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Full title: Roots and rhizospheric soil microbial community responses to tree species mixtures

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Abstract

Below-ground processes are crucial in determining the effects of plants on ecosystem function. The root-soil interface is a highly active zone due to root exudation and nutrient uptake. However, its role in determining effects of tree species and their interactions on the soil microbial community, ecosystem function and above-ground growth is less well known. We compared the effects of tree species monocultures and their mixture on rhizospheric microbial communities, specific functional genetic markers associated with processes in the nitrogen (N) cycle, and above-ground and below-ground growth and nutrient allocation. Two pairs of tree species were grown: *Pseudotsuga menziesii* and *Alnus rubra; Acer pseudoplatanus* and *Quercus robur.* Tree establishment altered soil microbial composition, but after 26 months differences amongst tree species and effects of species mixture were minor, suggesting functional redundancy in microbial communities. A greater abundance of fungi, bacteria, and specifically ammonia oxidizing and denitrifying bacteria in the rhizospheric soil of the N-fixing *A. rubra* was the most notable trend. Mixing *A. rubra* with *P. menziesii* did produce overyielding: trees grown in mixture attained a two-fold greater (Relative Yield Total 2.03 ± 0.52) above-ground biomass than in a mixture predicted from trees grown in monoculture. We did not observe strong trends in overyielding for *A. psuedoplatanus and Q. robur.* Inclusion of the N-fixing species *A. rubra* in admixture with *P. menziesii* promoted N cycling, and decreased the C:N ratios of leaf, branch, and root tissues but not soil C:N ratio for *P. menziesii*. Given the observed overyielding in the *A. rubra* with *P. menziesii* mixtures, we explored potential mechanistic links between functional genetic markers for nitrification and ammonification, however we found no statistically significant effects attributable to these genetic markers. We found root area index was significantly lower in *A. rubra* monocultures than in admixture with *P.*

menziesii. For both *P. menziesii* and *A. rubra*, the number of root tips was lower in mixture than monoculture, indicating physical partitioning of soil space as a result of growing in mixture. We documented additive and synergistic effects of tree species identity on above and belowground productivity, and rhizospheric microbial community development in these four tree species.

1. Introduction

Plant roots exude compounds belowground that mediate plant-soil interactions, which in turn play an essential role in ecosystem function and regulation (Wardle et al. 2004), and have cascading effects on the development of soil. These include tree species effects on the cycling of nutrients (Prescott et al. 2000a), soil physical properties (Binkley and Giardina, 1998), soil fertility (Augusto et al. 2014), and soil microbial community composition and function (Ribbons et al. 2016, 2018). One of the ways in which plants influence soils is through the physical presence of roots, with an immediately surrounding zone of soil strongly controlled by root activity, henceforth called the rhizosphere (Ryan et al. 2001; Pivato et al. 2017). Within the rhizosphere zone physically closest to plant roots, plant roots exude compounds that mediate plant-soil interactions both at the plant–microbe and the soil microbial community (soil–microbiome) levels (Jones 1998; Huang et al. 2014; Moreau et al. 2019).

Root exudates can play important roles in regulating ecosystem processes, including the fluxes of elements like carbon (C) (Farrar et al., 2003; Jones et al. 2009), nitrogen (N) (Jones et al. 2005), and phosphorus (P) (Oburger et al. 2011). Root exudates can also play important roles in mediating or regulating litter decomposition (Kuzyakov et al. 2007), and co-regulation along with mycorrhizal associates (Jones, Hodge and Kuzyakov 2004; Oburger and Jones 2018).

Another mechanism through which roots influence soils is priming, whereby an increase in soil organic matter (SOM) decomposition is mediated by inputs of labile C or N sources (Blagodatskaya and Kuzyakov, 2008; Prescott et al. 2020). To examine factors that regulate SOM decomposition, including plant C exudates and below-ground C and N turnover, Bengtson et al. (2012) experimentally tested the effects of priming in *Pinus ponderosa* (ponderosa pine), *Picea sitchensis* (Sitka spruce), and *Tsuga heterophylla* (western hemlock). They highlighted the importance of differentiating between real priming (which leads to a decrease in SOM) and apparent priming (an increase in microbial turnover but not leading to a decrease in SOM). Li et al. (2019) found context dependency for rhizosphere priming in pine and spruce forests through the use of novel stable isotope probing methods, which highlighted the importance of tree species identity in ecosystem functions like C and N cycling.

One functional approach to plant-soil interaction classification is to examine how well organisms can be grouped by characteristics that influence ecosystem functions, known as functional effect traits (Diaz and Cabido 1997, Diaz and Cabido 2001, Lavorel and Garnier 2002), or functional groups based on life history characteristics (Binkley and Fisher, 2012). Functional response traits determine how an organism responds to environmental conditions, and influence the abilities of species to colonize or thrive in a habitat and to persistent in the face of environmental changes (Diaz et al. 2008). Functional effect traits underlie impacts on ecosystem properties and ecosystem services, whether or not they confer an adaptive advantage (Lavorel and Garvier 2002). Some traits can serve as both functional effects and response traits, such as nitrogen content, which underlies multiple responses to the environment and effects on ecosystem properties (Suding et al. 2008).

Leaf litter is the dominant source of above-ground detritus input to the SOM pool in forest ecosystems, with litter nutrient content being a functional effect trait that influences decomposability (Moore et al. 1998, Prescott et al. 2000a,b, Kattge et al. 2011), soil nutrient content, C:N ratio and pH (Vesterdal et al. 2008, Schelfhout et al. 2017). Below-ground detritus inputs to the SOM pool through root and hyphal exudation and turnover are often neglected in models and experimental work despite mycorrhizal hyphal turnover being reported as a dominant pathway for C entering the SOM pool in a poplar plantation enriched atmospheric CO_2 (Godbold et al. 2006). Both roots and mycorrhizal hyphae were the dominant sources of SOM inputs to soil C in Swedish boreal forests (Clemmensen et al. 2013) and in temperate poplar plantations (Berhongaray et al. 2019). Whilst an evidence-base is emerging on the impact of tree species belowground inputs to SOM, Mayer et al. (2020) highlighted the need for a mechanistic explanation for the effect of tree species identity on below-ground C fluxes and specifically the greater soil C stocks observed in forests containing N-fixing tree species.

Amongst the many important root functional traits is the occurrence of symbiotic relationships with bacterial or fungal associates in some tree species which should lead to increased nutrient, water, or resource acquisition, and thus yield apparent benefits to the tree host including an increase in biomass. Nitrogen fixing tree species forms symbiosis in root nodules with the N-fixing bacterium *Frankia spp.* (Pawlowski et al. 2003), this should in turn reduce potential N limitation and increase biomass. Mycorrhizal symbioses exist in most tree species, and are highly variable in their function, with a major distinction between those that form ectomycorrhizal (EcM) versus arbuscular mycorrhizal (AM) symbioses (Genre et al. 2020). Roots that form

ectomycorrhizas may have greater access to organically-bound nutrients than do plant roots without mycorrhization, and this can increase the rate of plant nutrient uptake (Binkley et al. 1992). Arbuscular mycorrhizas, however, are thought to increase the efficiency of inorganic nutrient uptake from soil solution (Phillips et al. 2013).

A broad-scale question remains about the effect of tree-species mixtures compared with monocultures on the soil microbial community and nutrient cycling (Rothe and Binkley 2001; Prescott and Vesterdal 2013). There is considerable evidence that the above- and below-ground growth of juvenile trees can be greater when grown in species mixtures than in monocultures, a phenomenon referred to as overyielding (Forrester and Pretzsch 2015). This effect has been attributed to niche differentiation between species or niche complementarity resulting in improved resource use efficiency. Either of these phenomena might be indicated by differences in functional effect traits including foliar C:N ratio, concentrations of lignin and cellulose, and functional response traits like growth habit, shade tolerance, or root traits related to belowground exploitation of resources. However, the current evidence has largely focused on above-ground traits, though some studies have observed below-ground niche differentiation (Lei et al. 2012, Dawud et al. 2016).

In this study we examined whether the overyielding effect of tree species mixtures can be linked to below-ground functional traits associated with individual tree species, and if tree species mixture affected soil microbial community composition and functional genes. We aimed to quantify functional differences in pairs of tree species that have complementary mycorrhizal associations (sycamore maple (*Acer pseudoplatanus*) and pedunculate oak (*Quercus robur*)) and

foliar and root traits (Douglas fir (*Pseudotsuga menziesii*) and red alder (*Alnus rubra*)). Within each pair, we aimed to assess carbon allocation in above and belowground biomass pools and soil microbial communities. We generally expected complementary traits to lead to enhanced growth following the biodiversity and ecosystem function (BEF) model. The BEF model suggests general trends of increased productivity in more biodiverse systems (Loreau et al. 2001) via mechanisms like niche differentiation or complementary exploitation of soil resources.

As context, we first examined whether any observed overyielding in above-ground biomass was correlated with differences in C and N contents and ratios in above- and below-ground pools (leaves, branches, roots and rhizospheric soils). We examined the above- and below-ground pools of each tree species treatment on soil microbial community composition and abundance of functional genetic markers associated with specific processes in the N cycle. We then explored whether differences in growth between tree species treatments were linked to differences in soil microbial communities and functional types, with the objective of determining if the magnitude or direction of tree species mixture effects on rhizospheric soil are associated with effects on above-ground growth.

We had the following *a priori* hypotheses regarding soil microbial communities:

1) As a N-fixing species, alder would have the greatest abundance in N functional genetic markers *(nirK* and *nirS)*, which would confer increased productivity and biomass than the other tree species.

2) Alder and Douglas fir would have the most distinct microbial communities, in comparison with maple and oak, due to their contrasting functional traits and taxonomic distinction, respectively.

3) Douglas fir would have the highest fungal:bacteria ratio, and alder the lowest, with oak intermediate, and maple intermediate to low, in accordance with their respective functional traits associated with N cycling and fostering soil microbial bacterial dominance (maple and alder) or fungal dominance (Douglas fir).

2. Materials and Methods

We used a mesocosm-scale field experiment, establishing tree seedlings in a common initial soil substrate, to determine the influence of tree species identity and their mixture on soil microbial community composition and function, tree root characteristics, and C:N ratios.

2.1 Species selection

Tree species were selected based on their contrasting functional traits, and to represent a range of taxonomic, physiological, and ecological types, and to represent commonly occurring natural forest mixtures. The first tree species mixture was between two naturally co-occurring species (Binkley et al. 1992; Binkley, 2003) with contrasting functional traits, red alder and Douglas fir. We expected that the inclusion of the N-fixing deciduous red alder would facilitate an increase in the biomass of the Douglas fir seedlings relative to monoculture (Binkley, 2003), leading to an overyielding effect. This pair of trees also contains contrasting belowground characteristics. Alder is a fast-growing, short-lived, species with a shallow root system while Douglas fir is a slow-growing, long-lived species with a deep root system. We selected Douglas fir associates

with EcM (Harley and Harley, 1987), and red alder which associates with actinomycete *Frankia alni* and both EcM and AM (Pawlowski and Sirrenberg 2001, Tedersoo et al. 2016).

The second mixture was between two functionally more similar species, *Acer pseudoplatanus* (sycamore maple) and *Quercus robur* (pedunculate oak), which commonly co-occur in European forests. Maple and oak differ in several functional traits, including mycorrhizal type (AM vs EcM, respectively, see Harley and Harley 1987) and root morphology (heart-shaped vs tap-root respectively, see Evans et al. 2015), and several leaf traits including C:N ratio, base cation content, and decomposability (Vesterdal et al. 2008, 2012). Both tree species were selected to have similar growth forms and root structures, in the expectation that they would show a smaller mixture effect on both soil microbiomes and above-ground growth rates.

2.2 Site description and mesocosm study design

The experiment was carried out in the Malcom Cherrett Rhizotron, located at Treborth Botanic Garden in Gwynedd, Wales (53°13'00.5"N 4°10'22.9"W). Climate at the site is hyperoceanic and throughout the study period total annual rainfall was 934 mm and a mean annual temperature was 10.7 °C. The rhizotron enables replicated mesocosm-scale study of belowground processes and is comprised of twenty-four 1-m^3 compartmentalized soil bays, separated by metal partitions. Soils bays within the rhizotron were backfilled with a sandy loam textured Dystric Fluvic Cambisol collected adjacent to the rhizotron and repacked to mimic local soil profiles at a bulk density of 1.10 g cm^{-3}. Vegetation (i.e., *Poa* spp. and *Pteridium aquilinum*) previously grown in the rhizotron soil bays was carefully removed prior to the start of the experiment and the soil homogenized. We used a randomized block design, with six treatments randomly assigned to soil

bays within each of four blocks (the six treatments were: 1. alder, 2. Douglas fir, 3. mixture of alder and Douglas fir, 4. oak, 5. maple, 6. mixture of oak and maple). Sixteen tree seedlings were planted in an evenly-spaced square design in the 1 m^2 of each bay, with alternate seedlings of each species in the mixture treatments, in a replacement series design (i.e., the total density of seedlings was the same in the mixture as the monoculture treatments, see Supplementary Materials and Fig S1). The tree seedlings were obtained from Cheviot Trees (Berwick upon Tweed, UK), and were ~12 months old cell-grown seedlings from UK provenances, ranging from 20-40 cm in height at the time of planting.

2.3 Initial and intermediate data collection and conditions in soil bays

Prior to planting, initial soil samples from a depth of 0-10 cm were taken to determine baseline soil conditions, after removing the litter layer. One soil sample was collected per soil bay, and half was immediately sieved through a 2-mm-mesh sieve, frozen, and stored in a -80 °C freezer for subsequent analysis of pre-planting soil microbial community composition (DNA extraction and isolation and qPCR of marker genes). Soil pH and electrical conductivity was measured in deionized water according to Smith and Doran (1996) in a 1:2.5 v/v slurry, soil moisture content was measured gravimetrically, and organic matter content through loss on ignition at 550 °C. At the time of planting, we randomly sampled 10 seedlings from the planting stock of each of the four tree species to measure above- and below-ground biomass, and initial root:shoot ratios on those sacrificed seedlings. All materials were dried to a constant mass in an 80 °C oven for 72 hours to determine dry weights.

Tree heights were assessed at the time of planting (May 1st 2014), at two intermediate time points during the experiment (after 4 months: September 2014, and 19 months: December 2015), and at the final harvest (after 26 months: July 2016). A single bulk soil sample was taken in December 2015 from a depth of 25 cm within each soil bay, and used only to assess differences in bulk-soil microbial community composition and fungal:bacterial ratios using the same qPCR methods as the initial and final samples. Grasses (*Poa* spp.) and bracken (*Pteridium aquilinum*) were the two most abundant non-target species that grew in the rhizotron, and were removed across all treatments as they emerged.

3. Final harvest and data collection

In June 2016, roots were sampled using an 8-cm-diameter soil corer to a depth of 30 cm and divided into 10 cm soil core sections. Two soil cores were taken in each soil bay, consistently positioned 33 cm from each side of the bay and equidistant from the nearest trees. For each 10 cm section, soil cores were sieved through a 2-mm-mesh sieve and roots were washed to remove adhered soil prior to sorting into two size classes fine (<2 mm Ø) and coarse (>2 mm Ø). Live fine roots were scanned using an Epson 4990 scanner at a resolution of 300 dpi, after separated out by species prior to scanning. Images were analysed with WinRhizo (version 2005c, Reagent Instruments Inc, Quebec, Canada) to determine number of root tips, number of root forks, root area index (RAI, m^2 m^{-2}) and root length density (RLD, m m^{-3}).

Rhizosphere soils of fine roots, identified as the soil that adhered to roots after shaking, were collected from these same soil core samples prior to washing. Rhizosphere soils collected from within the same soil bay were subsequently pooled and homogenized such that, in the mixtures,

rhizospheric samples from each species were combined into a well-mixed representative sample per bay. For example, soils removed from the fine roots (<2 mm Ø) of both maple and oak grown in a mixture plot would have been combined and homogenized as one composite sample for that plot (n=4).

The entire experiment was destructively harvested after collecting final rhizospheric soil samples and root cores. Whole trees were cut at the root collar immediately above the ground and separated into stem, branch, and leaf fractions before being dried to constant mass at 80 °C for 72 h and weighed to determine biomass moisture content (data not presented here). There was no mortality of any planted seedlings by the end of the experiment.

We aimed to determine if the tree species mixtures led to an overyielding effect, that could correspond to overyielding of N-related functional genetic markers in the rhizosphere. To do this, we calculated the observed above-ground biomass in mixture and compared it to a theoretical mixture that was calculated from the mixture component species grown in monoculture. We then calculated the observed abundance of N-related genetic markers and compared them to theoretical values. This calculation is analogous to the use of Relative Yield Total (Hooper & Dukes, 2004; Weigelt & Jolliffe, 2003), which consists of mean actual biomass values for a given species grown in mixtures (biomass values from the mixed soil bays) divided by the theoretical values that would be produced if either of those conspecifics were grown in monocultures (predicted values).

2.4 Tree and rhizospheric soil C:N ratios

After the final harvest in 2016, a randomly-selected subset of three individual trees per species per bay (n=3 for each species) that had been separated into stem, branch, leaf and root fractions were selected for C:N analysis. All stem, branch, leaf, and root samples were ground using a MM200 ball-mill (Retsch GmbH, Haan, Germany) prior to C:N analysis. Rhizospheric soils for C:N analyses were collected from the roots of all diameters of each individual tree from the subset harvested in 2016. These soil samples were sieved through a 2-mm mesh sieve, and dried at 105 °C in preparation for total C and N analyses, ground using a MM200 ball-mill (Retsch GmbH, Haan, Germany), and weighed. Total elemental C and N concentrations of the plant biomass and rhizospheric soil samples were determined using a Truspec CN analyser (Leco Instruments UK Ltd., Stockport, UK.) with standards of known C and N concentrations analysed between every 12 samples. Carbon and N contents were calculated as biomass (grams) multiplied by C and N concentration separately for each fraction (stem, branch, leaf, root, and total biomass) for each harvested tree and for the combined rhizosphere soil sample for each bay.

2.5 Soil microbial community - DNA extraction and qPCR total gene copy analyses

Soil microbial communities were evaluated through comparisons of fungal:bacterial ratios and fungal *ITS* and bacterial and archaeal *16S* gene markers, as well as denitrifying microorganisms' *nirK* and *nirS*, and ammonia-oxidising bacterial and archaeal *amoA* AOB and AOA. Bulk soil (non-rhizospheric) microbial community composition was assessed for each soil bay by targeting total fungal *ITS*, bacterial *16S*, and fungal:bacterial ratios in soil samples taken in April 2014 (prior to the experiment, called "initial") and after 19 months in December 2015 (intermediate data point, data not presented here). Final rhizospheric soil samples (taken in June 2016) were analysed using the same measures and referred to in the figures and analyses as "final."

DNA was extracted using 0.25 g of frozen soil and a Mo-Bio PowerSoil DNA isolation kit (MoBio Laboratories, Inc., Carlsbad, CA), and quality and concentration were assessed using a nanodrop spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE) and electrophoresis in agarose gels (1% w/v in TBE). All extracts were stored at -20 °C prior to amplification, and ten-fold dilutions of DNA were used for quantitative polymerase chain reactions (qPCRs) to quantify gene copy numbers of two focal gene markers: bacterial *16S* and fungal *ITS*. All qPCRs were run in duplicate on a 7500 Fast Real-Time PCR System (Applied Biosystems, USA) machine with 20 μl reactions consisting of: 10.0 μl of SYBRGreen (2X) PCR Master Mix (Life Technologies Corp., Carlsbad, CA, USA), 0.25 μl each of forward and reverse primers, 1 μl of DNA template, and 8.5 μl of nuclease-free water.

PCR conditions for fungal *ITS* were 95 °C for 10 minutes followed by 40 cycles of 95 °C for 15 seconds, 55 °C for 30 seconds, and 72 °C for 20 seconds. Standard curves for fungal *ITS* were constructed using ten-fold serial dilutions of *Fusarium aveneceum* genomic DNA, which ranged from 10^3 to 10^9 gene copies. PCR conditions for bacterial *16S* were 95 °C for 10 minutes followed by 40 cycles of 95 °C for 15 seconds, 53 °C for 30 seconds, and 72 °C for 20 seconds, and we used ten-fold serial dilutions of *Pseudomonas putida* genomic DNA, which ranged from 10^2 to 10^7 gene copies. Fungal:bacterial ratios were calculated using these ratios of log gene copies. PCR conditions for both *nirK* and *nirS* were 10 min at 95 °C and 40 cycles of 95 °C for 60 seconds, 60 °C for 60 seconds and 72 °C for 60 seconds, with fluorescence quantified at extension (Levy-Booth and Winder, 2010). The standard curve for *nirS* and *nirK* used 10-fold serial dilutions of 10^1 to 10^7 gene copies from *Pseudomonas putida.* All gene copies were

calculated using exact soil extraction weights, and are presented in analyses as $\log_{10}$ gene copies/g of dry weight soil. All primers and references are provided in Appendix A.

2.6 Data analysis

Data were analysed separately for each grouping of tree species, such that Douglas fir, alder and alder-Douglas fir mixtures were grouped together, and maple, oak and oak-maple mixtures were grouped together. Tree species treatment effects (fixed effect, independent variable) on tree biomass variables (dependent variables), soil chemical properties (dependent variables) and soil microbial communities (dependent variables) were analysed using a one-way analysis of variance (ANOVA) with an alpha of 0.05, after meeting assumptions of normality and homoscedasticity. When significant differences were found in the main effects of ANOVA tests, a Tukey's honestly significant difference (HSD) pair-wise post-hoc test was performed. Principal component analysis was conducted on all plant biomass, soil and microbial data in order to discern if any patterns amongst treatments emerged across the multivariate dataset as a whole. All analyses, statistics, and figure production were conducted in the following packages: ggplot2 (graphs; Wickham, 2016), emmeans (least-squares means; Lenth et al. 2020), FactoMineR (PCA and multivariate analyses; Husson et al. 2016), gridExtra (to arrange graphs; Baptiste, 2017) within the R statistical program version 3.3.1 (The R Foundation for Statistical Computing, 2016).

3. Results

3.1 Soil chemistry

Soil analyses conducted prior to the establishment of treatments showed homogeneity in the bulk soil (Table 1). The 0-10 cm layer of soil in the bays had a mean pH of 6.74 ± 0.06 (standard error), C content of 6.0 ± 0.3%, and N content of 0.37 ± 0.01%. By the end of the experiment, at 26 months, rhizospheric soil pH was an average of 5.90 ± 0.15 across all soil bays, although no changes were statistically significant. The rhizospheric soil in the alder monoculture bays had the greatest decrease in pH by an average of 1.2 ± 0.3, compared with Douglas fir (0.8 ± 0.4), maple (0.7 ± 0.2) and oak (0.8 ± 0.4) monocultures, although these did not differ significantly between tree species with the exception of maple and alder (Tukey HSD p-value: 0.029). The mixture of alder and Douglas fir marginally decreased soil pH by an average of 0.9 ± 0.2, the same as for the oak and maple mixture, 0.9 ± 0.3 (p= 0.058).

3.2 Aboveground and belowground biomass and C:N ratios

Initial root:shoot ratios were highest for maple with 3.36 ± 0.94, followed by oak with 1.58 ± 0.27, alder with 0.29 ± 0.11, and Douglas fir with 0.26 ± 0.07. Alder produced the greatest above-ground biomass of the four tree species, followed by maple, oak, and Douglas fir (Table 2). Alder biomass was 64% larger in mixture than in monocultures, whereas there was no change in the biomass of Douglas fir (Table 2). Similarly, for alder the total N contents of the above-ground biomass per tree grown in mixture was 48% greater than in monoculture, and for Douglas fir it was 82% greater (Table 2) than in monoculture. In contrast, for both oak and maple there was less than 20% difference in their total above-ground biomass and its N contents per tree, between monoculture and mixture. Consequently, alder and Douglas fir mixtures exhibited an overyielding effect on above-ground biomass (Figure 1, Tukey's HSD P =0.038), while oak and maple mixtures did not (Tukey's HSD P =0.891).

Below-ground we observed some tree species root systems reacted to growth in mixtures. The number of root tips, number of root forks, RLD and RAI all showed trends towards differences between monocultures and mixtures for alder and Douglas fir (Figure 2A-D). Overall, the only root metric that had a significant main effect was RAI ($P < 0.01$), which was lower in the monocultures than in Douglas fir-alder mixtures (as indicated in Fig. 2C, $p = 0.05$). For both Douglas fir and alder, the number of root tips (Figure 2A) was lower in mixture than monoculture, although the main effect for root tips was not quite significant ($P = 0.08$). For alder the RAI (Figure 2C) was lower in mixture ($P<0.01$) than in monoculture. In monocultures, RAI was significantly greater in maple than in oak ($P=0.02$), in alder than in Douglas fir ($P<0.01$), and in alder than in oak ($P<0.01$). Root depth profiles showed trends towards increased RLD and RAI in both mixtures in the upper 10 cm of the soil profiles (Figure 3).

Tree species identity and mixing influenced tree tissue and rhizospheric soil C:N ratios at the end of the experiment (Figure 4.) The alder and Douglas fir treatments differed significantly in C:N ratios for leaves ($P< 0.001$), branches ($P< 0.001$), and roots ($P< 0.001$), but not in rhizospheric soils ($P= 0.675$). When grown in mixture with alder the C:N ratios of Douglas fir leaves, branches, and roots were significantly lower than when it was grown in monoculture (Figure 4 A-C). Alder had slightly elevated C:N ratios in branches, and significantly elevated C:N ratios in roots, when grown in mixture with Douglas fir, compared with its monoculture (Figure 4B, C). Rhizospheric soil C:N ratios for alder were significantly higher in monoculture than in mixture with Douglas fir (Figure 4D). For maple and oak monocultures, only leaf C:N ratio was significantly lower in oak ($P<0.001$) monocultures than in mixtures with maple. Leaf C:N was

higher for oak grown in mixture with maple than in monoculture (P= 0.015, Figure 4E). Oak had a higher rhizospheric soil C:N ratio in mixture than in monoculture (Figure 4 H), with weaker evidence for the same trend in roots (Figure 4G).

3.4 Soil microbial community

Fungal *ITS* abundances in rhizospheric soils were higher than those in initial bulk soils in all treatments (Figure S5A). Monoculture alder rhizospheric soils had the greatest increase in fungal *ITS* total abundance from initial bulk soils, although they did not differ significantly from Douglas fir monoculture or the mixture (ANOVA P = 0.697). In contrast, final bacterial and archaeal *16S* abundances decreased in all treatments from bulk (initial) to rhizospheric soil (26 months) during the experiment (Figure S5B). Rhizospheric soils in alder had significantly greater *16S* abundance than in Douglas fir monoculture (ANOVA P= 0.023).

During the experiment, *nirK* and *nirS* abundances increased from initial bulk to final rhizospheric soil for all treatments (Figure S5C, S5D). There were no significant differences among treatments in the rhizospheric soils for Douglas fir and alder (Figure 5C, D). Final *amoA* AOA increased from initial bulk to final rhizospheric soil during the experiment for all treatments (Figure S5E), but did not differ significantly amongst treatments in the rhizospheric soil (Figure 5E). Initial bulk soil *amoA* AOB was highly variable across all tree species. Final *amoA* AOB did not consistently increase from initial bulk to final rhizospheric soil during the experiment (Figure S5F). Final *amoA* AOB was significantly higher for oak monocultures than for the oak-maple mixture in the rhizospheric soil (ANOVA P= 0.024).

Fungal:bacterial ratios increased from initial bulk to final rhizospheric soil for all treatments during the experiment (Figure S5G), but there were no significant differences amongst the treatments in the rhizospheric soil (Figure 5G). *amoA* AOA:AOB ratio also increased for all treatments from initial bulk to final rhizospheric soil during the experiment (Figure S5H). In the rhizospheric soil, the *amoA* AOA:AOB ratio was significantly lower in alder than in Douglas fir monocultures (ANOVA P= 0.017) or alder-Douglas fir mixture (ANOVA P= 0.007), but there were no significant differences amongst the oak-maple treatments. We explored a new analytical approach to understanding mixture effects on soil microbial communities by calculating overyielding (or underyielding) for each suite of genetic markers. We adapted the same calculations used for aboveground biomass for each of the genetic markers and the 2 suites of ratios (Table 3).

The relationships amongst rhizospheric soil microbial community data and soil chemical variables (in the same samples) after 26 months of the experiment were assessed through PCA. Two PCAs were generated, one for bays containing the alder-Douglas fir treatments (Figure 6A), which explained about 55% of the variation in data, and one for the oak-maple treatments (Figure 6B), which explained about 50% of the variation in data. For these figures the ellipses are illustrative guides and are not based on statistical significance, although we observed trends in tree species and mixture separation. In both PCAs we observed a clear linkage between pH and biomass with PC1 axes, with positive associations to oak- maple and negative for the alder-Douglas fir PCA.

For alder-Douglas fir, PC 1 explained 34.8% of the variation and separated soil pH and C:N from the bacterial and archaeal *16S*, fungal *ITS* and microbial *nirK* and *nirS* abundance variables, while PC2 explained 20.7% of the variation and mainly separated C:N from *amoA* AOA abundance. Alder was associated with high *nirS*, *nirK*, fungal *ITS* and bacterial and archaeal *16S* abundance on PC1 in contrast to Douglas fir, which was associated with high pH, with the alder-Douglas fir mixture tending to lie between the monocultures (Figure 6A). For oak-maple, PC1 explained just 29.2% of the variation and separated bacterial and archaeal *16S* and fungal *ITS* abundance only from *amoA* AOA abundance, while PC2 explained 20.3% of the variation and predominantly partitioned soil pH from C:N (in a similar way to the alder-Douglas fir treatments). Maple was associated with high pH, *amoA* AOB, and bacterial and archaeal *16S*, and low C:N, but oak-maple mixture bays, like the oak monoculture bays, were widely scattered showing no consistent association with any of the soil variables (Figure 6B). In general, we observed stronger distinction between tree species in the alder and Douglas fir treatments than the mixture, whereas we observed greater overlap between monocultures and mixtures in the oak-maple treatments.

4. Discussion

4.1 Tree species and mixture effects on the rhizosphere soil microbial community

We observed tree-species specific effects on rhizospheric soil microbial communities most notably in both the monocultures and mixtures of alder and Douglas fir. We did not observe such consistent treatments effects in the maples and oaks. Final rhizosphere soil samples held an order of magnitude greater fungal *ITS*, *nirK*, *nirS*, *amoA* AOA and AOB, and slightly smaller bacterial and archaeal *16S* abundances, than initial soil samples. Initial soil conditions were

experimentally designed to be similar, and are often not collected in pre-afforestation experiments which only provide single end-point comparisons among treatments. Our microbial community analyses showed no differences among treatments in the initial sampling period, confirming similar starting conditions. By the final harvest, the differences across experimental treatments for six out of the eight functional genetic markers indicate the early influence of tree establishment, or afforestation, on rhizospheric soil microbial community structure and function. When these functional microbial data and additional belowground quantification of root traits were woven together, along with pH, soil C:N, aboveground biomass, and leaf biomass, we observed synergistic effects in alder-Douglas fir mixtures, but only additive effects in oak-maple mixtures.

For the functional genetic markers, there were no significant differences amongst the treatments in five of the eight rhizospheric soil microbial indicators after 26 months. We suggest that significant differences in the rhizosphere of mature trees may take several years to develop. Early afforestation effects on soil microbes may not be consistent with established forests that have developed over longer periods of time (see Ren et al. 2017). For example, simulation studies based on mature forests of red alder and Douglas fir, planted within established Douglas fir forests, suggested that the prolonged presence of red alder could lead to increased nitrification and NO_3^- leaching, which would ultimately decrease forest stand biomass because of acidification following NO_3^- production leaching (Verburg et al., 2001). However, initially high N availability in red alder soils could favor increased biomass of Douglas fir. That study underlined not only the possible temporal shifts in tree-species effects, but also found evidence of context-dependence of those effects as site history, including a land-use legacy on soil, was

found to influence forest nutrient cycle dynamics. A study of transboundary common garden transects in Denmark also showed that non-microbial based tree species effects of relatively similar species may be slight, even after decades of tree growth (Dawud et al. 2017).

4.2 Tree traits that relate to soil function

In addition to their natural co-existence in mixture, a contrast in functional traits was explicitly used in the selection of the tree species pairs. While they encompass a wide range of functional traits, since only four tree species were included in the experiment, we could not partition out which traits were dominant controlling factors of the observed responses. It is nonetheless notable that the N-fixing alder grew to an above-ground biomass more than 20 times that of the other three species. In this biomass alder accumulated a total N stock more than 22 times that of the other three species and, as a consequence, it had the lowest C:N ratios, especially in its roots. In contrast C:N ratios were highest for the conifer, Douglas fir. The alder-conifer mixture, which had larger alder trees than in monoculture, developed the lowest rhizospheric soil C:N ratio of all the treatments, illustrating how the presence of an N-fixing tree can rapidly start to affect soil N status. Evidence of how this might start to affect soil microbial function is provided by the rhizospheric soil of alder having the highest abundance of fungal *ITS*, bacterial and archaeal *16S*, and denitrifying *nirK* and ammonia-oxidising bacteria. The association between alder and rhizospheric microbial communities is likely due to high fluxes and abundance of NO_3^- and other forms of N, compared with Douglas fir or oak. While sycamore maple is also known to have higher N in litter and in soil than many other tree species (Withington et al. 2006; Vesterdal et al. 2008), this is of lower magnitude and in our experiment did not translate into the rhizosphere microbial community as clearly as for alder.

Alder is a fast-growing, light-demanding species, which in this study grew faster neighboring Douglas fir. This trend is similar to that observed by Ahmed et al. (2019) who showed that in a three-species polyculture comprised of *Alnus glutinosa*, *Betula pendula* and *Fagus sylvatica* the biomass of alder was greater in mixture than in monoculture after 6 years of growth. While no above-ground overyielding was observed in their study, this was explained by the suppression of the slow-growing *Fagus sylvatica* in mixture. Congruent with our study, Tobner et al. (2016) found positive functional diversity effects on stem biomass increment that were driven largely by fast-growing light-demanding species. They proposed an examination of functional identity and diversity metrics when evaluating biodiversity-ecosystem functioning relationships. Recently, Martin-Guay et al. (2020) have also stressed the importance of considering above- and below-ground biomass partitioning and niche differentiation effects on over-yielding. Both Tobner et al. (2016) and Martin-Guay et al. (2020) point to the need for careful consideration of which components of tree species mixtures contribute to overyielding.

Root traits complemented our rhizospheric soil microbial community analyses and can help explain trends in aboveground overyielding and carbon allocation. RLD and RAI almost doubled in the 0-10 cm layer in both mixtures, suggesting a change in root stratification through the soil profile (Figure S5). When we looked at root traits to potentially link the aboveground trends with belowground data, we observed a notably greater effect in the alder-Douglas fir mixture compared with the oak-maple mixtures. These trends might help explain observed aboveground overyielding by either increased exploration of the soil space (via increased root length density) or be linked to foraging strategies (via increased number of fine root tips).

4.3 Implications for tree species mixtures

We found that maple had more than twice the growth rate of pedunculate oak and a trend for the oak, which is a more light-demanding species (Hill et al. 1999), to grow more slowly in mixtures with maple than in monoculture. This aligns with previous concerns for oak woodland conservation that maples outcompete oaks in the seedling stage of establishment (Peterken, 1996). Pedunculate oak and sycamore maple are both widely distributed in the United Kingdom, but differ in their life history traits. Pedunculate oak is slow-growing with great longevity, while maple grows rapidly and reproduces quickly (Moorecroft and Roberts, 1999). In contrast, the results of this study provide evidence for the benefit of mixing two functionally contrasting species, such as the N-fixing broadleaved red alder and the conifer Douglas fir, during afforestation or forest restoration in terms of the establishment of the soil microbial community and its function in nutrient cycling (Binkley et al 1992; Cline et al. 2005; Gunina et al. 2017). More broadly, having a mixture between conifer and angiosperm species is considered a good approach to enhancing functional diversity, structural complexity (Kelty 2006), and ecosystem function (Cardinale et al. 2012; Gamfeldt et al. 2013) within forests.

Our alder-Douglas fir results confirm findings in Deal et al. (2017) which showed the inclusion of an N-fixing species with a non-N-fixing species can result in overyielding. This effect was associated with differences in some key functional attributes associated with nutrient cycling as observed in the increased abundance of *nirK, niS, amoA* AOA and AOB genetic markers in the alder rhizosphere soils. It is likely that the increased abundances of these functional genetic markers in alder reinforced the rapid biomass acquisition above- and below-ground for both the

alder and Douglas fir trees in mixture, which could have directly contributed to the observed overyielding effect. However, we cannot conclude with certainty that the over-yielding was due to the N fertilization effect of alder in the mixture, rather than other aspects of niche complementarity in this conifer-angiosperm mixture. One alternative interpretation of these data is that the high growth rate of alder contributed to trees in monocultures suffering from a higher above and belowground competition for light, space, and other resources from other alders than in mixture with Douglas fir.

Early-stage tree-species effects on soil microbial community composition and function were noticeable in our experiment, even though it is a relatively short period of growth over the expected lifetime of a tree. Future research should explore how this develops during the sapling to young mature tree stage of development in species monocultures and mixtures growing in common garden soils. Since the design of this study, there have been numerous methodological advances in collection techniques for rhizospheric compounds, which can shed additional light on the mechanistic underpinnings of plant-soil interactions (Williams et al. 2021). Many rhizospheric studies are focused on grass-based plant communities or agricultural settings (De Vries and Wallenstein, 2017), and highlight the importance of better understanding underground constituents for practical applied purposes (de Vries et al. 2020) and in response to a changing planet (Jansson and Hofmockel, 2020). The soil science community is quite collaborative and moving towards standardization and best practices in tracking and conserving biodiversity, and connecting these to the broader action for biodiversity protection measures (Guerra et al. 2021). Guerra et al. 2021 make explicit a proposal for linking larger scientific endeavors and important

policy targets to increase essential biodiversity variables, and highlight DNA technology and the integration of functional and diversity research in soil to these larger soil conservation efforts.

There are many other functionally important organisms that comprise soil biota aside from fungi, bacteria and archaea, including earthworms, ants, mites, and collembola, which are be the subject of similarly taxon-focused studies during tree-species establishment in monoculture and mixed-species forests (Peng et al. 2022). Future studies would benefit from focusing their design to test hypotheses relating to the mechanisms through which tree species influence rhizospheric soil microbial communities, and how these soil microbial communities influence both the bulk soil microbiome and its function, and the growth of the trees. For studies that are confined to shorter time periods, controlled manipulative laboratory experiments may be useful for isolating causal mechanisms linking plant and soil communities. One untapped area of research would be to examine the fine root C contents and fluxes in early-stage forest development through to a mature forest, building on the work of McCarthy-Neumann et al. (2019). Much also remains to be discovered about the role of mycorrhizas in tree species mixtures (Ferlian et al. 2018). Expanding on the growing body of research that explores the context in which interaction of above- and below-ground traits leads to overyielding would be a fruitful endeavor. Isotope-tracing studies have good potential to determine whether below-ground inputs from a tree actually lead to enhanced nutrient uptake and growth of a neighboring tree, and how this is influenced by whether it is a tree of the same or a different species.

4.4 Conclusions

Our study documented additive and synergistic effects of tree species identity on above and belowground productivity, and rhizospheric microbial community development for red alder and Douglas fir mixtures, and pedunculate oak and sycamore maple mixtures, in addition to their monoculture counterparts. The inclusion of an N-fixing species (alder) did increase biomass of the conspecific tree (Douglas fir) which led to overyielding in the biomass of both trees when grown in mixture. Although we found no mechanistic support for this when examining *nirS* and *nirK* functional genetic markers, we did observe differences in root characteristics that demonstrate belowground processes required to increase aboveground biomass. We found some support for our hypothesis that red alder and Douglas fir would have more distinct soil microbial communities, and expect that these distinctions would become more pronounced in forests after a longer period of time. We found support for our hypotheses that red alder would foster bacterial dominance, and the highest abundance of *nirK* and *amoA* AOB genetic markers, consistent with increased nitrogen cycling characteristics. We did not observe large differences in fungal:bacterial ratios amongst the tree species treatments, which also highlights the utility of examining specific functional genetic markers to isolate potential mechanisms leading to overyielding (e.g. increased nitrogen transformations) which may not be otherwise detected using *ITS:16S* ratios. There are logistical difficulties in assessing these types of soil rhizospheric microbial variables in intact forest systems, which highlights the utility of large rhizotron laboratories, such as the one at Treborth. Soil microbial communities within the tree rhizospheres are active regions that can inform our mechanistic understanding of key ecosystem processes like N and C cycling.

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Table legends

Table 1. Mean (± SE) characteristics of the soil at the start of the experiment (initial) and after 26 months (final rhizospheric soil in the bays of each tree species treatment (n=4).

Table 2. Mean (± SE) biomass (g) and nitrogen stock (mg /kg) per tree of stems (the main bole), branches, leaves, and the total above-ground biomass for each tree species grown in the monoculture and mixture treatments. Note that since these are all mean values, stems + branches +leaves will not equal the above-ground biomass values.

Table 3. Over- and under-yielding (relative yield total) of nitrogen cycle functional genetic markers determined from rhizosphere soil adhered to fine roots of oak and maple, and alder and Douglas fir mixtures after 26 months of growth. Values over 1 indicate overyielding, where tree species mixtures led to a higher than predicted abundance for each genetic marker: bacterial and archaeal *16S*, fungal *ITS*, *nirK*, *nirS*, *amoA* AOA, *amoA* AOB, and fungal:bactieral ratios and AOA:AOB ratios

Figure legends

Figure 1. Overyielding comparisons for aboveground biomass of alder-Douglas fir mixtures and oak-sycamore maple mixtures (standardized relative total yield +/- standard errors). **N= 4 treatments.** Values greater than 1 indicate overyielding.

Figure 2. Mean number of fine roots tips (1000s m^{-2}) (A), number of root forks (B), root area index (C: RAI), and root length density (D: RLD). Error bars denote one standard error for each tree species treatment, and colors indicate tree species identity grown across the treatments listed on the x-axis (N= 4).

Figure 3. Root traits across all treatments after 26 months of growth: Root Area Index, Root Length Density, and number of fine root tips by 10-cm soil depth increments (0-10 cm, 10-20 cm, and 20-30 cm) for monocultures and mixtures (N =4).

Figure 4. Carbon:nitrogen ratios for the tree mixtures and their component species grown in monoculture are shown side by side, leaf C:N (A, E) branch C:N ratio (B,F), root C:N ratio (C,G) rhizospheric soil C:N ratio (D,H) collected at the final harvest of the experiment (26 months) for alder, Douglas fir, and alder-Douglas fir mixtures (A-D) and oak, maple, and oak-maple mixtures (E-H). The points indicate the means for each treatment (n=4 for each treatment), the black line error bars denote one standard error, ANOVA main effects are shown in the top righthand corner of each panel. Note the y-axis labels differ between panels.

Figure 5. Soil microbial community gene abundance comparisons of rhizospheric soil at the end of the experiment (26 months, n=4) for Alder, Alder-Douglas fir mixture, Douglas fir, Oak, Oak-Maple mixture, and Maple tree species treatments (symbols and colors used to highlight different treatments). Panels refer to target genes for a) fungal *ITS,* b) bacterial *16S,* c) *nirK,* d) *nirS,* e) *amoA* AOA, and f) *amoA* AOB, and the ratios of g) fungi:bacteria, and h) AOA:AOB. The points indicate the means for each treatment and the black lines the SE. Please note that the y-axis differs between panels as gene quantities vary across large scales.

Figure 6. *a)* Principal components analysis of the alder, Douglas fir, and alder-Douglas fir mixture tree species treatments using the soil property and gene copy abundance data from the final (after 26 months) rhizospheric soil samples. *b)* Principal components analysis of the oak, maple, and oak-maple mixture tree species treatments using the soil property (pH, C: N) gene copy abundance (bacteria- *16S,* fungi- *ITS,* AOA *amoA*, AOB *amoA*, *niK*, and *nirS*) and root traits (RLD- Root Length Density, RAI- Root Area Index, and nTips- number of Tips) data from the final rhizospheric soil samples, combined with the final biomass harvest data (aboveground woody biomass in g, leaves biomass in g).

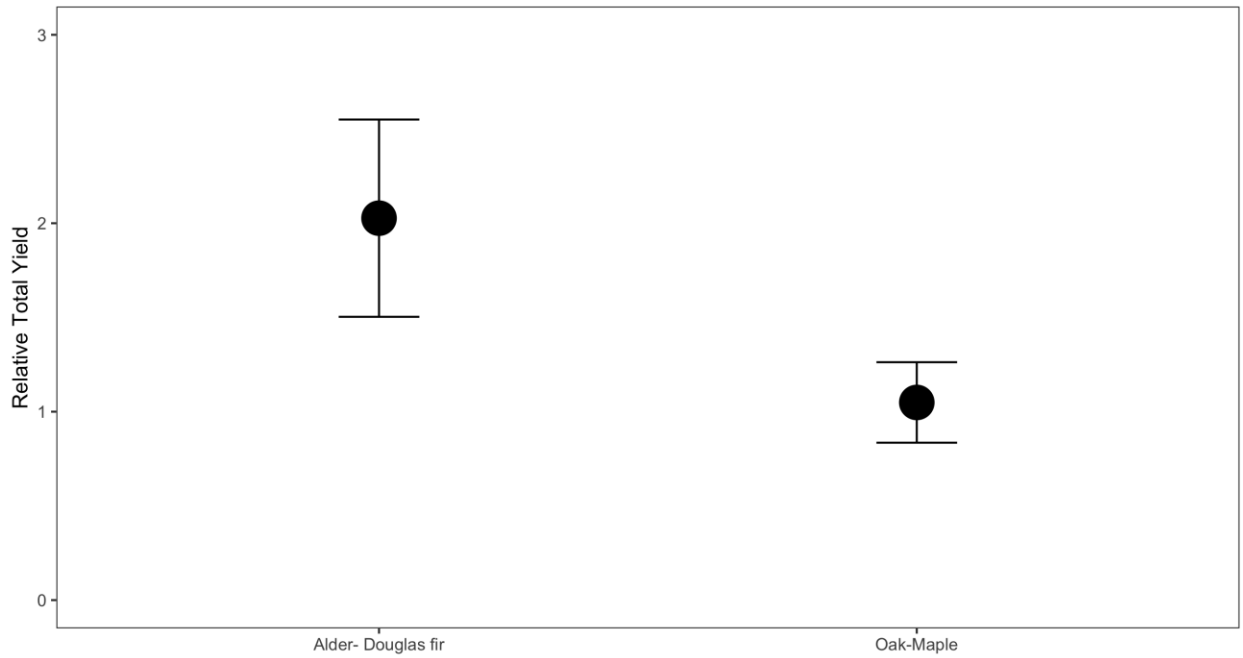


Fig. 1. Overyielding comparisons for aboveground biomass of alder-Douglas fir mixtures and oak-sycamore mixtures (standardized relative total yield +/- standard errors). Values greater than 1 indicate overyielding.

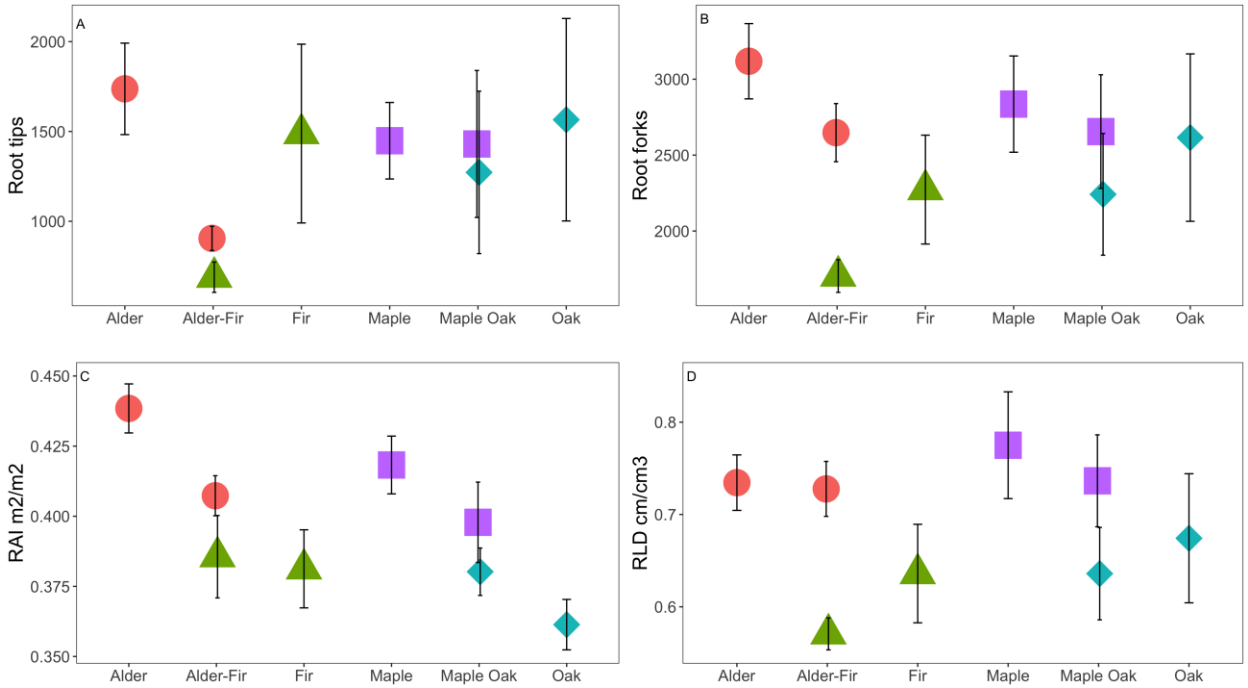
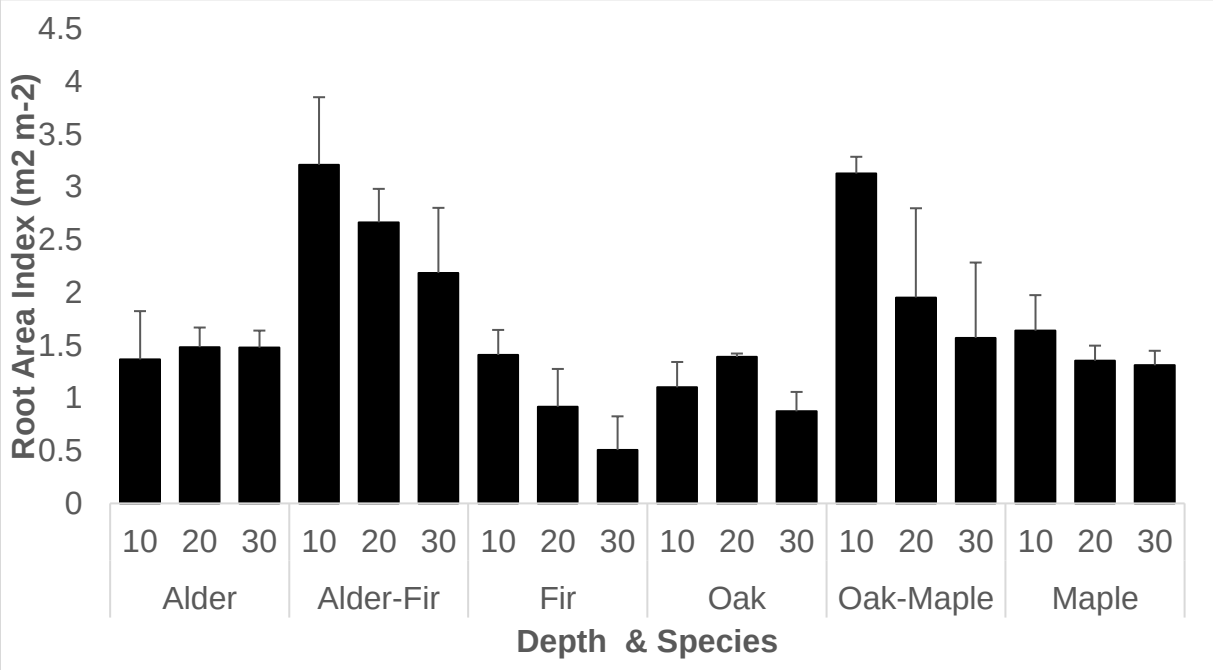
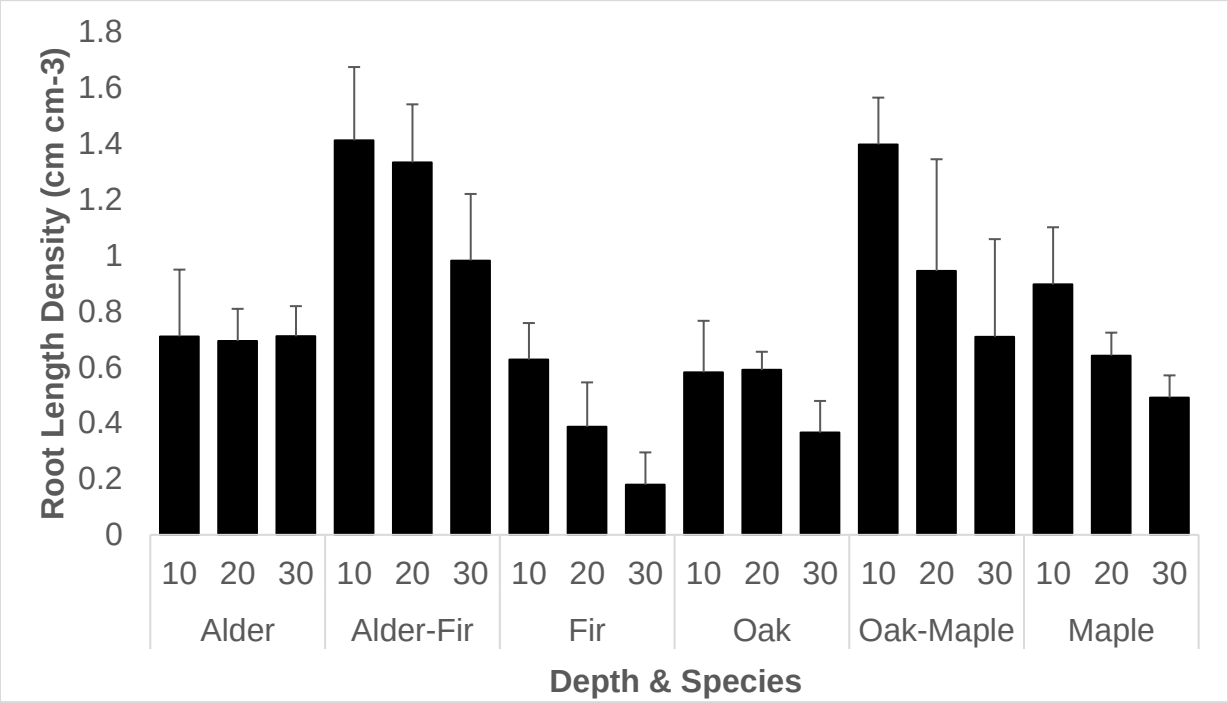


Fig. 2. Mean number of fine roots tips (1000s m^{-2}) (A), number of root forks (B), root area index (C: RAI), and root length density (D: RLD). Error bars denote one standard error for each tree species treatment.





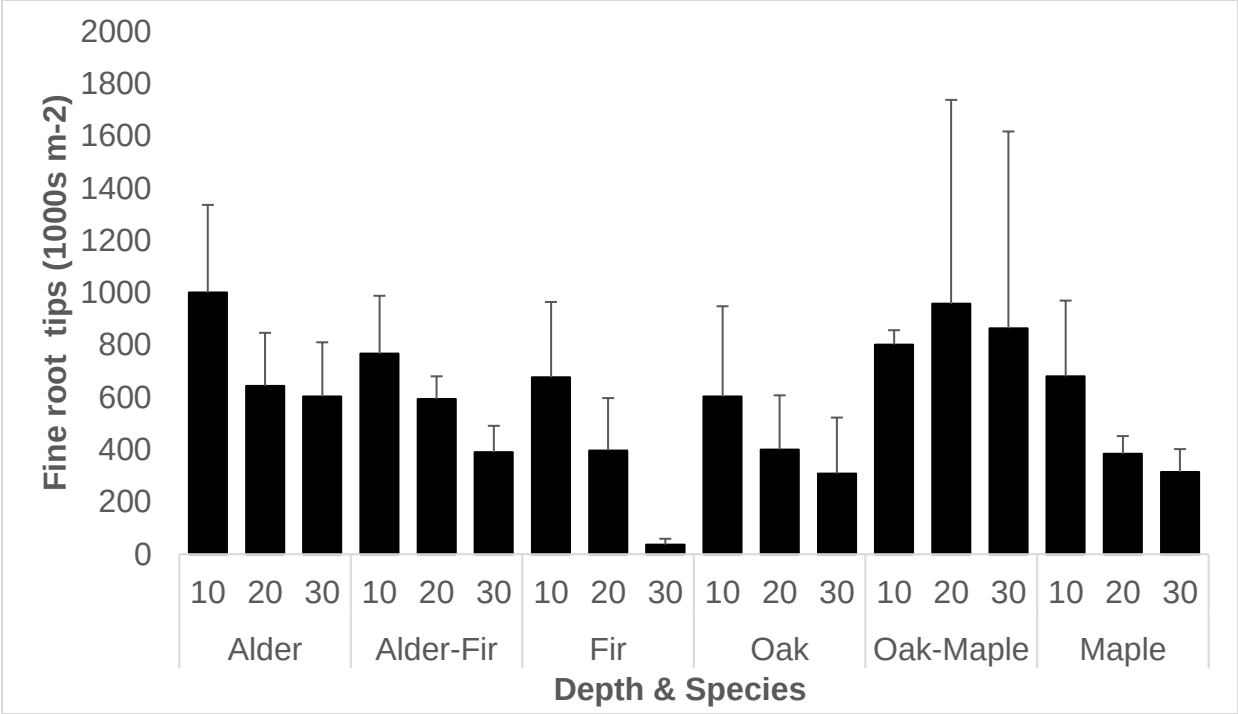
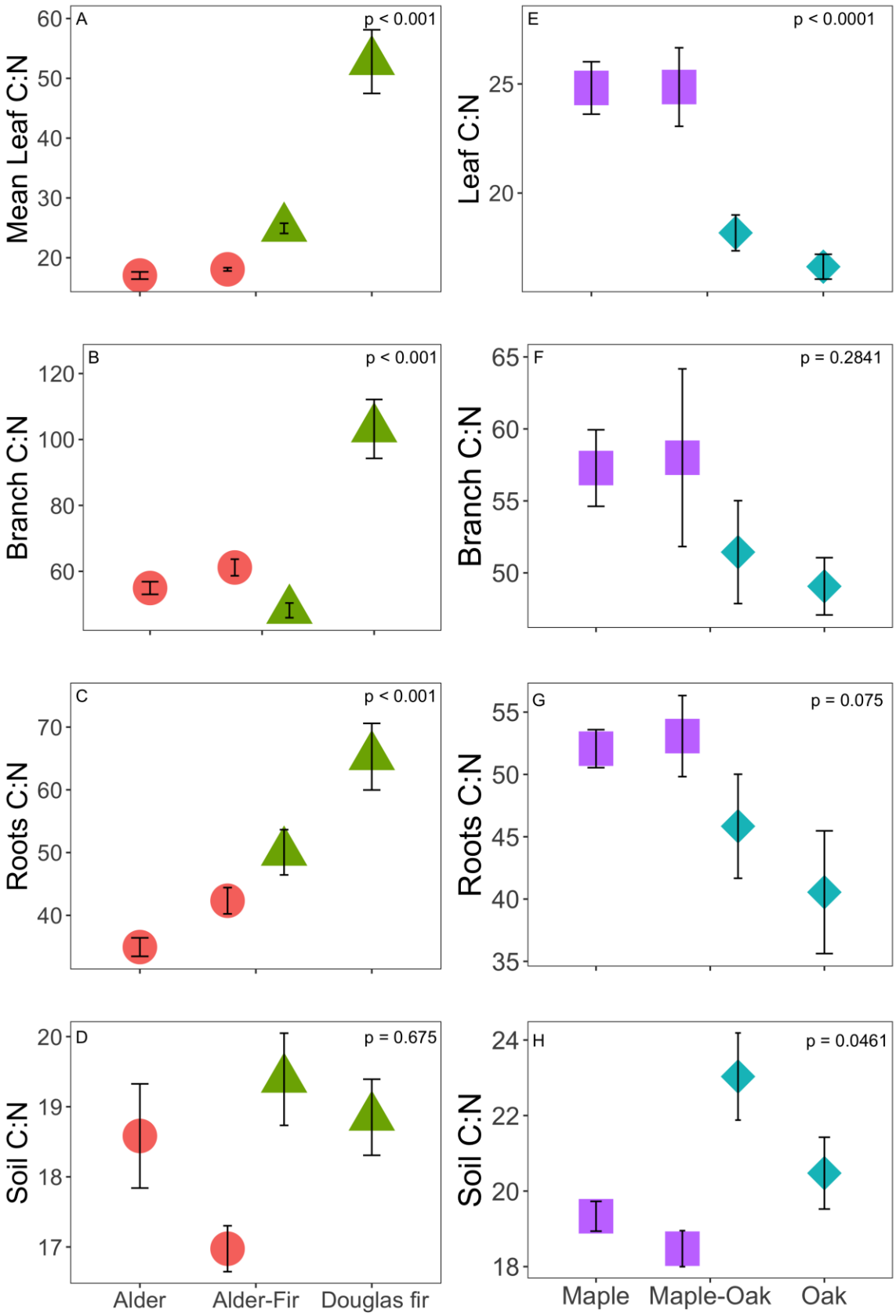
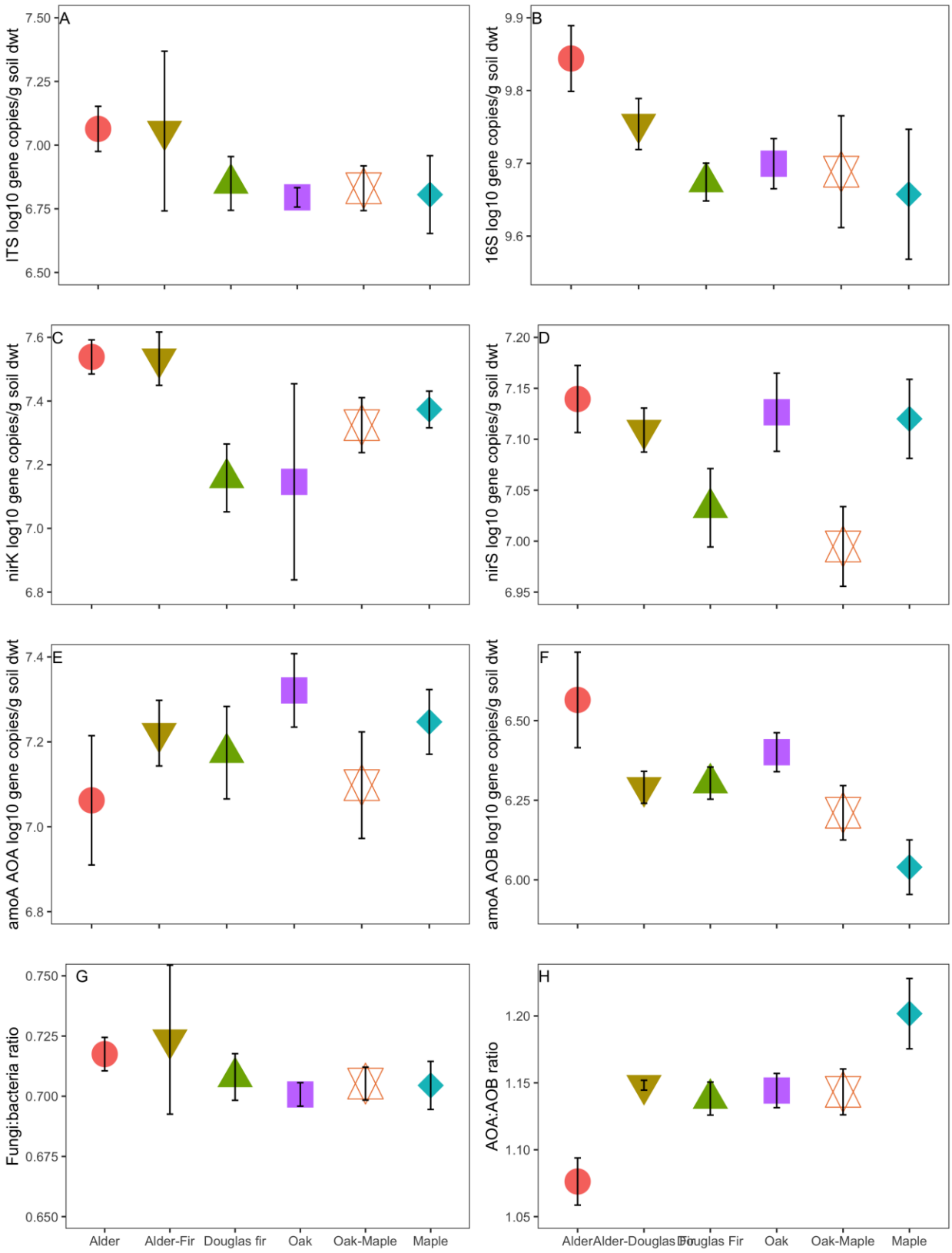


Fig. 3. Root traits across all treatments after 26 months of growth: Root Area Index, Root Length Density, and number of fine root tips by 10-cm soil depth increments (0–10 cm, 10–20 cm, and 20–30 cm) for monocultures and mixtures (N = 4).


A
p < 0.001
Mean Leaf C:N
60
50
40
30
20
E
p < 0.0001
Leaf C:N
25
20
B
p < 0.001
Branch C:N
120
100
80
60
F
p = 0.2841
Branch C:N
65
60
55
50
C
p < 0.001
Roots C:N
70
60
50
40
G
p = 0.075
Roots C:N
55
50
45
40
35
D
p = 0.675
Soil C:N
20
19
18
17
H
p = 0.0461
Soil C:N
24
22
20
18
Alder
Alder-Fir
Douglas fir
Maple
Maple-Oak
Oak

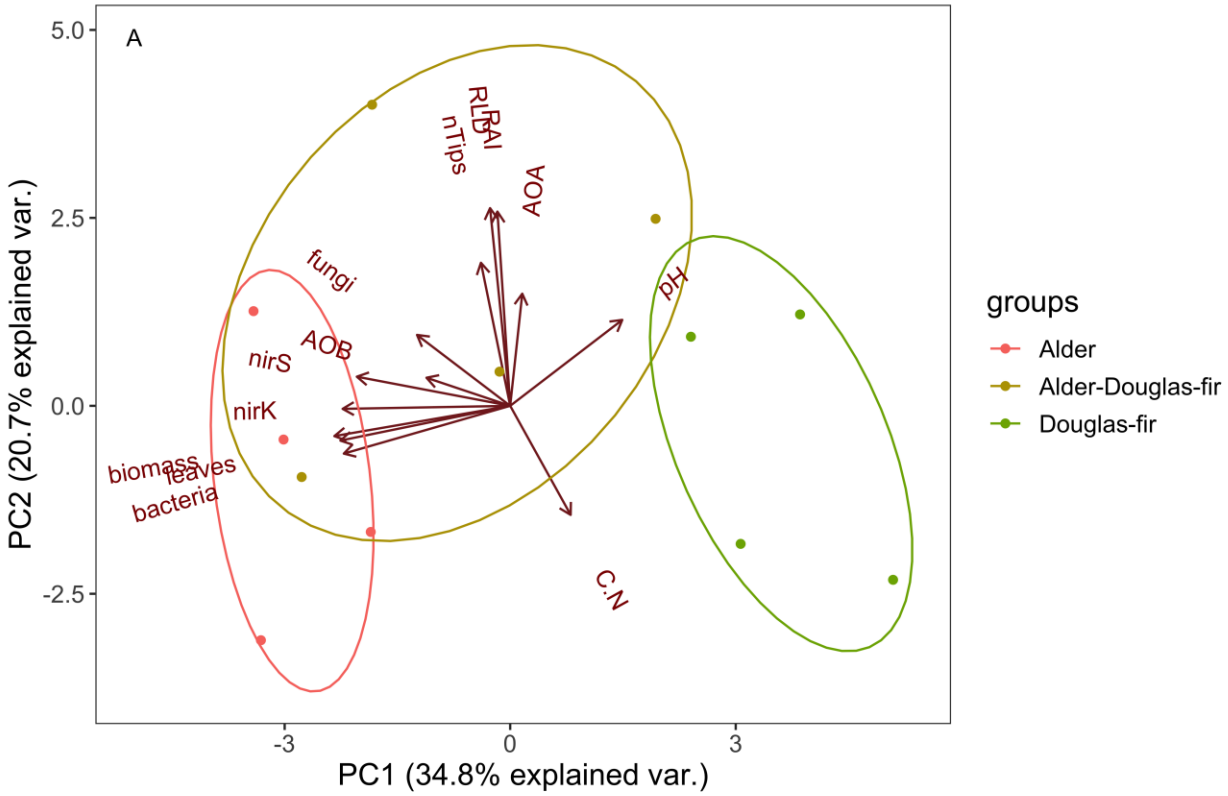
20

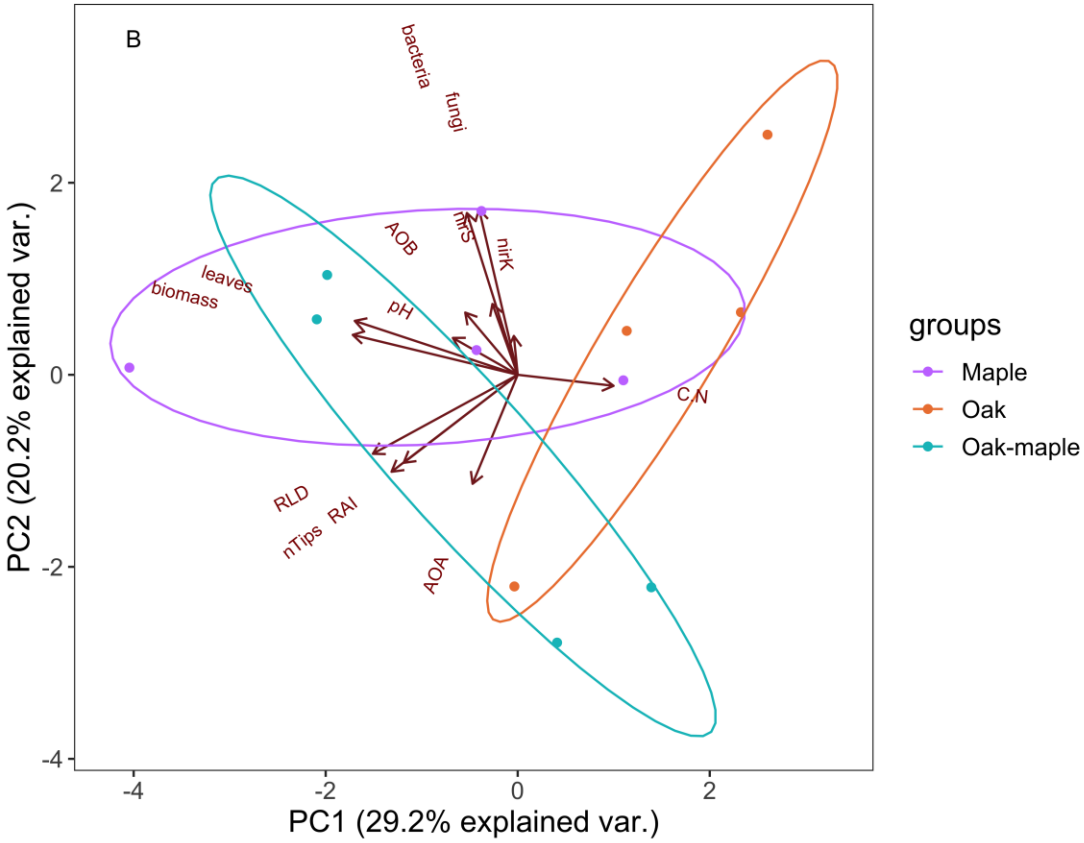
Fig. 4. Mean Carbon:nitrogen ratios for the tree mixtures and their component species grown in monoculture are shown side by side, leaf C:N (A, E) branch C:N ratio (B,F), root C:N ratio(C,G) rhizospheric soil C:N ratio (D,H) collected at the final harvest of the experiment (26 months) for alder, Douglas fir, and alder-Douglas fir mixtures (A-D) and oak, sycamore, and oak-sycamore mixtures (E-H) . The points indicate the means for each treatment, the black line error bars denote one standard error, ANOVA main effects are shown in the top righthand corner of each panel. Note the y-axis labels differ between panels.


A
ITS log10 gene copies/g soil dwt
7.50
7.25
7.00
6.75
6.50
B
16S log10 gene copies/g soil dwt
9.9
9.8
9.7
9.6
C
nirK log10 gene copies/g soil dwt
7.6
7.4
7.2
7.0
6.8
D
nirS log10 gene copies/g soil dwt
7.20
7.15
7.10
7.05
7.00
6.95
E
amoA AOA log10 gene copies/g soil dwt
7.4
7.2
7.0
6.8
F
amoA AOB log10 gene copies/g soil dwt
6.50
6.25
6.00
G
Fungi:bacteria ratio
0.750
0.725
0.700
0.675
0.650
H
AOA:AOB ratio
1.20
1.15
1.10
1.05
Alder
Alder-Fir
Douglas fir
Oak
Oak-Maple
Maple

30

Fig. 5. Soil microbial community gene abundance comparisons of initial bulk soil at the start of the experiment (grey symbols) and rhizospheric soil at the end of the experiment (26 months, black symbols) for Alder, Douglas fir, and Alder-Douglas fir mixture tree species treatments. Panels refer to target genes for a) fungal *ITS,* b) bacterial and archaeal *16S,* c) *nirK,* d) *nirS,* e) *amoA* AOA, and f) *amoA* AOB, and the ratios of g) fungi:bacteria, and h) AOA:AOB. The points indicate the means and the black lines the SE. Note that the y-axis differs between panels.


A
5.0
2.5
0.0
-2.5
-3
0
3
PC2 (20.7% explained var.)
PC1 (34.8% explained var.)
RLD
RAI
nTips
AOA
fungi
pH
AOB
nirS
nirK
biomass
leaves
bacteria
C.N
groups
Alder
Alder-Douglas-fir
Douglas-fir


B
2
0
-2
-4
-4
-2
0
2
PC2 (20.2% explained var.)
PC1 (29.2% explained var.)
bacteria
fungi
AOB
nirS
nirK
leaves
biomass
pH
C.N
RLD
nTips
RAI
AOA
groups
Maple
Oak
Oak-maple

39

Fig. 6. a) Principal components analysis of the alder, Douglas fir, and alder-Douglas fir mixture tree species treatments using the soil property and gene copy abundance data from the final (after 26 months) rhizospheric soil samples. b) Principal components analysis of the oak, maple, and oak-maple mixture tree species treatments using the soil property (pH, C:N) gene copy abundance (bacteria — 16S, fungi — ITS, AOA amoA, AOB amoA, niK, and nirS) and root traits (RLD — Root Length Density, RAI — Root Area Index, and nTips — number of Tips) data from the final rhizospheric soil samples, combined with the final biomass harvest data (aboveground woody biomass in g, leaves biomass in g).