reduces the risk of soil metal contaminant transfer.

Additional studies are needed to further explore the metal tolerance capabilities and niche variation in EMF species. For instance, there were several taxa that were identified by sequencing but not recognized during physical morphotyping. The tips of one morphotype were collected, but sequencing showed several taxa (species), sometimes from different genera and families, all present in the sample of that one morphotype. This could be a result of fine scale heterogeneity of resources in the soil matrix (Baxter and Dighton 2001). Conversely, multiple taxa may be converging in areas where there are abundant resources. *Inocybe* and *Tomentella* were frequently seen together in the same morphotype. The difficulty in differentiating the two taxa might suggest a structural relationship or dependency in the morphotype.

In conclusion, this study demonstrated that soil metal contamination does have an effect on EMF community composition, and it supported to the notion that EMF species have varying levels of tolerance for metals. Most novel was the observation that overall diversity of the EMF communities did not differ significantly, however the species composition of those communities was significantly different. The trends seen here can help guide future studies in determining additional biotic and abiotic factors and facultative/competitive relationships which may be driving EMF community composition in brownfield environments. Further characterization of metal tolerant EMF species and knowledge of the plant communities they support will facilitate restoration of brownfield sites.

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## Figure Legends

Figure 1. A map of the soil metal contamination levels across the interior section of the park, adapted from Gallagher et al. (2008b). The figure was amended to include a diagram of the site locations used in the present study.

Figure 2. The relationship between variance in ectomycorrhiza community explained by the first two factor scores of the Principle Components Analysis in 2012 (a) and 2013 (b). The factor score from each site is the mean score across the five pins at that site. For each site error bars indicate standard deviation, and n=5.

Figure 3. The relationship between Leaf Area Index and component scores 1 and 2 of the Principle Components Analysis in 2012 (a) and 2013 (b). Each vertical line corresponds to one site, and each point corresponds to the fungal community at one pin on one day.

589 The scatter plots represent significant correlations between LAI and PC2 (r=0.4484 and
590 P<0.05) in 2012, and between LAI and PC1 (r=-0.5138 and P<0.001) and PC2 (r=-
591 0.4079 and P<0.05) in 2013. Variance explained by the component scores is presented in
592 figure 2.

593 Figure 4. A comparison of the results of the Shannon Index for 2012 (a) and 2013 (b).
594 The graphs show the trend across sites. For each site error bars extend from the 75th
595 percentile to the maximum value (upper) and from the 25th percentile to the minimum
596 value (lower). There were no significant differences among the sites.
597
598
599

Table (No Excel Files. Please submit as MS Word.)

| **Table 1: Description of characteristics of each site.** | | | | | |
|---|---|---|---|---|---|
| | **L1** | **L2** | **H1** | **H2** | **H3** |
| Avg LAI 2012/2013 | 2.53/2.06 | 2.45/2.25 | 3.14/2.57 | 1.51/1.59 | NA/2.81 |
| †Shannon Index (2012/2013) | 1.19/.953 | 1.06/.888 | 0.83/.593 | 1.01/.92 | NA/1.06 |
| ‡TML (2005) | 1.56 | 1.64 | 3.56 | 3.08 | 4.31 |
| ‡As µg g-1 | 13 | 29 | 68 | 181 | 384 |
| ‡Cr µg g-1 | 142 | 19 | 452 | 115 | 66 |
| ‡Cu µg g-1 | 95 | 230 | 203 | 224 | 2200 |
| ‡Pb µg g-1 | 245 | 460 | 858 | 926 | 6673 |
| ‡Zn µg g-1 | 22 | 89 | 238 | 37 | 2327 |
| Soil pH (2009) | 5.9 | 7 | 4.8 | 5.2 | 6.2 |
| Organic Matter ppm (2009) | 9.6 | 10.9 | 19.5 | 4.3 | 41.6 |
| Total Soil Nitrogen % (2009) | 0.3 | 0.4 | 0.6 | 0.1 | 1.01 |
| P ppm (2009) | 3 | 9 | 8 | 3 | 4 |
| K ppm (2009) | 94 | 293 | 79 | 41 | 36 |
| †Describes average EMF diversity by site (n=5) | | | | | |
| ‡Adapted from Gallagher et al. 2008 | | | | | |

| **Table 2: Each EMF taxa identified by sequencing and BLAST analysis** | | | |
|---|---|---|---|
| **Known to be present at sites** | **Species** | **Blast Query Cover %** | **Blast Identity %** |
| L1, L2, H3 | *Inocybe lacera* | 97 | 96 |
| L1 | *Russula cerolens* | 97 | 98 |
| L1 | *Helotiaceae* (family) | 94 | 98 |
| L1 | *Cenococcum geophilum* | 98 | 96 |
| L1 | *Sebacina sp.* | 94 | 97 |
| L2 | *Rhizoscyphus sp.* | 96 | 92 |
| L2, H3 | *Phialocephala sp.* | 93 | 96 |
| H1, H2 | *Tomentella sp.* | 97 | 98 |
| H1, H3 | *Russula mariae* | 93 | 95 |
| H1 | *Russula parazurea* | 98 | 94 |
| H1 | *Peziza saccardoana* | 95 | 94 |
| H1, H3 | *Leptodontidium* | 87 | 99 |
| L2, H1, H2, H3 | *Helotiales* (order) | 94 | 99 |
| H2 | *Hebeloma mesophaeum* | 97 | 99 |
| H3 | *Cylindrocarpon sp.* | 90 | 99 |
| H3 | *Isaria fumosorosea* | 97 | 98 |
| H3 | *Phialocephala fortinii* | 90 | 83 |
| H3 | *Cadophora sp.* | 95 | 97 |
| H3 | *Cryptococcuz terricola* | 94 | 99 |
| H3 | *Meliniomyces sp.* | 92 | 97 |
| H3 | *Sordariomycete* | 98 | 97 |
| H3 | *Scleroderma bovista* | 98 | 99 |
| H3 | *Lecanoromycetidae* (family) | 95 | 95 |
| H3 | *Cylindrocarpon pauciseptatum* | 96 | 99 |
| H3 | *Lactarius glyciosmus* | 96 | 98 |
| H3 | *Fusarium oxysporum* | 96 | 99 |
| H3 | *Saccharomyces cerevisiae* | 94 | 99 |

| **Table 3: Relative abundance by site in 2013, as determined by morphotyping** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Site L1:** | | **Site L2:** | | **Site H1:** | | **Site H2:** | | **Site H3:** | |
| *Cenococcum geophilum* | 53.6% | *Cenococcum geophilum* | 54.5% | *Cenococcum geophilum* | 9.3% | *Cenococcum geophilum* | 41.0% | *Cenococcum geophilum* | 1.4% |
| *Leptodontidium* | 3.6% | *Leptodontidium* | 0.3% | *Leptodontidium* | 18.2% | *Leptodontidium* | 13.4% | *Leptodontidium* | 4.9% |
| *Inocybe lacera* | 1.3% | *Inocybe lacera* | 8.7% | *Inocybe lacera* | 22.2% | *Inocybe lacera* | 36.7% | *Inocybe lacera* | 25.9% |
| *Tomentella* | 0.2% | *Tomentella sublilacina* | 0.4% | *Russula parazurea* | 14.4% | *Tomentella* | 2.2% | *Tomentella* | 3.8% |
| *Sebacina* | 16.8% | *Sebacina* | 1.1% | *Russula mariae* | 6.1% | *Tomentella sublilacina* | 2.0% | *Saccharomyces cerevisiae* | 5.5% |
| *Saccharomyces cerevisiae* | 5.6% | *Phialocephala* | 29.1% | *Russula illota/laurocerasi* | 29.3% | *Russula parazurea* | 1.4% | *Russula parazurea* | 2.6% |
| Unknown 1 | 13.7% | Unknown 3 | 0.1% | Unknown 3 | 0.4% | *Russula mariae* | 0.8% | *Phialocephala* | 2.4% |
| Unknown 2 | 4.2% | Unknown 4 | 2.2% | Unknown 5 | 0.1% | Unknown 5 | 1.0% | *Scleroderma bovista* | 47.5% |
| Unknown 3 | 0.3% | Unknown 5 | 0.2% | | | Unknown 6 | 0.7% | *Fusarium oxysporum* | 3.9% |
| Unknown 4 | 0.7% | Unknown 6 | 1.2% | | | Unknown 7 | 0.8% | Unknown 5 | 0.6% |
| | | Unknown 7 | 1.2% | | | | | Unknown 9 | 1.5% |
| | | Unknown 8 | 1.0% | | | | | | |

Figure

Figure 1.

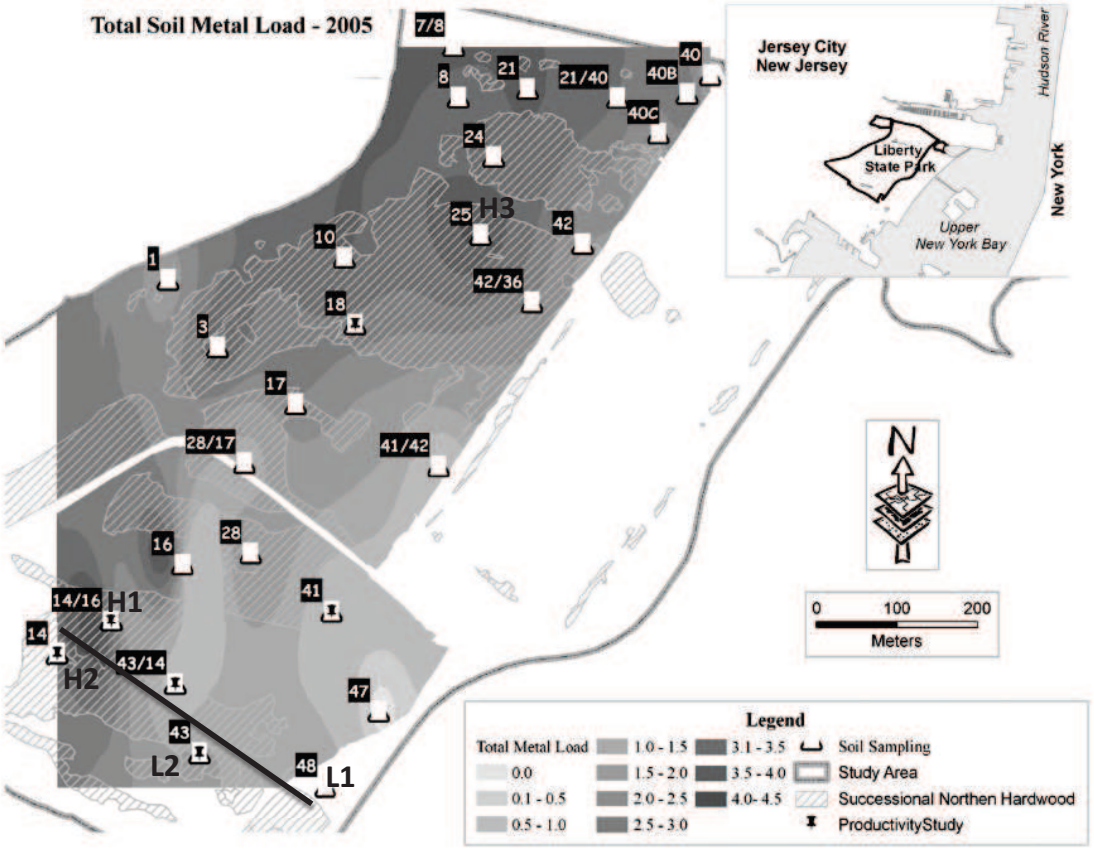
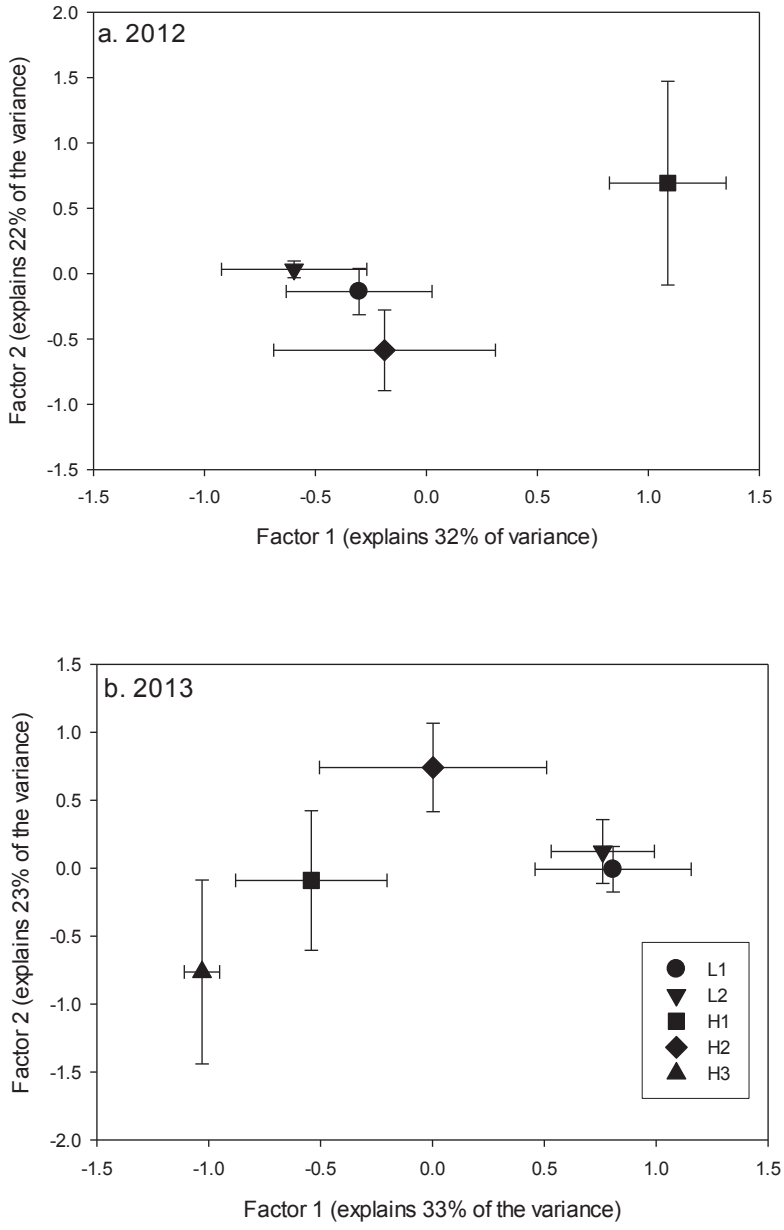


Figure 2.


a. 2012
Factor 2 (explains 22% of the variance)
Factor 1 (explains 32% of variance)
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5


b. 2013
Factor 2 (explains 23% of the variance)
Factor 1 (explains 33% of the variance)
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
L1
L2
H1
H2
H3

Figure 3.

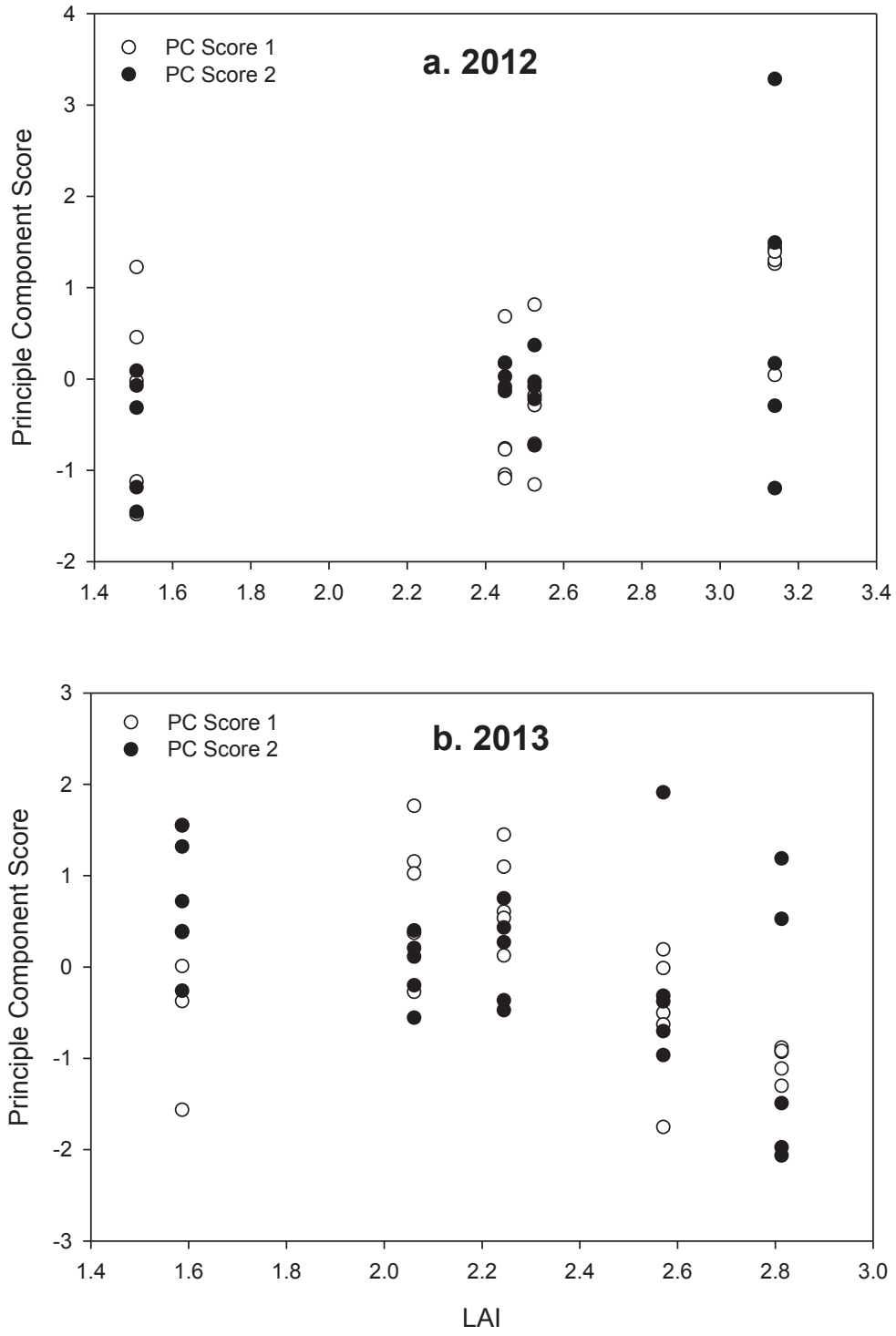
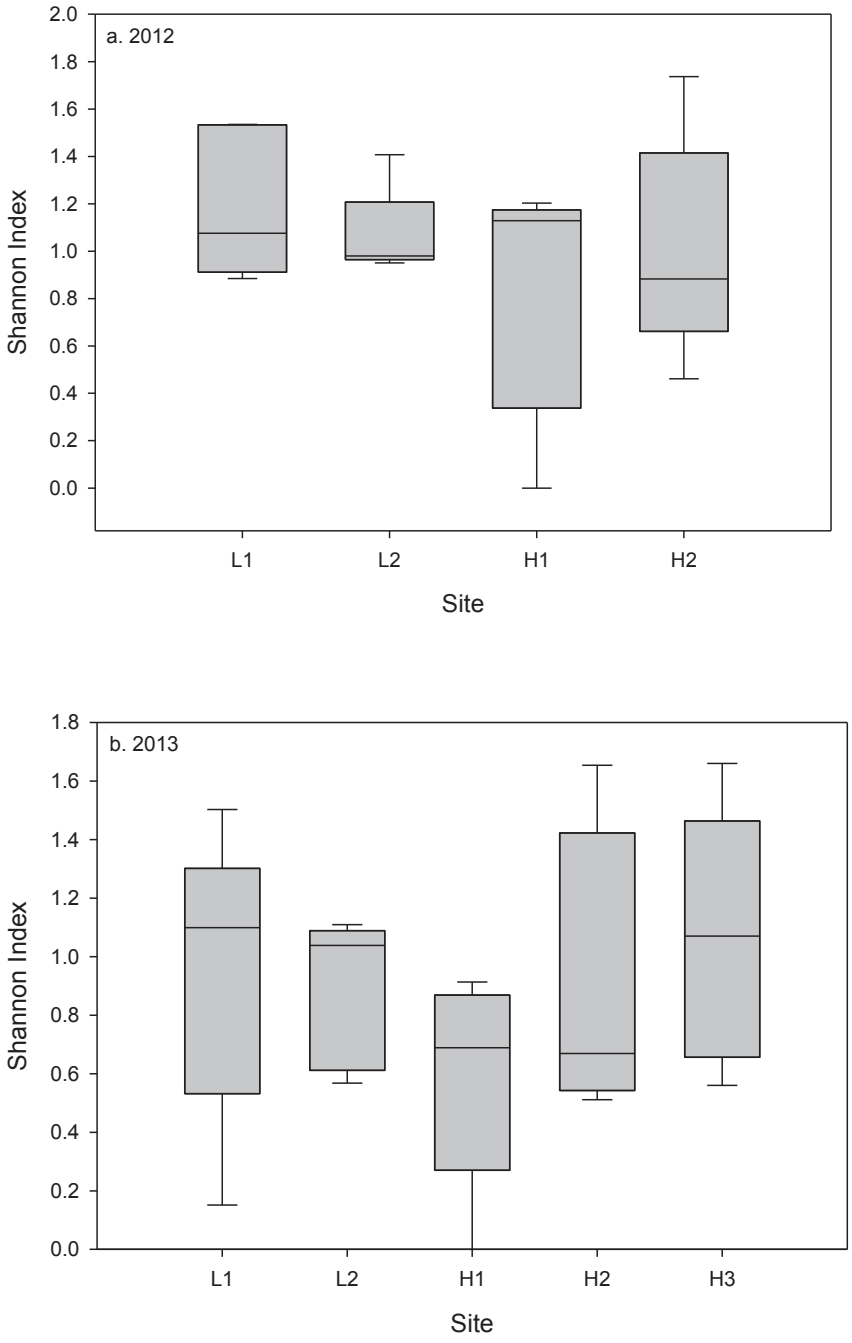

a. 2012
PC Score 1
PC Score 2
Principle Component Score
4
3
2
1
0
-1
-2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
3.0
3.2
3.4
b. 2013
PC Score 1
PC Score 2
Principle Component Score
3
2
1
0
-1
-2
-3
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
3.0
LAI

Figure 4.


a. 2012
Shannon Index
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
L1
L2
H1
H2
Site
b. 2013
Shannon Index
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
L1
L2
H1
H2
H3
Site

Evans et al.

**Response to Reviewers**

**SS-15-43 - "Plant Productivity, Ectomycorrhiza and Metal Contamination in Urban Brownfield Soils"**

***Please find our responses following each comment offset in italics.***

Guest Editor: The reviewers have raised two major issues that need addressing: the effects of moisture and other variables (vs metal content), and the inclusion on non-ectomycorrhizal fungal taxa. And some additional minor suggestions. Please see comments below.

Reviewer #1: This work utilizes an interesting setting for the study of ectomycorrhizal relationships under metal stress, however there are some inherent problems with the study that make it difficult to determine whether the results are due to metal stress or other factors.

As summarized in Table 1, in addition to metal load there are a number of other variables that change greatly across the site that may contribute to community shifts (organic matter, pH nutrient levels, among others). Additionally, soil moisture, which with pH may be the most influential factor affecting fungal proliferation in soil was not evaluated, nor was topography, which may influence moisture via drainage patterns.

- *The reviewer brings up a very important point, and we recognize the role of abiotic soil conditions in shaping microbial communities. Following this comment, we correlated both pH and soil moisture with the factor 1 scores for fungal community composition. Neither show significant correlations: pH, r= 0.297 with P=0.149 and soil moisture, r=0.291 and P=0.158. Further, to more rigorously test for pH and moisture interactions, we carried out a multiple regression procedure. The ANOVA found no significant effect where F= 2.09 and P= 0.147. Text was added to the manuscript at line 300 to address this point for readers. We appreciate the reviewer comment regarding topography. However, that has not been measured at this site, and it is beyond the scope of this study. We look forward to addressing this interesting hypothesis in the future.*

In terms of the methodology for EMF identification, it seems that the visual morphotyping process is very difficult to confirm accurate sorting. The use of the molecular tools is a much more accurate way to consider species diversity. While the ITS1F-ITS4 primer set will target a highly variable region of the 18S subunit, the fact that template DNA was not quantified prior to amplification means that different template concentrations may bias results between reactions. The quantification of DNA prior to amplification as well as the use of quantitative PCR would provide more reliable information regarding species distribution and abundance.

- *We recognize that the morphotyping process can be subjective and difficult to confirm accurate sorting. This is why we following up our morphotyping with cloning and sequence identification of individual tips. The exact composition of the community was not known prior to this study, so we did not have the ability to develop primers that would allow qPCR and a more accurate measure of relative abundance of the different types. However, we have inserted text at lines 205-207 to more clearly convey our goals with the molecular analysis. In short, the mycorrhizal morphotypes were given identifying names then microscopically counted. Representatives of each of those types was manually isolated under the microscope (thus eliminating bias), and their ITS region sequenced. Specifically, amplified products from each of the isolated EMF tips were cloned into a vector and competent cell. The ITS amplified DNA from each of those colonies was then sequenced. For this reason, quantification of our DNA was not relevant. We do recognize that more than one fungal taxon may be represented in each morphotype tip. The cloning and sequencing procedure allowed for resolution of this. Sequences were searched through BLAST. Percent sequence similarity for each identity was presented in Table 2. We very much appreciate the comment, and now knowing the sequence identification of so many EMF types from our site, we hope to be able to design primers and incorporate more rigorous enumeration methods like qPCR in the future. The work we present here is foundational for just such a study.*

Some minor issues include:
The introduction does not adequately utilize existing literature to define an existing knowledge gap.

- *We have developed the ideas and the place for our contribution to the literature in the introduction. We inserted new text and references throughout, but specifically please see lines 51, 78 and 92, which were added to clarify the need for further studies which examine diversity and community composition changes as they relate to the functioning of the above ground system.*

Principal components analysis should be spelled with 'pal' not 'ple'.

- *This has been fixed.*

The writing style of the manuscript is in a more informal first person style ("we").

*-The manuscript has been edited to address this.*

There are some punctuation issues regarding in-text citations and placement and number of parenthesis.

*-The manuscript as been edited to address this.*

Metal speciation generally refers to oxidative state and not metal type.

*- The manuscript has been edited to address this.*

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