Tree diversity is not always a strong driver of soil microbial diversity: a 7-yr-old diversity experiment with trees

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Abstract. Trees provide organic substrates in the form of root exudates, litterfall, and fine root turnover. They modify soil physical properties and support soil biological activities. Therefore, trees are hypothesized to control soil biodiversity in forested areas. We predicted that (1) experimental forest plantations with higher tree alpha-diversity have greater soil microbial alpha-diversity and (2) that plantations with more divergent tree community composition would have more divergent soil microbial assemblages (Whitaker’s beta-diversity). We tested these predictions by measuring soil bacteria and fungi in a 7-yr-old tree biodiversity experiment. The experimental plantation contained 37 different tree assemblages, which were composed of one to four native species from temperate mixed deciduous forests. Further, there was a gradient of functional diversity nested within each level of species diversity. Soil samples were assessed for bacteria and fungi by amplicon sequencing. Tree alpha-diversity weakly, but significantly, affected bacterial alpha-diversity, without affecting fungal alpha-diversity. Tree community composition was weakly, but significantly, linked to soil bacterial and fungal assemblages. In these 7-yr-old experimental plantations, tree diversity was not the most influential driver of soil microbial diversity.

Key words: 16S-rRNA gene sequencing; aboveground–belowground interactions; biodiversity–ecosystem functioning; forest plantation; IDENT; ITS-rRNA gene sequencing; temperate forest; TreeDivNet.

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Introduction

Changes in soil biological diversity may explain up to 50% of the variation in an ecosystem’s multifunctionality (Wagg et al. 2014, Delgado-Baquerizo et al. 2016). It is thus important to understand what drives such diversity. Soil biological diversity is likely explained by a mixture of neutral and niche processes. Among the niche processes, some have proposed that edaphic and climatic conditions are important drivers of soil biodiversity (Tedersoo et al. 2014, Prober et al. 2015), but in forest ecosystems, it could also be influenced by tree community structure, that is, diversity and composition (Hooper et al. 2000). Tree community structure is hypothesized to control belowground community structure through (1) the provision of organic substrates that originate from root exudates, litter, and fine root turnover; (2) the creation of physical habitats; and (3) the roles of trees as hosts to soil biota (Wardle 2006). From this tree-as-driver hypothesis, it can be deduced that the alpha- and beta-diversity (i.e., the...
turnover in taxa composition of a community along a gradient; Whittaker 1972) of soil biota would match the alpha- and beta-diversity of trees at the plot scale (1–50 m²). Vegetation has also been considered as a factor controlling the alpha- and beta-diversity of soil biota in grassland ecosystems, as postulated by Prober et al. (2015).

Evidence for this tree-as-driver hypothesis is equivocal. On one hand, results from some studies suggest that a strong relationship will occur between tree and soil microbial diversity. At a landscape scale, Hiiesalu et al. (2017) observed that tree diversity was a key predictor of fungal diversity in low-productivity Scots pine forests. Similarly, Gao et al. (2013) found that ectomycorrhizal fungi were positively correlated with host plant diversity in tropical and temperate forests.

On the other hand, experimental studies have found less evidence for the tree-as-driver hypothesis. For example, Yamamura et al. (2013) provide evidence that tree species identity and richness exerted few effects on the structure of bacterial communities within the bulk soil of a tropical tree plantation. Tree diversity experiments in the boreal and temperate regions found that tree species identity and richness could affect soil fungal diversity, but it was largely context-dependent and depended upon taxonomic group (Nguyen et al. 2016, Tedersoo et al. 2016). It is challenging to detect tree interactions with soil microorganisms in model forest experiments, compared to other plant-based models such as grassland experiments (Tobner et al. 2014). For example, in tree diversity experiments, the understory vegetation layer furnishes additional organic substrates, micro-habitats, and hosts for soil biota that could obscure the effects of tree identity and diversity on microbial diversity (Tedersoo et al. 2016). Effectively controlling for confounding effects of understory vegetation in tree diversity experiments would allow us to gain a better understanding of the effects of tree diversity on soil microbial diversity.

In the current study, we investigated the effect of tree diversity and composition on soil bacterial and fungal diversity, and composition in a seven-year-old, weed-free high-density plantation experiment (IDENT-Montreal). The common garden experiment, which is part of TreeDivNet (Grossman et al. 2018), consists of deciduous and evergreen tree species monocultures, and mixtures of identical species richness varying in functional diversity. Further, it is the first tree diversity experiment to have continuously eliminated understory vegetation, allowing us to isolate effects of tree diversity and composition on soil microbial diversity. In the IDENT-Montreal experiment, Laforest-Lapointe et al. (2017) reported that tree species identity and functional diversity were the main drivers of tree leaf bacterial community structure and diversity. Furthermore, Jewell et al. (2017) found that soil respiration rates responded positively to taxonomic and functional richness of tree species, possibly due to tree community effects on the soil moisture and chemical properties (Rivest et al. 2015). Khlifa et al. (2017) found that microbial communities associated with mixed species had greater microbial biomass and used a greater number of carbon sources than those in monocultures. Overall, this suggests that these tree communities with diverse functional traits and species composition successfully produced different soil resources, habitats, and hosts, making the site suitable for testing the hypothesis that trees are strong drivers of soil biodiversity. Based on this hypothesis, we predicted that (1) plots with higher tree alpha-diversity would have greater soil microbial alpha-diversity and (2) plots with more divergent tree community composition (beta-diversity) would have more divergent soil microbial communities (beta-diversity).

**Methods**

**Site description and experimental treatments**

The experimental site (IDENT-Montreal) was established in May 2009 on an agricultural field, which was located in Ste-Anne-de-Bellevue, QC (45°26′ N, 73°56′ W, 39 m asl). The site is characterized by a cold, humid temperate climate, where monthly temperature over the last ten years averages from −9.1°C in January to 21.3°C in July, with mean annual precipitation of 903 mm (Ste-Anne-de-Bellevue Climate Station; Environment Canada 2018). In the Canadian system of classification, the soil is a Humic Gleysol (7th Approx., Typic Endoaquent), with a sandy loam texture (780 g/kg sand, 60 g/kg silt, 160 g/kg clay). In 2015, average (±SD) soil pH_{water} was 6.48 (±0.23), organic carbon was 2.62% (±0.41),

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total nitrogen was 0.24% (±0.043), and extractable nitrate and ammonium were, respectively, 3.64 (±0.93) mg/kg and 2.06 (±0.62) mg/kg.

The experiment was designed as a split-plot, completely randomized block design with four blocks and two factors under investigation: species diversity (1, 2, 4, and 12 species per plot) and functional diversity (continuous gradient grouped in eight levels). The functional diversity gradient was nested within the assemblages of two- and four-tree species (for a complete description of the experimental design, see Tobner et al. 2014). Thirty-seven different species assemblages were created and replicated in each of the four blocks, which each contained 12 monocultures of deciduous and evergreen North American temperate tree species, 14 combinations of two-species mixtures, 10 combinations of four-species mixtures, and one mixture with all 12 species. Plots with the 12-species mixture were omitted from most analyses, given the low relative number of replicates for this treatment and its high undue influence. Therefore, 148 plots (16 m²) were sampled in this study. Species mixtures were established to create a functional diversity gradient over each of the fixed and independent species richness levels. Tree community plots, which measured 4 × 4 m, consisted of 64 individual trees that had been planted every 50 cm in eight rows. Rows were separated by 1.25 m wide corridors to reduce interactions among adjacent plots and to allow personnel and equipment movement without disturbing the plots. The corridors were trenched to a depth of 30 cm in the third and fourth growing seasons (2011 and 2012) to prevent roots of neighboring tree communities from interacting. Growth on the site was rapid and, together with the high density at which they were planted and negligible mortality, the trees already had formed a closed canopy by the fourth year following planting. The rapid growth of the model stands resulted in significant biodiversity effects among tree communities (Tobner et al. 2016). The distribution of trees within plots was identical in all four blocks. However, the distribution of plots was randomized for each block. Around the outermost rows of the experiment, buffer trees were planted every 50 cm in three rows to minimize edge effects. Tree communities were periodically weeded manually to maintain desired composition and diversity.

**Soil sampling and analysis**

Soil samples were collected in mid-September 2015, when the trees were 7 yr old, to assess soil chemical properties, and soil bacterial and fungal diversity. To minimize edge effects, soils were sampled using the core area, which contained 36 trees (Fig. 1). In each sample plot, 20 soil cores (2 cm diameter, 0–10 cm depth) were collected to form a composite sample for the determination of soil chemical properties. An additional five soil cores were taken from the central area of the plot and bulked into a composite sample for DNA extraction of soil bacteria and fungi. The auger was rinsed with 70% ethanol before collecting each core in the samples that were designated for soil bacterial and fungal analyses. Soil samples were transported on ice and stored within 24 h at either 4°C (chemical properties) or −20°C (bacteria and fungi). Soil chemical analyses performed on air-dried and sieved (<2 mm) samples for pH (1:2 soil:deionized water slurries), or finely ground with a ball mill to determine total carbon and nitrogen concentrations using a TruMac CNS analyzer (LECO Corporation, Saint Joseph, Michigan, USA). About 10 g of field-moist soil was extracted in 100 mL of 2 M KCl solution (Maynard and Kalra 1993), shaken for 1 h on a reciprocal shaker, and gravity-filtered through cellulose papers (Whatman No. 5) for colorimetric determination of NH₄⁺ and NO₃⁻ (converted to NH₄⁺ using Devarda’s alloy) concentrations at 650 nm using a modified indophenol microplate method (Sims et al. 1995).

**DNA extraction and sequencing of soil bacteria and fungi**

Soil samples were defrosted at room temperature for 4 h and homogenized, after which DNA was extracted using the MoBio kit (Power Soil) following manufacturer’s instructions (Qiagen, Toronto, Ontario, Canada). Amplicon libraries were made using primers 341F and 805R for bacteria (Klindworth et al. 2013) and primer 517fITS7, coupled to primer rITS4 for fungi (Ihrmark et al. 2012). Those primers were selected because they are less biased than standard alternatives. Adapters fCS1 and rCS2 were added to primers to enable sequencing on a MiSEQ 300 (Illumina, San Diego, California, USA). MiSEQ300 is known to yield sequences with lower quality scores, but it was chosen due to the expected...
length of the region that was covered by the primers. Consequently, 65% of the sequences were filtered out (see details below).

Sequences were separated into amplicons of bacterial and fungal origin. All the subsequent steps were accomplished using DADA2 (Callahan et al. 2016). Forward and backward sequences were then trimmed and truncated. The first 25 nucleotides were removed from both forward and backward sequences. Forward sequences were trimmed to a length of 280 bp, while backward sequences were trimmed to a length of 200 bp. Dozens of trimming lengths were tested on 12 samples to find the best values. Consistent with the default for the filterAndTrim function that is provided by the package DADA2, sequences were trimmed at the first instance of a quality score \( \geq 2 \). Sequences with an expected error \( \geq 2 \) were omitted from subsequent analyses. Sequencing errors were corrected with the DADA2 function (Callahan et al. 2016, R Core Team 2018). Forward and reverse sequences were merged if they matched on \( \geq 12 \) base pairs; otherwise, the sequence was removed from the dataset. After checking for chimeras and removing singletons, 35% of the sequences remained. Finally, we rarefied to a depth of 1500 sequence reads for fungi and 7000 for bacteria. For bacteria, three samples (three experimental plots) were treated as missing data because the samples did not contain at least 7000 sequences; for fungi, five samples were treated as missing data.

**Diversity metrics**

Tree diversity was measured in three ways: (1) species counts; (2) functional diversity; and (3) phylogenetic diversity. We present mostly the results from species counts given that these were very similar across diversity estimates. For alpha-diversity, functional dispersion with community-weighted means using tree basal area as a measure of abundance was used to estimate tree functional diversity. The traits that were used included root growth rate, wood density, specific leaf area, leaf nitrogen, leaf longevity, branching intensity, specific root length, fine root diameter, shade tolerance, drought tolerance, leaf litter carbon, mean rooting depth, and leaf dry matter content. Phylogenetic diversity was estimated using Faith's phylogenetic diversity (Faith 1992). Again, tree basal area was used as a measure of abundance. We used the database Time-tree.org (Hedges et al. 2006) to construct the phylogenetic tree. For tree beta-diversity, functional dissimilarities between communities of trees were measured as the Euclidean distance between two communities’ weighted means of
traits. Tree basal area was used to weight the trait means. Phylogenetic dissimilarities between communities of trees were measured with UniFrac, which was weighted using tree basal area (Lozupone and Knight 2005).

As recommended by Jost (2006), alpha-diversity was calculated as the exponential of the Shannon entropy ($e^{H}$) of bacterial and fungal amplicons. Beta-diversity was calculated as Bray-Curtis pair-wise dissimilarity (Bray and Curtis 1957) between community compositions for all biological communities (i.e., bacteria, fungi, and trees). Sequence number was used as the measure of abundance for bacteria and fungi, while tree basal area was used for trees. Sequence number was used to weight the measurement, rather than using the usual species abundance. Sequence number has been shown to positively correlate (~0.6) with organism abundance (Kembel et al. 2012).

**Statistical analyses**

To test the prediction (1) that plots with higher tree alpha-diversity have greater soil biota alpha-diversity, the effect of tree species, functional, or phylogenetic diversity on the $e^{H}$ of soil bacteria or fungi was evaluated with a linear mixed model, where tree diversity was a fixed effect and the experimental block was a random effect. The analysis was implemented with the function lme from the R package nlme (R Core Team 2018, Pinheiro et al. 2017). To test the prediction (2) that plots with more divergent tree community composition (beta-diversity) have more divergent soil biotic communities (beta-diversity), Bray-Curtis pair-wise dissimilarity (Bray and Curtis 1957) between community compositions of bacteria or fungi was evaluated with three distinct linear models against tree Bray-Curtis pair-wise dissimilarity between community compositions of trees and statistically tested with three partial Mantel tests. These two steps were performed, as implemented in the function multi.mantel from the R package phytools version 0.5-64 (Revell 2012).

**RESULTS**

Alpha-diversity of bacteria, but not fungi, was related to all three measures of tree alpha-diversity (Fig. 2, Table 1). Tree species richness, tree functional diversity, and tree phylogenetic diversity all yielded very similar results (Table 1, Fig. 2; Appendix S1: Figs. S1, S2). The alpha-diversities of bacteria and fungi were not related to soil properties (Table 2). Although soil bacterial alpha-diversity significantly increased as tree diversity increased from one to four species, this trend did not extrapolate accurately to the plots with 12-tree species. The alpha-diversities for bacteria in the 12-species plot were significantly lower than what was predicted by the linear regressions that had been developed using plots containing one- to four-tree species (predicted = 501, observed mean = 233 [$n = 4$], confidence interval [CI] = 166–299, $P = 0.001$).

Tree beta-diversity (community pair-wise dissimilarity), respectively, explained only 0.9% and 0.7% of the variance in bacterial and fungal beta-diversity (community pair-wise dissimilarity; Mantel’s $R_{bacteria}^2 = 0.009$, $P < 0.001$; Mantel’s $R_{fungi}^2 = 0.007$, $P < 0.001$; Fig. 3). Tree community beta-diversity that was based upon functional traits and phylogenetic distances yielded
results that were very similar to those obtained with tree composition (Appendix S1: Figs. S3, S4); with the exception of functional beta-diversity for fungi, the relationship was not significant (Appendix S1: Fig. S3). Changes in bacterial and fungal beta-diversity were not related to pairwise dissimilarity in soil properties ($R_{\text{bacteria}}^2 = 0.009, P = 0.001; R_{\text{fungi}}^2 = 0.007, P = 0.001$).

Table 1. Linear mixed model relating the alpha-diversity of soil microbes to tree alpha-diversity.

| Dependent variable | Tree diversity metric | Intercept | Tree species richness coefficient | Degrees of freedom | $P$-value | $R^2$ marginal | $R^2$ conditional |
|--------------------|-----------------------|-----------|-------------------------------|--------------------|-----------|---------------|-----------------|
| Bacteria           | Species richness      | 249       | 21                            | 134                | 0.04      | 0.029         | 0.045           |
|                    | Functional dispersion | 257       | 22                            | 134                | 0.02      | 0.042         | 0.058           |
| Fungi              | Phylogenetic diversity| 232       | 0.12                          | 134                | 0.03      | 0.034         | 0.051           |
|                    | Species richness      | 143       | $-3$                          | 132                | 0.39      | 0.005         | 0.008           |
|                    | Functional dispersion | 135       | 1                             | 132                | 0.65      | 0.002         | 0.004           |
|                    | Phylogenetic diversity| 141       | $-0.007$                      | 132                | 0.65      | 0.001         | 0.004           |

Table 2. Linear mixed model of the exponential of the Shannon diversity index ($e^{H_0}$) for soil microbial alpha-diversity in relation to soil properties in plantations.

| Dependent variable | Independent variables | Parameter value | Standard error | Degree of freedom | t-value | P-value |
|--------------------|-----------------------|-----------------|----------------|-------------------|---------|---------|
| Bacterial alpha-diversity ($e^{H_0}$) | (Intercept) | 603 | 403 | 122 | 1.50 | 0.14 |
|                    | Total carbon | $-35$ | 31 | 122 | $-1.15$ | 0.25 |
|                    | pH | $-28$ | 61 | 122 | $-0.46$ | 0.65 |
|                    | Nitrate content | $-196$ | 342 | 122 | $-0.57$ | 0.57 |
| Fungal alpha-diversity ($e^{H_0}$) | (Intercept) | 154 | 120 | 120 | 1.28 | 0.20 |
|                    | Total carbon | $-3$ | 9 | 120 | $-0.32$ | 0.75 |
|                    | pH | $-1$ | 18 | 120 | $-0.03$ | 0.98 |
|                    | Nitrate content | $-27$ | 103 | 120 | $-0.26$ | 0.79 |

Fig. 3. (A) Bacterial and (B) fungal composition significantly change as tree composition changes. Changes in taxonomic composition (beta-diversity) for all taxa (trees, bacteria, and fungi) were measured with the Bray-Curtis index, which is a measure of dissimilarity between pairs of experimental plots. Lines represent linear regressions and partial Mantel tests were used to calculate the $P$-values. ($R_{\text{bacteria}}^2 = 0.009, P = 0.001; R_{\text{fungi}}^2 = 0.007, P = 0.001$).
DISCUSSION

Soil bacteria and fungi from the bulk soil were weakly related to tree properties. Soil microbes responded significantly to tree communities (beta-diversity), and bacterial alpha-diversity responded significantly to tree species richness (alpha-diversity). The fact that those results were significant speaks more to the statistical power of our experiment than to the biological significance of the relationship. In all cases, the slope of the relationships between microbes and trees was small and the amount of variance that was unexplained was always above 95%. Observational studies from several ecosystems in different biomes (Barberán et al. 2015, Prober et al. 2015, Scheibe et al. 2015, Wang et al. 2016) have generally found a significant weak relationship for beta-diversities, with no relationship for alpha-diversities. The grassland study that was conducted by Chen et al. (2017) is the only exception thus far. Here, we show that this relationship can be partly caused by a manipulation of tree composition, which is consistent with the tree-as-driver hypothesis. However, the relationship was subtle. For alpha-diversity, we show that tree diversity can cause a small increase in soil bacterial diversity, but not in fungal diversity. This result is also consistent with our tree-as-driver hypothesis for bacteria, but not for fungi. It is important to emphasize that the relationships between trees and microbes were always weak, which is counter to our expectations. We suggest that only a small proportion of the soil microbial community responded to changes in tree composition. Thus, we propose that even if tree community composition drives the presence of some soil bacteria and fungi, most are insensitive to tree composition. This conjecture, if true, would imply that most soil bacteria and fungi accept a wide range of carbon sources (e.g., litter and root exudates) or that there is high redundancy in microbes that can optimally use these ecological niches.

Another reason that trees were not strong drivers of soil biotic diversity may be that some underlying soil properties were more important in structuring soil biotic assemblages than the vegetation. Yet, soil chemical properties (pH, total C, total N, NH\textsubscript{4}+ and NO\textsubscript{3}− concentrations) in the experimental plantation did not explain variation in the diversity and composition of either bacteria or fungi, probably because these chemical properties did not reflect micro-gradients of relevance for soil biological composition and diversity. For example, soil pH was stable across the experimental plantation, on average 6.48 ± 0.23 (SD, standard deviation). In another plot-scale study, Stursová et al. (2016) reported no effect of pH on bacterial and fungal diversity when pH was 3.50 ± 0.15 (SD). Hiiesalu et al. (2017) proposed that variables affecting biotic diversity and composition may differ from study to study according to the magnitude of abiotic gradients, and these gradients were relatively minor in this study.

Given the relatively close proximity among plots, root and leaf litter (especially broadleaf species) substrate dispersion may have partially obscured the actual effect of tree identity and diversity on soil microbial diversity. Another limitation of this tree experiment is that trees were regularly spaced within each plot, which does not reflect the possible effect of more heterogeneous spacing, as is found in natural forests, on soil microbial diversity.

The age of our experiment (7 yr old) could have prevented us from measuring strong coupling between tree and soil biodiversity. This is consistent with grassland diversity experiments, which showed no effect of vegetation on soil properties, such as microbial respiration, until 5 yr after the initiation of the experiment (Eisenhauer et al. 2010). Moreover, earlier studies at IDENT-Montreal found that soil respiration was related to tree species richness (Khelifa et al. 2017) and tree functional diversity (Jewell et al. 2017). Although specific groups of soil biota can obviously respond to the experimental treatments in forest plantations within 7 yr following their installation, it may take more time for entire soil microbial communities, which include many more species with multi-functional roles, to change following tree establishment.

In summary, aboveground plant diversity and composition weakly affected bacteria and fungi in the bulk soil of 7-yr-old experimental tree plantations, as indicated by the low or absent alpha-diversity coupling and low beta-diversity coupling. In the short term, it appears that tree inputs to the soil, such as root exudates, root biomass (as a food source), and leaf litter leachates, cannot induce changes in the diversity of soil bacteria and fungi that dwell in the bulk soil. In
the long term, it remains possible that some aspects of tree species composition and diversity will affect the soil biota, either directly or indirectly, but responses will likely be context-dependent (Hendershot et al. 2017). We conclude that policies to enhance biodiversity that focus on increasing the diversity of tree plantations do not disrupt or significantly alter soil life in the short term, showing the resilience of soil organisms to tree-planting activities.

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