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Tree species mixing affects soil microbial functioning indirectly via root and litter traits and soil parameters in European forests

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Abstract

1. Plant community composition influences soil microbial communities through plant trait variations that lead to changes in nutrient and organic carbon inputs into the soil by root exudates and plant litter. Although plant litter and living roots are known to influence microbial functioning independently, their relative effects are rarely measured simultaneously in naturally occurring plant communities.

2. Here, we sought to evaluate how forest floor litter and absorptive roots affect broad functions of soil microbial communities, and how this may be influenced by tree species mixing. To do so, forest floor litter, absorptive roots, and soil were sampled from mono-specific and 3-species mixed stands in four mature, natural forest ecosystems across Europe. The direct effects of tree species mixing, its indirect effects via litter and root traits, and the effects of soil parameters on microbial biomass, catabolic activity and diversity, and denitrification were analyzed.
3. Results did not show direct tree mixture effects on the soil microbial parameters we measured but did suggest indirect influences via tree mixture effects on traits of aboveground litter and absorptive roots and soil parameters. Mixed forests composed of any three tree species modified soil microbial functioning by influencing nutrient availability in forest floor litter and root resource acquisition. Tree mixing also modified soil microbial functioning and catabolic diversity by influencing soil fertility and physicochemical properties.
4. Our findings suggest an indirect but present influence of tree species mixing on the activity of heterotrophic soil microbial communities across four different forest ecosystems ranging from Mediterranean to boreal forests. Our study contributes to a better mechanistic understanding of mixed tree species effects on soil microbial functioning via the modification of forest floor litter properties and traits of absorptive roots represented by the tree community beyond simple species numbers consideration, and potentially via soil properties.

Key words: Soil microorganisms, functioning, forest floor litter, absorptive tree roots, tree species mixtures

Introduction

Soils harbor a highly diverse community of microorganisms that play a crucial role in many ecological processes and impact terrestrial ecosystem functioning and stability (Bardgett & van der Putten, 2014; Fierer, 2017). Microorganisms control the rate nutrients and carbon are released from dead organic matter and made available to organisms (Crowther et al., 2019) and concurrently release greenhouse gases, notably CO₂ and N₂O, into the atmosphere (Robertson & Groffman, 2007; Singh, Bardgett, Smith, & Reay, 2010). However, soil microbial activity and its subsequent influence on ecosystem functioning are dependent on many extrinsic factors such as soil properties, climatic conditions, and the plant community (Bardgett & Caruso, 2020).

Different plant species, and thus different plant community assemblages, can exert significant and contrasting effects on soil microbial communities and associated processes (Eisenhauer et al., 2010; Scheibe et al., 2015; Urbanová, Šnajdr, & Baldrian, 2015) through differences in litter quality and diversity (Thoms, Gatteringer, Jacob, Thomas, & Gleixner, 2010), rhizodeposition (Steinauer, Chatzinotas, & Eisenhauer, 2016), root symbionts (Baldrian, 2017), above- and belowground litter production, and microclimatic conditions (e.g. temperature and soil humidity) (Prescott & Grayston, 2013). Plant diversity effects can be expressed for example, through disproportional effects (either positive or negative) of specific plant species on soil microorganisms, i.e. the sampling effect hypothesis (Aarssen, 2016; Huston, 1997; Tilman, Lehman, & Thomson, 1997). Species with complementary traits can also lead to non-additive effects on soil microorganism activities, for example, through the combination of varying litter qualities (Barantal, Schimann, Fromin, & Hättenschwiler, 2014; Handa et al., 2014) and/or increased root exudate diversity or rate of diffusion (Cesarz et al., 2013; Jones, Hodge, & Kuzyakov, 2004; Prescott & Grayston, 2013). Consequently, higher plant exudate and litter diversity and/or quantity could potentially support a more diverse (Cesarz et al., 2013;

Eisenhauer et al., 2017; Prescott & Grayston, 2013; Steinauer et al., 2016) and more abundant/active soil microbial community.

Numerous studies have evaluated plant effects on soil microbial processes with a particular focus on effects through litter production and quality (Fanin, Hättenschwiler, & Fromin, 2014; Hättenschwiler, Tiunov, & Scheu, 2005; Hatton, Castanha, Torn, & Bird, 2015; Joly, Fromin, Kiikkilä, & Hättenschwiler, 2016; Pfeiffer et al., 2013; Thoms et al., 2010), root activity (Cesarz et al., 2013; Eisenhauer et al., 2017; Landi et al., 2006), or microclimatic conditions (Kara, Bolat, Çakiroğlu, & Öztürk, 2008; Lange et al., 2014; Wu et al., 2012). Indeed, plant litter inputs and live root processes are the two primary pathways by which plants affect soil microbial communities (Baldrian, 2017; Lladó, López-Mondéjar, & Baldrian, 2017, 2018), yet their relative importance is poorly understood and most studies do not take these factors into account simultaneously.

Plant litter is an important source of organic carbon (C), nitrogen (N), phosphorus (P), and micronutrients for soil microorganisms (Becher, Bernhardt, Fuchs, & Riedel, 2013; Thomas & Packham, 2007). Litter can also provide cations that reduce soil acidification, pH being a key driver in microbial community composition and low pH being linked to slow litter decomposition (Lladó et al., 2017; Pfeiffer et al., 2013). Since many leaf characteristics, e.g. lignin and polyphenol concentrations, persist after senescence, plant leaf economic strategies (LES, Wright et al., 2004) can dictate species litter effects on decomposition rates and release of resources into the soil (Coq, Souquet, Meudec, Cheynier, & Hättenschwiler, 2010; Cornwell et al., 2008; Prescott, 2005). For example, conservative leaf traits, associated with higher energy investment in resource conservation and resource immobilization in long-living plant tissues, could negatively affect microbial activity by producing defense compounds (lignin or polyphenols) that can inhibit microorganisms (Freschet, Aerts, & Cornelissen, 2012; Prescott, 2005). Plants with acquisitive traits, associated with higher investment in growth and resource

acquisition, tend to have higher quality litter (e.g. lower C:N ratio, higher P content, lower lignin and tannin/polyphenol contents) which is correlated to increased microbial activity and faster litter turnover and influences microbial community composition and biomass (Freschet et al., 2012; Lladó et al., 2017).

Living roots influence soil microbial activity by altering soil physical structure, water flow, and exudation of ions and organic compounds (McCormack et al., 2015; Prescott & Grayston, 2013). Similar to the leaf economics spectrum, accumulating evidence from root research suggests a universal root economics spectrum (RES) (Bergmann et al., 2020; Roumet et al., 2016). Generally, it seems that higher specific root length (SRL) and nitrogen (N) concentration combined with lower root diameter and tissue density are associated with an acquisitive root resource strategy, while the inverse is associated with a conservative strategy (Kong et al., 2019; Weemstra et al., 2016). In herbaceous species, acquisitive plant species have been found to produce higher amounts of root exudates than conservative species (Guyonnet, Cantarel, Simon, & Haichar, 2018; Henneron, Cros, Picon-Cochard, Rahimian, & Fontaine, 2019), which can lead to higher soil microbial biomass and activity (Kaštovská, Edwards, Pícek, & Šantrůčková, 2015). However, the distinction between acquisitive and conservative root strategies and associated traits is presently less clear for woody species (Weemstra et al., 2016), and their effects on the microbial community is not well understood. This is likely due to a combination of spatial and temporal variability in fine-root traits (e.g. season, age, and soil depth), as well as mycorrhizal colonization. The organic matter provided to the soil microbial community in the form of root litter may also stimulate soil microbial activity more efficiently than aboveground litter (Freschet et al., 2013; Hatton et al., 2015; Jackson, Mooney, & Schulze, 1997) because of tight spatial proximity.

In this study, we evaluate the influences of tree species mixing by comparing single tree species stands to stands with three tree species and their associated litter and root traits on soil

microbial functioning (microbial respiration, denitrification potential, and catabolic diversity estimated from 15 carbon substrates) in four mature, natural forest ecosystems across Europe (including a total of 13 tree species, 34 different species combinations, and varying soil types). By incorporating a wide range of climate, soil, and forest types we were able to explore general trends of tree characteristics and species mixture influences on microbial functioning beyond site and forest community-specific variations. We hypothesize that, tree species mixtures promote higher soil microbial activity across forest ecosystems irrespective of the biotic and abiotic conditions. We further hypothesize that, while more diverse above-ground litter and below-ground root traits and associated resource inputs to the soil both contribute to these mixture effects, roots have a more dominant role because of their intimate contact with microbial communities in the three-dimensional topsoil space whereas aboveground litter remains largely on the soil surface.

Materials and Methods

Study sites and sampling protocol: The studied sites are part of a permanent network of mature forest plots established in 2011 and 2012 (detailed site descriptions can be found in Baeten et al., 2013) across Europe: Colline Metallifere (Italy), Râșca (Romania), Białowieża (Poland), and North Karelia (Finland). These forests represent four major European forest types including Mediterranean thermophilous, montane mixed beech, hemiboreal mixed broadleaved-coniferous, and boreal forests (see Table S1 and Figure S1 in Supporting Information). The forests are managed to variable degrees, but species composition is mostly influenced by selective logging, not planting, of naturally established trees. Within each site, 30 m × 30 m forest plots were selected that had stands composed of either one dominant tree species (mono-specific plot) or three co-dominating tree species (mixed plot); species were defined as co-dominant when it composed >15% of the stand. Tree species identity and mixed

plot tree species combinations varied among sites. Each tree species at each site had two replicate mono-specific plots (with the exception of *Picea abies* L. and *Quercus robur* L. plots with one replicate each and *Betula pendula* Roth with no mono-specific plot in Białowieża, Poland). Mixed plots had a minimum of three replicates per site, but the tree species compositions of these replicates included any three target species present at that site, i.e. mixed plot replicates were not necessarily composed of the same tree species. The final selection of 64 plots included 13 tree species and 34 different species combinations, as well as a wide range of soil types (Table S1).

Within each plot, five tree triplets were identified following the approach described by Vivanco & Austin (2008), a tree triplet being a triangle of three canopy tree individuals with less than 8 m distance from one another and with no other tree individuals within the triangle. In mono-specific plots, tree triplets were composed of the same tree species, and in mixed plots, each triplet was formed by one individual from each of the three co-dominant trees species of the plot. At the estimated center of each triplet, weighted by tree individual size (individual diameter at breast height), four 15 × 15 cm forest floor litter layer samples were collected and dried at 60°C. All litter material within the square, which was predominantly leaf litter but sometimes included other plant parts such as reproductive structures and small (< 25 mm diameter) branches, was collected down to the mineral soil, meaning that multiple years of litter accumulation were sampled. We considered the entire decomposing forest floor more representative of how aboveground litter accumulation may affect underlying soil microbial communities than only annual fresh litter fall. Such higher realism came at the cost of distinguishing litter material originating from different tree species within mixture plots, which impeded us from considering functional diversity of litter traits. One soil core (5.3 cm diameter) was sampled from the top 10 cm of soil within each of the four squares where the litter had been removed. This means that the soil cores included the mineral layers starting with the A-

horizon. The four soil cores sampled within each tree triplet were then combined and sieved through a 2 mm sieve and air-dried immediately after sampling for soil microbial analyses. One additional soil core was taken in the same location using the same methods and kept intact for root measurements. There were thus five replicate samples for each of the 64 plots for a total of $n = 320$ samples (mono-specific plot samples $n = 150$, mixed plot samples $n = 170$) of forest floor, sieved soil, and soil for root measurements.

Soil measurements: The soil bulk density, carbon (C) concentration, carbon:nitrogen (C:N) ratio, and pH were measured during the FunDivEUROPE project in 2012 (Baeten et al., 2013; Dawud et al., 2016). For the FunDivEUROPE soil sampling methods see (Dawud et al., 2016). Another set of unground soil subsamples (10 g per sample) were used to determine soil texture. The soil was pre-treated for organic and carbonate removal (ISRIC & FAO, 2002) if present, the texture was then measured by laser granulometry (Malvern Mastersizer S, Malvern Instruments Limited, Worcestershire, United Kingdom). Soil data are provided in Table S2.

Forest floor litter characterization: Each subplot litter sample (five per plot) was dried at 60°C, weighed, and the weight was divided by the surface area sampled to be expressed as kg dry weight litter per square meter of soil surface. After weighing, the entire litter sample was ground to approximately 2 mm (Retsch cutting mill SM1, Haan, Germany) for homogenization and then a subsample of litter was ground to 1 mm (Cyclotec 1093 cyclone grinder, Tecator, Höganäs, Sweden) for chemical analyses.

The total C and nitrogen (N) concentrations (%) of each individual litter sample were measured using the Pregl-Dumas method with a CHN Elemental Analyzser (Flash EA1112 Series, ThermoFinnigan, Milan, Italy) on 3.7 ± 0.4 mg of litter. The C concentration was then divided by the N concentration to obtain the litter C:N ratio.

For the other litter quality parameters based on methods that take much more time than C and N analyses, we used the near infrared spectrum (NIRS) approach for chemical

characterization of each individual sample. We first determined the NIR spectrum of each of the 320 samples using Fourier-transformed infrared spectroscopy (FTIR) with a NIRFlex N-500 spectrometer (BUCHI Corporation, New Castle, DE, United States). The litter samples were scanned in a cuvette ($W \times D \times H$: $12.5 \times 12.5 \times 45$ mm) with a spectral range from 1000 nm – 2500 nm and spectral resolution of 8 cm^{-1} for 16 scans. Each sample was scanned twice, and the two spectra were averaged. Based on the bulk of obtained spectra, a selection program (NIRWare Management Console, BUCHI Corporation) identified the required number and spread of a subset of samples on which a calibration data base was constructed using the NIRWare NIRCal software (BUCHI Corporation). A total of 87 spectra out of the 320 were selected to accurately represent the sample spectra distribution. The P, lignin, condensed tannins, and total phenolics concentrations were then measured for these 87 samples, which were subsequently used to predict the values for the remaining 233 samples based on their individual near infrared spectra (NIRWare NIRCal software). The program tested multiple methods as well as multiple transformations to obtain the best regression coefficient, using two-thirds of the spectra for calibration and one-third for validation. The calibration methods, transformations, and calibration and validation results are detailed in Table S3; the r^2 values for all litter quality parameters were all larger than 0.76.

The P concentration was measured colorimetrically using the molybdate blue method (Grimshaw, Allen, & Parkinson, 1989). First, 84.0 ± 4.0 mg of litter was mineralized by adding 8 ml of HNO_3 (2.24 mol L^{-1}) and, over 10 min, heated to 120°C , then over 20 minutes, heated to 175°C and kept at this temperature for 10 min in an ETHOS One microwave (Milestone, Via Fatebenefratelli 1/5-24010 Sorisol, Italy). Once the sample cooled, 100 μl was deposited in each well of a 96-DeepWell Microplate (Fisher Scientific E39199) and 100 μl NaOH (2 mol L^{-1}), 50 μl sodium molybdate (7 g L^{-1}), and 50 μl ascorbic acid (10 g L^{-1}) was added in that order.

The plate was incubated at 40°C for 30 minutes then the optic density was read (wavelength 720 nm) with a Victor 1420 Multilabel Counter (PerkinElmer, Massachusetts, USA).

Cellulose, hemicellulose, and lignin fractions were measured with the FIBERSAC® method 12 (Fibersac 24, Ankom, Macedon, NJ, USA; Ankom Technology, 2017) adapted from Van Soest (1963). Following this protocol, plant tissue constituents were extracted and measured gravimetrically by sequentially exposing 510.0 ± 10.0 mg dry weight of the litter sample to neutral detergent (NDS), acid detergent (ADS), and H_2SO_4 (72%).

The concentration of condensed tannins was measured by spectrophotometry with the butanol-HCl method (Porter, Hrstich, & Chan, 1985; Waterman & Mole, 1994) as described in detail by Coq et al. (2010). Total phenolic concentration was measured colorimetrically, using the method described by Ribéreau-Graydon (1972) and using the Hach TanniVer™ reagent (Hach Company, Loveland, CO, USA), according to the detailed description in Coq et al. (2010). Extractions were diluted when necessary. Forest floor litter data are provided in Table S2.

Absorptive root traits: Roots were sorted from the soil cores and all fine roots (< 2 mm in diameter) were subsequently classified as absorptive (the first three root orders) or transport roots (4th and 5th order roots) according to the functional classification approach by McCormack et al. (2015). On average, absorptive roots of the target species made up $53.5 \pm 2.4\%$ of all fine roots (absorptive and transport roots combined). For further details on the root sorting and measurement methods see (Wambsganss, Beyer, Freschet, Scherer-Lorenzen, & Bauhus, 2021). This order-based approach was used, as opposed to the still commonly applied traditional diameter classification, because studies have shown that the first three most distal root orders (i.e. the absorptive roots according to McCormack et al. (2015)) significantly differ in their functions from higher order roots. The absorptive roots are responsible for most of the resource uptake (absorption), and thus exudation, and are therefore more relevant in affecting

microorganisms than higher order roots (Guo et al., 2008; McCormack et al., 2015). The morphological absorptive root traits reflect root growth, resource capture strategies, and associated functioning (Bardgett, Mommer, & De Vries, 2014; McCormack & Iversen, 2019; Weemstra et al., 2016). Absorptive root trait data are provided in Table S2.

Soil microbial parameters: Soil microbial analyses were done on soils that were air-dried immediately after sampling, because it was not possible to work on fresh soils for logistical reasons (geographical spread of the sampling sites, time required for sampling all the plots, sample shipping constraints). Air-drying has been found to not significantly impact microbial community composition and structure (Wang et al., 2021), but even if shifts in microbial parameters occur, the relative differences in C and N transformations between samples are generally preserved (Makarov, Mulyukova, Malysheva, & Menyailo, 2013).

Classical substrate induced respiration (SIR) method was used to measure the potentially active microbial biomass (Beare, Neely, Coleman, & Hargrove, 1990). This method allows to determine glucose-induced respiration activity as the amount of CO₂ produced under optimal conditions over a short duration (4 hours), to measure the active enzyme pool respiration before new enzymes can be synthesized or new microbial growth (Fanin, Hättenschwiler, Barantal, Schimann, & Fromin, 2011 for methods). The active microbial biomass ($\mu\text{g C}_{\text{mic}} \text{g}^{-1} \text{dry soil}$) was then estimated using the calculation proposed by Anderson and Domsch (1978): SIR rate ($\mu\text{l C-CO}_2 \text{g}^{-1} \text{dry soil h}^{-1}$) * 40.04 + 0.37.

The MicroRespTM method described by Shiha et al. (2017) was used to determine the catabolic diversity of soil microorganisms, based on the ability of the soil microbial community to respire on a range of various C substrates ($\mu\text{g C-CO}_2 \text{g}^{-1} \text{dry soil h}^{-1}$). We used the same 15 different C sources as described in detail by Shiha et al. (2017). Three technical replicates were run per substrate with approximately 0.39 g of soil dry weight per replicate. The substrate addition equated to 1.5 mg of C per g dry weight of soil. The SIR rates of all 15 substrates were

summed to obtain a global catabolic respiration value (Sum15). The Shannon metabolic diversity index for each subplot was calculated using the formula: $H' = -\sum_{i=1}^{15} p_i \times \ln(p_i)$ where p_i is the standardized respiration rate for the substrate 'i', i.e. the respiration rate of substrate 'i' divided by the Sum15 value. For the respiration rate of each of the 15 C sources, a substrate was considered 'used' by the microbial community when the respiration rate was 15% higher relative to the respiration without the addition of a C substrate (i.e. just water), all respiration rates below this threshold were considered not-used and were replaced by zeros for the ANOSIM and GLMM analyses (see below).

Potential microbial denitrification enzyme activity (DEA, $\mu\text{g N-N}_2\text{O g}^{-1}$ dry soil h^{-1}) was measured using the acetylene inhibition method described by Smith & Tiedje (1979) as described by Pinay et al. (2007). This is a measure of the potential denitrification activity since it is conducted under optimal conditions and the enzyme concentration is the only activity-limiting factor.

Statistical Analysis: The R software (R Development Core Team, 2008) (version 3.5.3) was used for all figures and statistical analyses, figures were made using the 'pirateplot' function from the YaRrr! Package (version 0.1.5, Phillips, 2018), the function 'fviz_pca_biplot' from the factoextra package (version 1.0.6, Kassambara & Mundt, 2019), and the function 'radarchart' in the fmsb package (version 0.7.0, Nakazawa, 2019). The QGIS software (version 3.12.3) was used to create the sampling locations map (Figure S1) with a basemap from www.naturalearthdata.com.

To take into account the site-specificity of the soil parameters in subsequent analyses, the soil variables were incorporated into a principal component analyses (PCA) using the function 'prcomp' from the factoextra package (version 1.0.6, Kassambara & Mundt, 2019) (Fig. 1a) and the PC scores of the first two axes were extracted. The extracted PCA scores were then

included in the general linear models and structural equation models as explanatory variables. This was also done for the chosen forest floor litter characteristics and the absorptive root traits.

Generalized mixed-effects linear models were run, using the lme4 package (version 1.1-21; Bates et al., 2019), on each response variable (C_{mic} , Sum15, H' , and DEA) testing the effect of the explanatory variables (Litter PC1, Litter PC2, Root PC1, Root PC2, Sol PC1, Soil PC2, and tree mixture). Response variables were transformed (log2) when necessary, and extreme values ($> \pm 3$ times the interquartile range) were removed (the number of removed values never exceeded 10% of the total number of values). The site, which takes into account all associated site-specific differences such as climatic variables, and plot were included as random variables. The model structure was as follows: response variable $\sim$ Litter PC1 + Litter PC2 + Root PC1 + Root PC2 + Soil PC1 + Soil PC2 + Tree species number + (1|Site/Plot). In order to identify the most parsimonious models and the most consistent predictors we used a model averaging approach via the 'dredge' and 'model.avg' functions in the MuMIn package (Bartoń, 2019) which uses the lowest Akaike Information Criteria (AIC) to rank all possible models with all possible combinations of the explanatory variables in the full model. A 95% confidence set was used to select a subset of the models to be averaged, i.e. average of the estimates, calculated using the zero method (Burnham & Anderson, 2002), with the standard error, importance value, z-value, and p-value. The importance value is calculated by summing the model weights of the models where the variable appears.

Before testing the respiration rates of the substrates considered to be 'used' (see definition above) at a univariate level, we first tested them at the multivariate level. An analysis of similarities (ANOSIM) was performed on the 'used' substrate respiration rates using the function 'anosim' in the vegan package (version 2.5-6; Oksanen et al., 2019) to explore the influence of the explanatory variables listed above. Data were averaged at the plot level since 'anosim' can only accept one random variable, i.e. pooling the five within-plot measurements

to a single mean value with site as the only random variable. Since the ANOSIM results showed a marginally significant tree species mixing effect on the multi-substrate use (Table 2), we ran univariate GLMMs, using the same model structure and method as before, on each individual substrate. The data were analyzed at the sub-plot level for the GLMMs since this analysis can accept multiple random variables.

Structural Equation Modelling (SEM; Grace et al., 2015) was used to test the support for a network of hypothesized causal relationships between tree species mixing, forest floor litter characteristics, absorptive root traits, and soil parameters on soil microbial functioning. The piecewiseSEM package (version 2.1.0; Lefcheck et al., 2019) was used to build SEMs for each microbial response variable (C_{mic} , Sum15, H' , DEA) excluding the individual 15 C substrate values, see Figure S2 for the model structure. Tree species mixture was included as an exogenous variable with influence on the microbial functional response variable directly and indirectly via Soil PC1, Litter PC1, and Root PC1. The response variables were transformed ($\log_2$) when necessary before running the SEM. Because there were insufficient data points to include all axes simultaneously, we constructed a second identically structured SEM with the second axes (i.e. Litter PC2, Root PC2, and Soil PC2). Additional SEMs were also created to explore whether litter and root parameters indirectly influenced the microbial variables via the soil parameters. Model goodness of fit was analyzed using the test of directed separation by combining all p-values across the basis set in the Fisher's C test statistic and comparing it to a χ^2 -distribution with 2k degrees of freedom. The model has a good fit when p of the χ^2 test > 0.05. We tested all PC1 and PC2 combinations possible, and mixing PC1 and PC2 variables within a SEM did not change the results or, in the case of Soil PC1 or PC2 effects on opposite Litter or Root PC axes (e.g. Soil PC1 effect on Litter PC2), were not significant. We therefore present the SEM results as a SEM constructed with PC1 variables and a second SEM with the PC2 variables.

Results

Soil parameters, forest floor litter traits, and traits of absorptive root varied widely among the four studied forests across Europe (Fig. 1). The first axis of the soil parameter PCA (Soil PC1) accounted for 52.5% of the variance and was mostly determined by soil physicochemical properties (Fig. 1a). High bulk density was associated with negative PC scores, and high pH, C, and clay concentrations were associated with positive scores. The second axis (Soil PC2) explained 24.9% of variance, with negative PC scores correlated with low C:N ratio. The first axis of the forest floor litter trait PCA (Litter PC1) accounted for 50.2% of the variance, with negative PC scores associated to high concentrations of phenolic compounds and positive scores to high concentrations of lignin (Fig. 1b). The second axis (Litter PC2) accounted for 23.7% of the variance, with negative PC scores associated with high C:N ratio and low P and positive PC scores to low C:N ratio and high P. The first axis of the PCA on traits of absorptive roots (Root PC1) accounted for 42.2% of the variance, with negative scores associated with high SRL, RLD, and surface area and positive scores associated with high diameter and RTD (Fig. 1c). The second axis (Root PC2) captured 26.9% of the variance and was mainly related to the root surface area (negative scores) and ECM colonization intensity (positive scores; Fig. 1c).

Overall, mixed forest plots had higher Litter PC2 scores (higher P concentration and lower C:N ratio), lower Root PC1 (high SRL, RLD, and surface area), and higher Root PC2 scores (higher ECM colonization intensity and lower root surface area) compared to mono-specific plots. Between sites, Litter PC1, Litter PC2, Root PC1, and Root PC2 were reasonably comparable, with slight deviation for the Finnish Litter PC1, and had generally consistent patterns between mono-specific and mixed plots (Figure S3). Slightly more deviation was found

for soil PC scores, particularly for Soil PC1 where the sites in Italy and Romania were distinct from the sites in Finland and Poland.

Across all 320 collected soil samples, we measured an average C microbial biomass (C_{mic}) of $166.0 \pm 92.7 \mu\text{g } C_{mic} \text{ g}^{-1}$ dry soil, with a very large 20-fold range between 28.8 and $568.5 \mu\text{g } C_{mic} \text{ g}^{-1}$ dry soil (Fig. 2a). The average denitrification enzyme activity (DEA) was $0.01 \pm 0.014 \mu\text{g N-N}_2\text{O g}^{-1}$ dry soil h^{-1} , ranging between 0.00 and $0.06 \mu\text{g N-N}_2\text{O g}^{-1}$ dry soil h^{-1} (Fig. 2b). The average sum of the microbial catabolic respiration rate induced by 15 different C-sources (Sum15) was $62.7 \pm 34.5 \mu\text{g C-CO}_2 \text{ g}^{-1}$ dry soil h^{-1} and ranged between 26.1 and $204.3 \mu\text{g C-CO}_2 \text{ g}^{-1}$ dry soil h^{-1} (Fig. 2c). We measured an average Shannon metabolic diversity index (H') of 2.69 ± 0.014 (unitless), with a range between 2.64 and 2.71 (Fig. 2d). Across the 15 substrates included, the average ‘used’ C substrate respiration rate was $2.51 \pm 3.51 \mu\text{g C-CO}_2 \text{ g}^{-1}$ dry soil h^{-1} and was lowest for vanillic acid ($0.68 \pm 2.0 \mu\text{g C-CO}_2 \text{ g}^{-1}$ dry soil h^{-1}) and highest for oxalic acid ($5.47 \pm 5.22 \mu\text{g C-CO}_2 \text{ g}^{-1}$ dry soil h^{-1}) (Fig. 3). The microbial variables (i.e. biomass, DEA, sum15, H' , and substrate use) were generally comparable between sites and had similar patterns between mono-specific and mixed plots, except for higher DEA rates in mixed compared to monospecific stands in Finland and Romania (Supplementary Figs 4).

The GLMMs showed no direct tree species mixing effects, i.e. no difference between soils from mono-specific and mixed tree species stands, on any of the measured microbial activity parameters (Table 1, Fig. 2). The ANOSIM results showed a marginally significant difference between mono-specific and mixed stands for the respiration rates calculated from the ‘used’ C substrates only (Table 2, Fig. 3). However, none of the univariate GLMMs run for each substrate individually showed a tree species mixing effect (Table S5).

Variations in forest floor litter and absorptive roots traits showed some effects on soil microbial variables but appeared response variable-dependent (Table 1). There was no significant Root PC1 effect, but both C_{mic} and Sum15 rates showed a coherent negative

correlation with Root PC2, i.e. higher C_{mic} and Sum15 associated with higher root surface area and lower ECM colonization intensity (the two variables that best represent Root PC2; Fig. 1c). DEA was the only microbial parameter we assessed that was significantly affected by litter traits (Table 1). DEA was positively correlated with Litter PC2 scores, indicating that DEA was higher when the litter layer had high litter P concentrations and low C:N ratios (Fig. 1b). The multivariate analysis on the ‘used’ substrates (with respiration rates above the threshold of 15% higher than that of pure water addition; see Materials & Methods) showed that the ‘used’ substrate profiles were not affected by litter parameters nor Root PC1 but, as for microbial biomass and Sum15, were related to Root PC2 (Table 2). The univariate GLMM analyses run on the ‘used’ substrate respiration rates for each individual substrate showed no correlation between litter or root PC1 or PC2 scores and substrate use (Table S5).

Soil parameters had the most consistent effects on soil microbial response variables. Microbial biomass, Sum15, H' , and DEA all varied significantly along the first soil PCA axis (Table 1) associated with variation in pH, clay and organic matter content (C), and bulk density (Fig. 1a). While microbial biomass, Sum15, and DEA increased with Soil PC1 scores corresponding to soils with finer texture and higher pH, we observed the opposite for H' (Table 1). None of the tested microbial variables varied significantly along the soil PC2 axis (Table 1). The use of the different C substrates was affected by both Soil PC1 and Soil PC2 scores (Table 2). At univariate substrate level, higher Soil PC1 scores were correlated with higher respiration rates of one carbohydrate (D-glucose), three amino acids (L-asparagine, L-serine, and L-glutamine), and two carboxylic acids (oxalic acid and malic acid), while lower Soil PC2 scores were related to higher oxalic acid use only (Table S5).

With structural equation modeling (SEM), we found an indirect tree species mixing effect on the measured microbial response variables (Fig. 4). There were strong and consistent tree species mixture effects on soil parameters (Soil PC1 and PC2), forest floor litter nutrient

characteristics (Litter PC2) and traits of absorptive roots (Root PC1 and PC2) (Fig. 4). Mixed tree species stands were related to lower Soil PC1, Soil PC2, and Root PC1 scores, and to higher Litter PC2 and Root PC2 scores, regardless of which of the four microbial response variables was fitted (see also Figure S3). These tree mixture effects on soil physicochemical parameters, forest floor litter characteristics, and absorptive root traits had some cascading effects on microbial activity. Tree species mixing indirectly, negatively influenced microbial biomass, DEA, and Sum15 through its negative affect on Soil PC1 (i.e. lower soil pH, clay and C content and higher soil density). Microbial H' however, was positively, indirectly influenced by tree species mixing through its effects on Soil PC1, but negatively, indirectly influenced by its effects on Soil PC2, i.e. lower soil fertility in mixed stands leading to lower H' . Tree species mixing indirectly, positively influenced DEA through its positive effect on Litter PC2, that is to say mixed tree species stands had higher nutrient availability (lower C:N and higher P) leading to higher potential denitrification activity. In addition, tree species mixing indirectly, negatively influenced microbial biomass and Sum15 through its positive effect on Root PC2 (Fig. 4a,c), meaning mixed tree species stands had lower root surface area and higher ECM colonization leading to lower microbial biomass and catabolic activity. Soil PC1 also had a consistent positive correlation with Litter PC1 (lower soil density and higher pH, clay and C contents leading to higher litter lignin and lower litter tannin/phenolics concentrations), while Soil PC2 had a negative effect on Litter PC2 in the DEA model (lower soil C:N and C content and higher bulk density leading to higher litter P concentration and lower C:N). These may have also been pathways by which tree species mixing influenced H' and DEA.

Discussion

With the measurements of soil microbial functioning in our study, we did not detect direct tree species mixture effects over four mature natural forests across Europe, encompassing a

wide range of climate, soil, and forest types. However, despite the absence of direct tree species mixture effects, we found that tree mixing indirectly affected soil microbial parameters through changes in tree functional traits, which partly confirms our first hypothesis, and potentially through changes in soil parameters.

These results from natural forest stands of varying site conditions differ from those obtained in synthetic single site experiments. For example, the long-term Jena experiment, manipulating herbaceous species diversity, showed a positive correlation between species richness (Eisenhauer et al., 2010; Lange et al., 2015) or root exudate diversity (Steinauer et al., 2016) and soil microbial biomass and activity. Similarly, the findings from a young tree plantation experiment support enhanced soil microbial biomass and activity in soil from communities with higher tree species richness (Khlifa, Paquette, Messier, Reich, & Munson, 2017). Compared to these single site experiments, our four study sites from different climate zones and with mature trees and distinct soil properties introduced more variation in a range of factors, making the detection of species mixing effects more difficult. However, in a recent meta-analysis covering a wide range of species (herbaceous and woody), habitat types (natural, artificial/planted, in container, forest, grassland, and cropland), successional stages, and climate zones, Chen et al. (2019) reported an overall higher soil microbial biomass and activity with increasing plant species richness. The studies they considered in their meta-analysis covered a much wider diversity gradient (from one up to 60 species combinations) and increasing plant species richness was the main driver of the observed generally positive biodiversity effects on soil microbial parameters. It seems therefore likely that the comparatively small difference from one to three tree species in the forests we studied did not allow the detection of a more general species richness effect potentially expressed at wider species richness gradients. Species identity effects may further outweigh mixture effects on soil microbial community composition and/or functioning (L. Chen et al., 2019; Dijkstra, West, Hobbie, & Reich, 2009; Scheibe et al.,

2015), particularly in a design with a low diversity gradient such as ours. However, since we did not have the same species at the four sites, we could not introduce species identity as a co-variable in our statistical models, which is an unavoidable trade-off when working on natural forests and may have contributed to obscuring direct diversity effects.

Despite the wide range of tree species and forest ecosystems covered by our study, tree mixtures had some general influence on the traits of forest floor litter and absorptive roots and on soil parameters, which appear to consequently affect soil microbial activity indirectly. Mixed stands generally displayed higher Litter PC2 scores (Fig. 1b, Figure S3), related to higher litter P concentrations and lower C:N ratios, indicating higher nutrient availability and potentially faster litter decomposition (Prescott, 2005). Higher decomposition rates lead to faster cycling and lower immobilization of nutrients, which could lead to a more balanced soil nutrient stoichiometry beneficial to soil microorganisms (Cleveland & Liptzin, 2007; Thomas & Packham, 2007). Tree mixtures also increase the probability of including complementary traits or substrates with non-additive effects on the microbial functioning (Joly et al., 2016). Higher forest floor litter P concentrations and lower C:N ratios associated with mixed stands are potentially an indirect stimulus of the higher DEA rates, as seen in the hierarchical PC2 SEM (Fig. 4b). Higher nitrogen mineralization rates have been correlated with mixed tree species stands (Forrester, 2017) and could lead to higher soil N availability for DEA. In correlation, higher litter turnover could increase C and nitrate availability, which typically limits denitrification (Robertson & Groffman, 2007). This lends support to the hypothesis that tree mixtures indirectly influence DEA through influences on forest floor litter quality and decomposability. These findings are in line with those of Thoms et al. (2010), who showed that aboveground tree species diversity (up to three species) stimulated soil microbial diversity mostly through indirect interactions with specific plant traits rather than by the tree species diversity itself.

Mixture effects on leaf defense-related compounds (Litter PC1) or forest floor nutrient availability (Litter PC2) did not translate into an effect on microbial biomass (C_{mic}), metabolic respiration (Sum15), metabolic diversity (H'), or the range of C substrates used by the soil microbial community. This could mean that soil microbial biomass and C-use are insensitive to the observed differences in forest floor chemistry between single and mixed species forests. The forest floor chemistry variability among the forests composed of different tree species was likely too large for such differences to be expressed, as indicated by the considerable overlap in forest floor characteristics between single species forests and tree mixtures (Fig. 1b). Litter trait diversity is what usually affects microbial biomass and activity in the litter layer, potentially leading to altered decomposition (Handa et al., 2014; Kou et al., 2020) and higher microbial abundance and diversity (Santónja et al., 2017). However, the impact on soil microbial communities is less understood. Controlled laboratory (Fanin et al., 2014; Pfeiffer et al., 2013) and field (Thoms et al., 2010) studies showed that soil microbial communities responded differently to various leaf litters decomposing at the soil surface and that tree litter leachate mixtures had non-additive, short-term effects on soil microbial activity (Joly et al., 2016). Our results from a field setting suggest that such litter effects may not be easily distinguished from numerous other sources of variation playing out at broader spatial scales, such as changing tree species identity.

Contrary to our second hypothesis, traits of absorptive roots did not show more pronounced mixture effects on the measured soil microbial responses than the forest floor litter traits. Higher Root PC2 scores in mixed stands (Fig. 4, Figure S3), meaning lower root surface area and higher ECM colonization intensity (Fig. 1c), lead to lower microbial biomass and respiration potential (Sum15). This may indicate that mixed stands are higher on the fungal collaboration gradient (i.e. higher reliance on mycorrhizal partners for soil space exploration/exploitation and therefore resource acquisition; Bergmann et al., 2020). However, this effect is subtle and on

average microbial biomass and Sum15 rates were not significantly different between stand types (Fig. 2a,c). Furthermore, metabolic diversity (H') was not affected by Root PC2 scores. Root PC2 effects on microbial respiration but not on catabolic diversity may indicate microbial communities with different activity levels but equally diverse metabolic capabilities. This would entail changes in substrate use, which was seen for overall ‘used’ substrate respiration rate (ANOSIM results) but not at the individual substrate level (GLMM results).

Despite the strong, negative tree mixture effect on Root PC1, appearing to represent a gradient in economic strategy (Fig. 1c), Root PC1 in turn only affected Sum15 but no other microbial variable (Fig. 4). The distinction between acquisitive and conservative root strategies is presently less clear for woody species than for herbaceous species, and mycorrhizal interactions were a proposed reason offsetting the presence of a RES (Bergmann et al., 2020; Kong et al., 2019; Ma et al., 2018; McCormack & Iversen, 2019). This is likely applicable to the findings here considering the robust Root PC2 effect found, the Root PC2 axis being strongly associated to mycorrhizal colonization intensity. Indeed, the Root PC2 effect may lay along the “do it yourself” (i.e. roots that efficiently explore the soil space by themselves with a typically high SRL) vs. “outsourcing” (i.e. roots that rely more on mycorrhizal partners for soil resource acquisition correlated with a large root diameter) gradient correlated to microbial root associations (Bergmann et al., 2020).

We acknowledge that the forest floor and tree root traits measured here are not exhaustive, and missing traits could have influenced findings. For example, the composition and quantity of root exudates could elucidate possible root effects on soil microbial functioning associated with tree species mixing (Steinauer et al., 2016). A more detailed analysis of carbon quality from forest floor and root exudates or decomposing roots may allow a better understanding of how tree mixtures affect soil microbial activity (Sun et al., 2018).

Soil parameters had the strongest and most consistent effects on microbial responses, especially those defining the variance of Soil PC1 with increasing scores associated to higher

pH, organic matter and clay concentrations and lower bulk density. Indeed, these soil parameters are principal factors determining soil microbial community composition and functioning (Fierer & Jackson, 2006; Paul, 2007; Thomas & Packham, 2007). In our study, higher Soil PC1 scores were correlated with higher potential respiration rates (Sum15 and DEA) and microbial biomass, while inversely correlated with catabolic diversity (H'). Clay content strongly affects microbial community structure due to its often higher nutrient stocks and desiccation protection for bacteria (Frey, 2015; Scheibe et al., 2015; Thoms et al., 2010). In addition, the use of the 15 C-substrates was overall influenced by these soil parameters but effects on individual substrates were not universal. SEM results also showed a possible indirect influence of tree species mixing on soil microbial functioning via its correlation with soil parameters, physicochemical parameters (Soil PC1) in particular. However, potential patterns seen in the SEMs were not always supported by GLMM results or in contradiction to one another. For example, the indirect, negative mixed tree species effect on DEA via its negative correlation with Soil PC1 is contradictory to the indirect, positive tree species mixing effect on DEA via Litter PC2. This complex dynamic is possibly the reason for the non-significant, direct tree species mixing effect on soil microbial functioning; a combination of positive and negative mixing effects may have cancelled each other. Indeed, the studied system is intricate, with multi-directional, hierarchal pathways by which trees species mixing can affect microbial functioning. This complexity is not even taking into consideration the reciprocal influences between the studied parameters. Notably, although tree species diversity influences soil properties (Reich et al., 2005), soil properties also determine plant species composition and diversity (Lafleur, Paré, Munson, & Bergeron, 2010; van Breemen, Finzi, & Canham, 1997). The predominant direction of this reciprocal influence is not clear in the forests studied here, because although they are mostly naturally established, i.e. soil properties influenced forest

establishment, they are also mature stands, meaning the trees have had time to significantly alter soil parameters.

The soil C:N ratio (related to Soil PC2), a potential indicator of soil fertility or nutrient limitation (Cleveland & Liptzin, 2007), did not affect microbial biomass, activity (Sum15 or DEA), or metabolic diversity (H'). These variables are measured under non-limiting conditions (i.e. substrate additions) and would therefore obscure any C- and to a lesser degree N-limitation effects. The soil C:N ratio had a strong correlation with overall 'used' C substrate respiration dissimilarity (i.e. ANOSIM results). However, this correlation appears to be primarily driven by the negative relationship between soil C:N ratio and oxalic acid use, which may indicate that soils less limited in N permit a larger microbial response to the addition of oxalic acid.

Conclusion

The main interest of our study is that it covers a wide range of forests, tree species, and environmental conditions seeking to understand whether there are any general patterns of tree species mixing on broad functions of soil microbial communities. A strong result of our study was that, compared to single tree species forests, mixed forests composed of any three tree species modify soil microbial biomass and functioning indirectly through traits of the forest floor litter and of absorptive roots and potentially through soil parameters across forests as different as Mediterranean and boreal forests. This result helps for a better mechanistic understanding of mixed tree species effects on soil microbial functioning beyond simple species number considerations. The studied system is however, complex and disentangling the effects of individual parameters is difficult at the large spatial scale of our study. The consequences of changes in tree species composition in response to species loss, climate change, or management decisions for soil microbial functioning may thus be largely determined by the modification of

soil properties, forest floor litter properties, and the traits of absorptive roots represented by the newly established tree communities.

Author contributions

L.G., N.F., S.H., and A.M. developed the study design, planned and assisted in field sampling, and participated in article redaction. L.G. performed the forest floor litter analyses, J.W. performed the root measurements and assisted in article redaction, and A.S. performed the soil microbial activity measurements.

Competing Interests statement

The author declares no competing interests.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request. This data is stored as excel files on a data portal associated with the FunDivEUROPE and SoilForEUROPE projects and available after a 1- year embargo (data url: <https://data.botanik.uni-halle.de/fundiveurope/datasets/523>).

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944 Table 1. GLM model averaging results: R^2 marginal (R^2_m), and R^2 conditional (R^2_c), estimated
945 slope (Est.), standard error (SE), importance (Imp.), z-value, and p-values for the response
946 variables: microbial biomass ($\mu\text{g C}_{\text{microbial}} \text{ g}^{-1} \text{ dry soil}$), potential denitrification enzyme activity
947 (DEA; $\mu\text{g N-N}_2\text{O g}^{-1} \text{ dry soil h}^{-1}$), sum of the microbial catabolic respiration induced by 15
948 different C-sources (Sum15; $\mu\text{g C-CO}_2 \text{ g}^{-1} \text{ dry soil h}^{-1}$), and Shannon metabolic diversity index
949 (H'). Blue and red estimate values indicate positive and negative relationships, respectively.
950 Explanatory variables are abbreviated as: 3-species tree mixture stands (Tree mix.), first and
951 second forest floor litter PCA axis (Litter PC1 and Litter PC2), first and second absorptive root
952 PCA axis (Root PC1 and Root PC2), and first and second soil parameters axis (Soil PC1 and
953 Soil PC2). P-values are coded as such: $p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$.

	Microbial Biomass						Denitrification					
	$R^2_m = 0.35$		$R^2_c = 0.77$		AIC= 566		$R^2_m = 0.23$		$R^2_c = 0.79$		AIC= 875.6	
	Est.	SE	Imp.	z-value	p-value		Est.	SE	Imp.	z-value	p-value	
Tree mix.	0.04	0.11	0.30	0.40	0.69		-0.10	0.21	0.33	0.46	0.65	
Litter PC1	0.04	0.04	0.68	1.08	0.28		0.05	0.06	0.53	0.76	0.45	
Litter PC2	0.05	0.05	0.65	1.01	0.31		0.22	0.07	1.00	3.00	0.003	**
Root PC1	-0.01	0.02	0.27	0.35	0.73		0.02	0.04	0.34	0.46	0.65	
Root PC2	-0.11	0.04	1.00	2.96	0.003	**	-0.02	0.05	0.35	0.47	0.64	
Soil PC1	0.37	0.07	1.00	5.04	5.00E-07	***	0.60	0.14	1.00	4.24	0.00002	***
Soil PC2	0.00	0.04	0.20	0.05	0.96		-0.02	0.08	0.24	0.23	0.82	
	Sum15						H'					
	$R^2_m = 0.30$		$R^2_c = 0.67$		AIC= 396.4		$R^2_m = 0.14$		$R^2_c = 0.35$		AIC= -1905.7	
	Est.	SE	Imp.	z-value	p-value		Est.	SE	Imp.	z-value	p-value	
Tree mix.	0.04	0.08	0.37	0.51	0.61		-0.0004	0.001	0.61	0.37	0.71	
Litter PC1	0.00	0.01	0.26	0.27	0.79		-0.0003	0.0004	0.30	0.70	0.48	
Litter PC2	0.01	0.02	0.38	0.54	0.59		0.00004	0.0003	0.48	0.15	0.88	
Root PC1	-0.02	0.03	0.60	0.91	0.36		0.0005	0.0005	0.22	0.96	0.34	
Root PC2	-0.08	0.03	0.97	2.42	0.0154	*	-0.001	0.001	0.62	1.56	0.12	
Soil PC1	0.23	0.04	1.00	5.22	2.00E-07	***	-0.002	0.001	0.84	3.78	0.0002	***
Soil PC2	0.00	0.02	0.22	0.07	0.94		0.001	0.001	1.00	0.92	0.36	

955 Table 2. ANOSIM results for the respiration rates of the ‘used’ C sources (defined as at least
 956 15% higher than the respiration rates measured with pure water addition). Explanatory variables
 957 are abbreviated as: 3-species tree mixture stands (Tree mixture), first and second forest floor
 958 litter PCA axes (Litter PC1 and Litter PC2), first and second absorptive root PCA axes (Litter
 959 PC1 and Litter PC2), and first and second soil parameters axes (Soil PC1 and Soil PC2). P-
 960 values are coded as such: $p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$.

	Df	Sums of sqs	Mean sqs	F model	R ²	Pr(>F)	
Tree mixture	1	0.25	0.25	2.28	0.03	0.098	.
Litter PC1	1	0.03	0.03	0.26	3.49E-03	0.96	
Litter PC2	1	0.17	0.17	1.50	0.02	0.67	
Root PC1	1	0.15	0.15	1.35	0.02	0.69	
Root PC2	1	0.39	0.39	3.54	0.05	0.04	*
Soil PC1	1	0.61	0.61	5.51	0.07	0.01	*
Soil PC2	1	0.64	0.64	5.80	0.08	1.60E-03	**
Residuals	55	6.07	0.11		0.73		
Total	62	8.30			1.00		

961

962 **Figures:**

963

964 Figure 1. Principal component analyses (PCA) ordination of **a.** soil parameters, **b.** forest floor
965 characteristics, and **c.** absorptive root traits. Soil parameters (for the first 10 cm of the A
966 horizon): BD= bulk density (g cm^{-3}), C= carbon content (mg g^{-1} soil), Clay= clay content (%),
967 C:N= carbon to nitrogen ratio, pH= soil pH. Forest floor characteristics: C:N ratio = carbon to
968 nitrogen ratio, Lignin = lignin concentration (g kg^{-1} dry litter), Mass = litter mass (kg m^{-2}),
969 Phosphorous = phosphorous concentration (%), Total phenolics = total phenolic concentration
970 (mg g^{-1} dry litter), Condensed tannins = condensed tannin concentration (%). Absorptive root
971 traits: Diameter = root diameter (mm), Ectomycorrhizal colonization = ectomycorrhizal
972 colonization intensity (number cm^{-1}), RLD = root length density (cm cm^{-3}), RTD = root tissue
973 density (g cm^{-3}), SRL = specific root length (m g^{-1}), Surface area = root surface area (cm^2).
974 Triangles and circles indicate tree triplets. PCA loadings can be found in Table S4.

Figure 2. Variations between soil from mono-specific tree stands (gray) or mixed tree stands (green) for microbial biomass ($\mu\text{g C}_{\text{mic}} \text{ g}^{-1} \text{ dry soil}$), denitrification potential (DEA; $\mu\text{g N-N}_2\text{O g}^{-1} \text{ dry soil h}^{-1}$), sum of the microbial catabolic respiration induced by 15 different C-substrates (Sum15; $\mu\text{g C-CO}_2 \text{ g}^{-1} \text{ dry soil h}^{-1}$), and Shannon metabolic diversity index (H'). Marginal R^2 (R^2_m) and conditional (R^2_c) values are from GLM results. There were no statistically significant differences between mono-specific and mixed stand plots (indicated by the same letter “a”). Data presented by site and by forest stand type can be found in Figure S4.

983
 984 Figure 3. Average 'used' C substrate respiration rates ($\mu\text{g C-CO}_2 \text{ g}^{-1} \text{ dry soil h}^{-1}$; defined as at
 985 least 15% higher than the respiration rates measured with pure water addition) of the fifteen
 986 substrates belonging to five substrate groups for soil from mono-specific tree stands (black) or
 987 mixed tree stands (green). Abbreviations: D-glucose (GLU), xylan (XYL), cellulose (CELL),
 988 L-asparagine (ASP), L-serine (SER), L-lysine (LYS), L-glycine (GLY), L-glutamine (GLUT),
 989 N-acetylglucosamine (N-AC), oxalic acid (OX), uric acid (UR), malic acid (MAL), caffeic acid
 990 (CAF), syringic acid (SYR), and vanillic acid (VAN). ANOSIM results showed a marginally
 991 significant difference in substrate utilization pattern between mono-specific and mixed stands
 992 (Table 2). Data presented by site and by forest stand type can be found in Figure S5.

(A) Microbial biomass:

(B) DEA:

Figure 4. Structural equation models (SEM) quantifying the relative importance of the directional causal relationships between 3-species tree mixtures (Tree mixture), forest floor litter characteristics (Litter PC1 and PC2), absorptive root traits (Root PC1 and PC2), and soil parameters (Soil PC1 and PC2) on soil microbial functioning: **a.** microbial biomass ($C_{microbial}$; $\mu\text{g } C_{microbial} \text{ g}^{-1} \text{ dry soil}$) and **b.** potential denitrification enzyme activity (DEA; $\mu\text{g N-N}_2\text{O g}^{-1} \text{ dry soil h}^{-1}$). Positive relationships are indicated by blue arrows, negative relationships by red arrows, and non-significant relationships by black arrows. Estimate values are positioned on the corresponding arrow, and p-values are coded as such: $p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$.

(C) Sum15:

(D) Shannon diversity:

Figure 4 Continued. Structural equation models (SEM) quantifying the relative importance of the directional causal relationships between 3-species tree mixtures (Tree mixture), forest floor litter characteristics (Litter PC1 and PC2), absorptive root traits (Root PC1 and PC2), and soil parameters (Soil PC1 and PC2) on soil microbial functioning: **c.** sum of the microbial catabolic respiration induced by 15 different C-sources (Sum15, $\mu\text{g C-CO}_2 \text{ g}^{-1} \text{ dry soil h}^{-1}$) and **d.** Shannon metabolic diversity index (H').

1009 **Supporting information:**

1010 Additional supporting information may be found in the online version of this article.

1011 Figure S1 Field sampling locations.

1012 Table S1 Sampling site information

1013 Table S2 Soil, forest floor litter, and absorptive root analysis results

1014 Table S3 Method and pretreatment used for NIRS calibration

1015 Figure S2 SEM model structure

1016 Table S4 Litter, Root, and Soil PCA Loadings

1017 Table S5 ‘Used’ C SIR GLMM results

1018 Figure S3 PCA country and stand type variance

1019 Figure S4 Microbial variable country and stand type variance

1020 Figure S5 ‘Used’ C country and stand type variance