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

3

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16

17 **Abstract**

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

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

44

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

47

48 **Introduction**

49 Soils harbor a highly diverse community of microorganisms that play a crucial role in many
50 ecological processes and impact terrestrial ecosystem functioning and stability (Bardgett & van
51 der Putten, 2014; Fierer, 2017). Microorganisms control the rate nutrients and carbon are
52 released from dead organic matter and made available to organisms (Crowther et al., 2019) and
53 concurrently release greenhouse gases, notably CO₂ and N₂O, into the atmosphere (Robertson
54 & Groffman, 2007; Singh, Bardgett, Smith, & Reay, 2010). However, soil microbial activity
55 and its subsequent influence on ecosystem functioning are dependent on many extrinsic factors
56 such as soil properties, climatic conditions, and the plant community (Bardgett & Caruso,
57 2020).

58 Different plant species, and thus different plant community assemblages, can exert
59 significant and contrasting effects on soil microbial communities and associated processes
60 (Eisenhauer et al., 2010; Scheibe et al., 2015; Urbanová, Šnajdr, & Baldrian, 2015) through
61 differences in litter quality and diversity (Thoms, Gattinger, Jacob, Thomas, & Gleixner, 2010),
62 rhizodeposition (Steinauer, Chatzinotas, & Eisenhauer, 2016), root symbionts (Baldrian, 2017),
63 above- and belowground litter production, and microclimatic conditions (e.g. temperature and
64 soil humidity) (Prescott & Grayston, 2013). Plant diversity effects can be expressed for
65 example, through disproportional effects (either positive or negative) of specific plant species
66 on soil microorganisms, i.e. the sampling effect hypothesis (Aarssen, 2016; Huston, 1997;
67 Tilman, Lehman, & Thomson, 1997). Species with complementary traits can also lead to non-
68 additive effects on soil microorganism activities, for example, through the combination of
69 varying litter qualities (Barantal, Schimann, Fromin, & Hättenschwiler, 2014; Handa et al.,
70 2014) and/or increased root exudate diversity or rate of diffusion (Cesarz et al., 2013; Jones,
71 Hodge, & Kuzyakov, 2004; Prescott & Grayston, 2013). Consequently, higher plant exudate
72 and litter diversity and/or quantity could potentially support a more diverse (Cesarz et al., 2013;

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

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

85 Plant litter is an important source of organic carbon (C), nitrogen (N), phosphorus (P), and
86 micronutrients for soil microorganisms (Becher, Bernhardt, Fuchs, & Riedel, 2013; Thomas &
87 Packham, 2007). Litter can also provide cations that reduce soil acidification, pH being a key
88 driver in microbial community composition and low pH being linked to slow litter
89 decomposition (Lladó et al., 2017; Pfeiffer et al., 2013). Since many leaf characteristics, e.g.
90 lignin and polyphenol concentrations, persist after senescence, plant leaf economic strategies
91 (LES, Wright et al., 2004) can dictate species litter effects on decomposition rates and release
92 of resources into the soil (Coq, Souquet, Meudec, Cheynier, & Hättenschwiler, 2010; Cornwell
93 et al., 2008; Prescott, 2005). For example, conservative leaf traits, associated with higher energy
94 investment in resource conservation and resource immobilization in long-living plant tissues,
95 could negatively affect microbial activity by producing defense compounds (lignin or
96 polyphenols) that can inhibit microorganisms (Freschet, Aerts, & Cornelissen, 2012; Prescott,
97 2005). Plants with acquisitive traits, associated with higher investment in growth and resource

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

102 Living roots influence soil microbial activity by altering soil physical structure, water flow,
103 and exudation of ions and organic compounds (McCormack et al., 2015; Prescott & Grayston,
104 2013). Similar to the leaf economics spectrum, accumulating evidence from root research
105 suggests a universal root economics spectrum (RES) (Bergmann et al., 2020; Roumet et al.,
106 2016). Generally, it seems that higher specific root length (SRL) and nitrogen (N) concentration
107 combined with lower root diameter and tissue density are associated with an acquisitive root
108 resource strategy, while the inverse is associated with a conservative strategy (Kong et al., 2019;
109 Weemstra et al., 2016). In herbaceous species, acquisitive plant species have been found to
110 produce higher amounts of root exudates than conservative species (Guyonnet, Cantarel,
111 Simon, & Haichar, 2018; Henneron, Cros, Picon-Cochard, Rahimian, & Fontaine, 2019), which
112 can lead to higher soil microbial biomass and activity (Kaštovská, Edwards, Picek, &
113 Šantrůčková, 2015). However, the distinction between acquisitive and conservative root
114 strategies and associated traits is presently less clear for woody species (Weemstra et al., 2016),
115 and their effects on the microbial community is not well understood. This is likely due to a
116 combination of spatial and temporal variability in fine-root traits (e.g. season, age, and soil
117 depth), as well as mycorrhizal colonization. The organic matter provided to the soil microbial
118 community in the form of root litter may also stimulate soil microbial activity more efficiently
119 than aboveground litter (Freschet et al., 2013; Hatton et al., 2015; Jackson, Mooney, & Schulze,
120 1997) because of tight spatial proximity.

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

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

135

136 **Materials and Methods**

137 **Study sites and sampling protocol:** The studied sites are part of a permanent network of
138 mature forest plots established in 2011 and 2012 (detailed site descriptions can be found in
139 Baeten et al., 2013) across Europe: Colline Metallifere (Italy), Râșca (Romania), Białowieża
140 (Poland), and North Karelia (Finland). These forests represent four major European forest types
141 including Mediterranean thermophilous, montane mixed beech, hemiboreal mixed
142 broadleaved-coniferous, and boreal forests (see Table S1 and Figure S1 in Supporting
143 Information). The forests are managed to variable degrees, but species composition is mostly
144 influenced by selective logging, not planting, of naturally established trees. Within each site,
145 30 m × 30 m forest plots were selected that had stands composed of either one dominant tree
146 species (mono-specific plot) or three co-dominating tree species (mixed plot); species were
147 defined as co-dominant when it composed >15% of the stand. Tree species identity and mixed

148 plot tree species combinations varied among sites. Each tree species at each site had two
149 replicate mono-specific plots (with the exception of *Picea abies* L. and *Quercus robur* L. plots
150 with one replicate each and *Betula pendula* Roth with no mono-specific plot in Białowieża,
151 Poland). Mixed plots had a minimum of three replicates per site, but the tree species
152 compositions of these replicates included any three target species present at that site, i.e. mixed
153 plot replicates were not necessarily composed of the same tree species. The final selection of
154 64 plots included 13 tree species and 34 different species combinations, as well as a wide range
155 of soil types (Table S1).

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

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

179 **Soil measurements:** The soil bulk density, carbon (C) concentration, carbon:nitrogen
180 (C:N) ratio, and pH were measured during the FunDivEUROPE project in 2012 (Baeten et al.,
181 2013; Dawud et al., 2016). For the FunDivEUROPE soil sampling methods see (Dawud et al.,
182 2016). Another set of unground soil subsamples (10 g per sample) were used to determine soil
183 texture. The soil was pre-treated for organic and carbonate removal (ISRIC & FAO, 2002) if
184 present, the texture was then measured by laser granulometry (Malvern Mastersizer S, Malvern
185 Instruments Limited, Worcestershire, United Kingdom). Soil data are provided in Table S2.

186 **Forest floor litter characterization:** Each subplot litter sample (five per plot) was dried at
187 60°C, weighed, and the weight was divided by the surface area sampled to be expressed as kg
188 dry weight litter per square meter of soil surface. After weighing, the entire litter sample was
189 ground to approximately 2 mm (Retsch cutting mill SM1, Haan, Germany) for homogenization
190 and then a subsample of litter was ground to 1 mm (Cyclotec 1093 cyclone grinder, Tecator,
191 Höganäs, Sweden) for chemical analyses.

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

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

198 characterization of each individual sample. We first determined the NIR spectrum of each of
199 the 320 samples using Fourier-transformed infrared spectroscopy (FTIR) with a NIRFlex N-
200 500 spectrometer (BUCHI Corporation, New Castle, DE, United States). The litter samples
201 were scanned in a cuvette ($W \times D \times H$: $12.5 \times 12.5 \times 45$ mm) with a spectral range from 1000
202 nm – 2500 nm and spectral resolution of 8 cm^{-1} for 16 scans. Each sample was scanned twice,
203 and the two spectra were averaged. Based on the bulk of obtained spectra, a selection program
204 (NIRWare Management Console, BUCHI Corporation) identified the required number and
205 spread of a subset of samples on which a calibration data base was constructed using the
206 NIRWare NIRCal software (BUCHI Corporation). A total of 87 spectra out of the 320 were
207 selected to accurately represent the sample spectra distribution. The P, lignin, condensed
208 tannins, and total phenolics concentrations were then measured for these 87 samples, which
209 were subsequently used to predict the values for the remaining 233 samples based on their
210 individual near infrared spectra (NIRWare NIRCal software). The program tested multiple
211 methods as well as multiple transformations to obtain the best regression coefficient, using two-
212 thirds of the spectra for calibration and one-third for validation. The calibration methods,
213 transformations, and calibration and validation results are detailed in Table S3; the r^2 values for
214 all litter quality parameters were all larger than 0.76.

215 The P concentration was measured colorimetrically using the molybdate blue method
216 (Grimshaw, Allen, & Parkinson, 1989). First, 84.0 ± 4.0 mg of litter was mineralized by adding
217 8 ml of HNO_3 (2.24 mol L^{-1}) and, over 10 min, heated to 120°C , then over 20 minutes, heated
218 to 175°C and kept at this temperature for 10 min in an ETHOS One microwave (Milestone, Via
219 Fatebenefratelli 1/5-24010 Sorisol, Italy). Once the sample cooled, 100 μl was deposited in
220 each well of a 96-DeepWell Microplate (Fisher Scientific E39199) and 100 μl NaOH (2 mol L^{-1})
221 1), 50 μl sodium molybdate (7 g L^{-1}), and 50 μl ascorbic acid (10 g L^{-1}) was added in that order.

222 The plate was incubated at 40°C for 30 minutes then the optic density was read (wavelength
223 720 nm) with a Victor 1420 Multilabel Counter (PerkinElmer, Massachusetts, USA).

224 Cellulose, hemicellulose, and lignin fractions were measured with the FIBERSAC[®] method
225 12 (Fibersac 24, Ankom, Macedon, NJ, USA; Ankom Technology, 2017) adapted from Van
226 Soest (1963). Following this protocol, plant tissue constituents were extracted and measured
227 gravimetrically by sequentially exposing 510.0 ± 10.0 mg dry weight of the litter sample to
228 neutral detergent (NDS), acid detergent (ADS), and H₂SO₄ (72%).

229 The concentration of condensed tannins was measured by spectrophotometry with the
230 butanol-HCl method (Porter, Hrstich, & Chan, 1985; Waterman & Mole, 1994) as described in
231 detail by Coq et al. (2010). Total phenolic concentration was measured colorimetrically, using
232 the method described by Ribéreau-Graydon (1972) and using the Hach TanniVer[™] reagent
233 (Hach Company, Loveland, CO, USA), according to the detailed description in Coq et al.
234 (2010). Extractions were diluted when necessary. Forest floor litter data are provided in Table
235 S2.

236 **Absorptive root traits:** Roots were sorted from the soil cores and all fine roots (< 2 mm in
237 diameter) were subsequently classified as absorptive (the first three root orders) or transport
238 roots (4th and 5th order roots) according to the functional classification approach by McCormack
239 et al. (2015). On average, absorptive roots of the target species made up 53.5 ± 2.4% of all fine
240 roots (absorptive and transport roots combined). For further details on the root sorting and
241 measurement methods see (Wambsganss, Beyer, Freschet, Scherer-Lorenzen, & Bauhus,
242 2021). This order-based approach was used, as opposed to the still commonly applied
243 traditional diameter classification, because studies have shown that the first three most distal
244 root orders (i.e. the absorptive roots according to McCormack et al. (2015)) significantly differ
245 in their functions from higher order roots. The absorptive roots are responsible for most of the
246 resource uptake (absorption), and thus exudation, and are therefore more relevant in affecting

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

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

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

266 The MicroRespTM method described by Shihan et al. (2017) was used to determine the
267 catabolic diversity of soil microorganisms, based on the ability of the soil microbial community
268 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
269 different C sources as described in detail by Shihan et al. (2017). Three technical replicates
270 were run per substrate with approximately 0.39 g of soil dry weight per replicate. The substrate
271 addition equated to 1.5 mg of C per g dry weight of soil. The SIR rates of all 15 substrates were

272 summed to obtain a global catabolic respiration value (Sum15). The Shannon metabolic
273 diversity index for each subplot was calculated using the formula: $H' = -\sum_{i=1}^{15} p_i \times \ln(p_i)$
274 where p_i is the standardized respiration rate for the substrate 'i', i.e. the respiration rate of
275 substrate 'i' divided by the Sum15 value. For the respiration rate of each of the 15 C sources,
276 a substrate was considered 'used' by the microbial community when the respiration rate was
277 15% higher relative to the respiration without the addition of a C substrate (i.e. just water), all
278 respiration rates below this threshold were considered not-used and were replaced by zeros for
279 the ANOSIM and GLMM analyses (see below).

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

285 **Statistical Analysis:** The R software (R Development Core Team, 2008) (version 3.5.3)
286 was used for all figures and statistical analyses, figures were made using the 'pirateplot'
287 function from the YaRrr! Package (version 0.1.5, Phillips, 2018), the function 'fviz_pca_biplot'
288 from the factoextra package (version 1.0.6, Kassambara & Mundt, 2019), and the function
289 'radarchart' in the fmsb package (version 0.7.0, Nakazawa, 2019). The QGIS software (version
290 3.12.3) was used to create the sampling locations map (Figure S1) with a basemap from
291 www.naturalearthdata.com.

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

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

298 Generalized mixed-effects linear models were run, using the lme4 package (version 1.1-21;
299 Bates et al., 2019), on each response variable (C_{mic} , Sum15, H' , and DEA) testing the effect of
300 the explanatory variables (Litter PC1, Litter PC2, Root PC1, Root PC2, Sol PC1, Soil PC2, and
301 tree mixture). Response variables were transformed (\log_2) when necessary, and extreme values
302 ($> \pm 3$ times the interquartile range) were removed (the number of removed values never
303 exceeded 10% of the total number of values). The site, which takes into account all associated
304 site-specific differences such as climatic variables, and plot were included as random variables.
305 The model structure was as follows: response variable \sim Litter PC1 + Litter PC2 + Root PC1 +
306 Root PC2 + Soil PC1 + Soil PC2 + Tree species number + (1|Site/Plot). In order to identify the
307 most parsimonious models and the most consistent predictors we used a model averaging
308 approach via the 'dredge' and 'model.avg' functions in the MuMIn package (Bartoń, 2019)
309 which uses the lowest Akaike Information Criteria (AIC) to rank all possible models with all
310 possible combinations of the explanatory variables in the full model. A 95% confidence set was
311 used to select a subset of the models to be averaged, i.e. average of the estimates, calculated
312 using the zero method (Burnham & Anderson, 2002), with the standard error, importance value,
313 z-value, and p-value. The importance value is calculated by summing the model weights of the
314 models where the variable appears.

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

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

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

346

347 **Results**

348 Soil parameters, forest floor litter traits, and traits of absorptive root varied widely among
349 the four studied forests across Europe (Fig. 1). The first axis of the soil parameter PCA (Soil
350 PC1) accounted for 52.5% of the variance and was mostly determined by soil physicochemical
351 properties (Fig. 1a). High bulk density was associated with negative PC scores, and high pH,
352 C, and clay concentrations were associated with positive scores. The second axis (Soil PC2)
353 explained 24.9% of variance, with negative PC scores correlated with low C:N ratio. The first
354 axis of the forest floor litter trait PCA (Litter PC1) accounted for 50.2% of the variance, with
355 negative PC scores associated to high concentrations of phenolic compounds and positive
356 scores to high concentrations of lignin (Fig. 1b). The second axis (Litter PC2) accounted for
357 23.7% of the variance, with negative PC scores associated with high C:N ratio and low P and
358 positive PC scores to low C:N ratio and high P. The first axis of the PCA on traits of absorptive
359 roots (Root PC1) accounted for 42.2% of the variance, with negative scores associated with
360 high SRL, RLD, and surface area and positive scores associated with high diameter and RTD
361 (Fig. 1c). The second axis (Root PC2) captured 26.9% of the variance and was mainly related
362 to the root surface area (negative scores) and ECM colonization intensity (positive scores; Fig.
363 1c).

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

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

372 Across all 320 collected soil samples, we measured an average C microbial biomass (C_{mic})
373 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 μg
374 $C_{mic} \text{ g}^{-1}$ dry soil (Fig. 2a). The average denitrification enzyme activity (DEA) was 0.01 ± 0.014
375 $\mu\text{g N-N}_2\text{O g}^{-1}$ 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).
376 The average sum of the microbial catabolic respiration rate induced by 15 different C-sources
377 (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 $\mu\text{g C-}$
378 $\text{CO}_2 \text{ g}^{-1}$ dry soil h^{-1} (Fig. 2c). We measured an average Shannon metabolic diversity index (H')
379 of 2.69 ± 0.014 (unitless), with a range between 2.64 and 2.71 (Fig. 2d). Across the 15 substrates
380 included, the average 'used' C substrate respiration rate was $2.51 \pm 3.51 \text{ C-CO}_2 \text{ g}^{-1}$ dry soil h^{-1}
381 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
382 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,
383 DEA, sum15, H' , and substrate use) were generally comparable between sites and had similar
384 patterns between mono-specific and mixed plots, except for higher DEA rates in mixed
385 compared to monospecific stands in Finland and Romania (Supplementary Figs 4).

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

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

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

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

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

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

441

442 **Discussion**

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

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

449 These results from natural forest stands of varying site conditions differ from those obtained
450 in synthetic single site experiments. For example, the long-term Jena experiment, manipulating
451 herbaceous species diversity, showed a positive correlation between species richness
452 (Eisenhauer et al., 2010; Lange et al., 2015) or root exudate diversity (Steinauer et al., 2016)
453 and soil microbial biomass and activity. Similarly, the findings from a young tree plantation
454 experiment support enhanced soil microbial biomass and activity in soil from communities with
455 higher tree species richness (Khlifa, Paquette, Messier, Reich, & Munson, 2017). Compared to
456 these single site experiments, our four study sites from different climate zones and with mature
457 trees and distinct soil properties introduced more variation in a range of factors, making the
458 detection of species mixing effects more difficult. However, in a recent meta-analysis covering
459 a wide range of species (herbaceous and woody), habitat types (natural, artificial/planted, in
460 container, forest, grassland, and cropland), successional stages, and climate zones, Chen et al.
461 (2019) reported an overall higher soil microbial biomass and activity with increasing plant
462 species richness. The studies they considered in their meta-analysis covered a much wider
463 diversity gradient (from one up to 60 species combinations) and increasing plant species
464 richness was the main driver of the observed generally positive biodiversity effects on soil
465 microbial parameters. It seems therefore likely that the comparatively small difference from
466 one to three tree species in the forests we studied did not allow the detection of a more general
467 species richness effect potentially expressed at wider species richness gradients. Species
468 identity effects may further outweigh mixture effects on soil microbial community composition
469 and/or functioning (L. Chen et al., 2019; Dijkstra, West, Hobbie, & Reich, 2009; Scheibe et al.,

470 2015), particularly in a design with a low diversity gradient such as ours. However, since we
471 did not have the same species at the four sites, we could not introduce species identity as a co-
472 variable in our statistical models, which is an unavoidable trade-off when working on natural
473 forests and may have contributed to obscuring direct diversity effects.

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

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

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

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

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

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

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

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

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

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

579

580 **Conclusion**

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

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

595

596 **Author contributions**

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

601

602 **Competing Interests statement**

603 The author declares no competing interests.

604

605 **Data availability**

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

610

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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 ($\mu\text{g C}_{\text{microbial}} \text{g}^{-1}$ dry soil), potential denitrification enzyme activity
 947 (DEA; $\mu\text{g N-N}_2\text{O g}^{-1}$ dry soil h^{-1}), sum of the microbial catabolic respiration induced by 15
 948 different C-sources (Sum15; $\mu\text{g C-CO}_2 \text{g}^{-1}$ 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

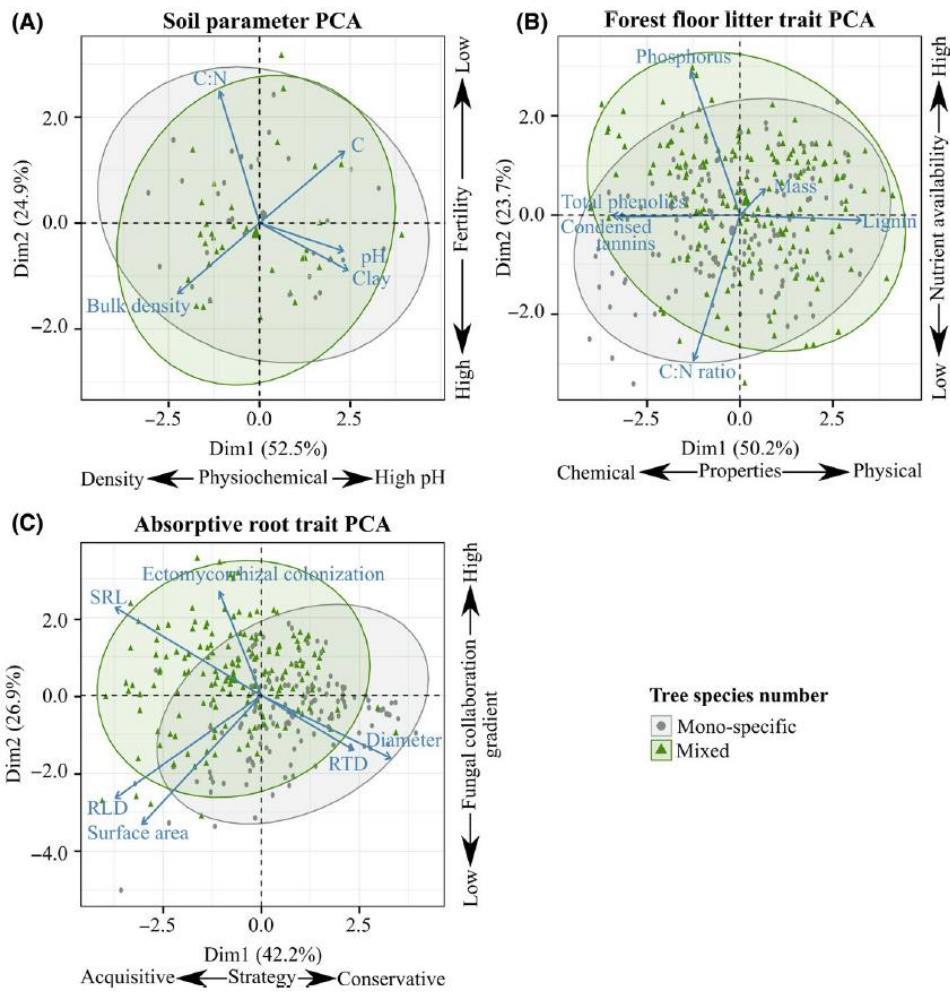
954

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		

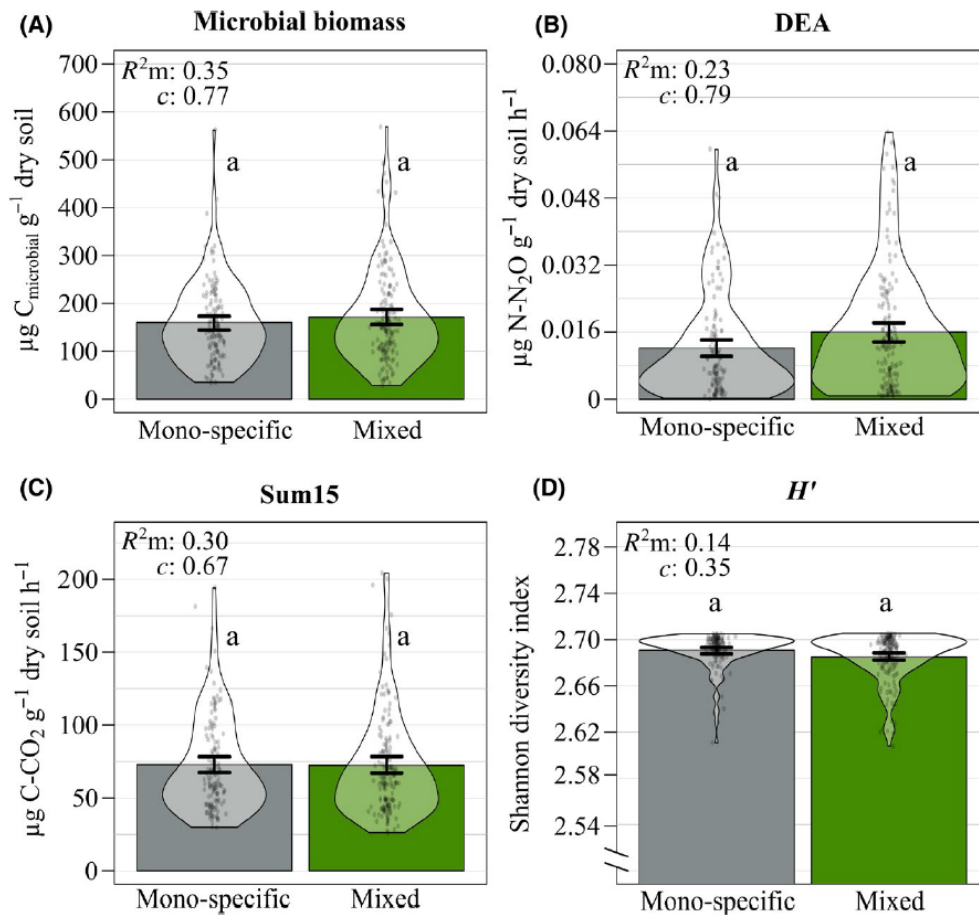
961

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.



975

976 Figure 2. Variations between soil from mono-specific tree stands (gray) or mixed tree stands

977 (green) for microbial biomass ($\mu\text{g C}_{\text{mic}} \text{g}^{-1} \text{dry soil}$), denitrification potential (DEA; $\mu\text{g N-N}_2\text{O}$

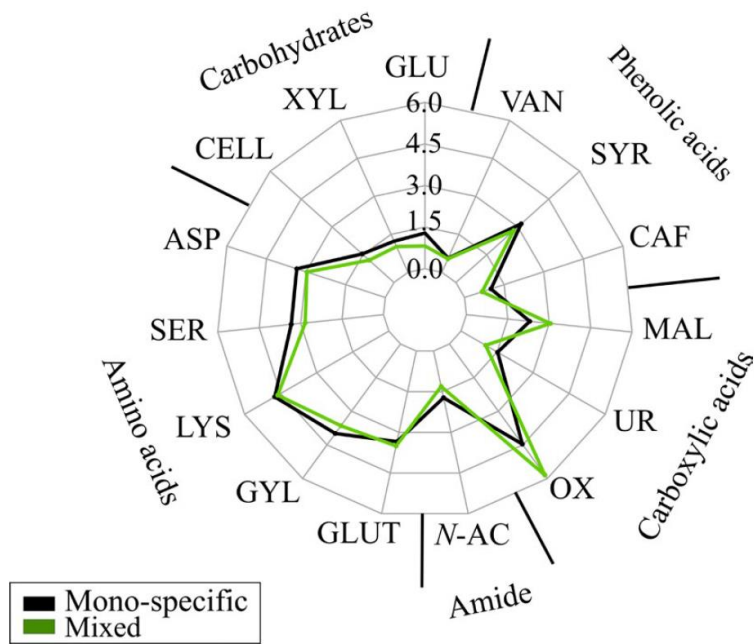
978 $\text{g}^{-1} \text{dry soil h}^{-1}$), sum of the microbial catabolic respiration induced by 15 different C-substrates

979 (Sum15; $\mu\text{g C-CO}_2 \text{g}^{-1} \text{dry soil h}^{-1}$), and Shannon metabolic diversity index (H'). Marginal R^2

980 (R^2_m) and conditional (R^2_c) values are from GLM results. There were no statistically significant

981 differences between mono-specific and mixed stand plots (indicated by the same letter "a").

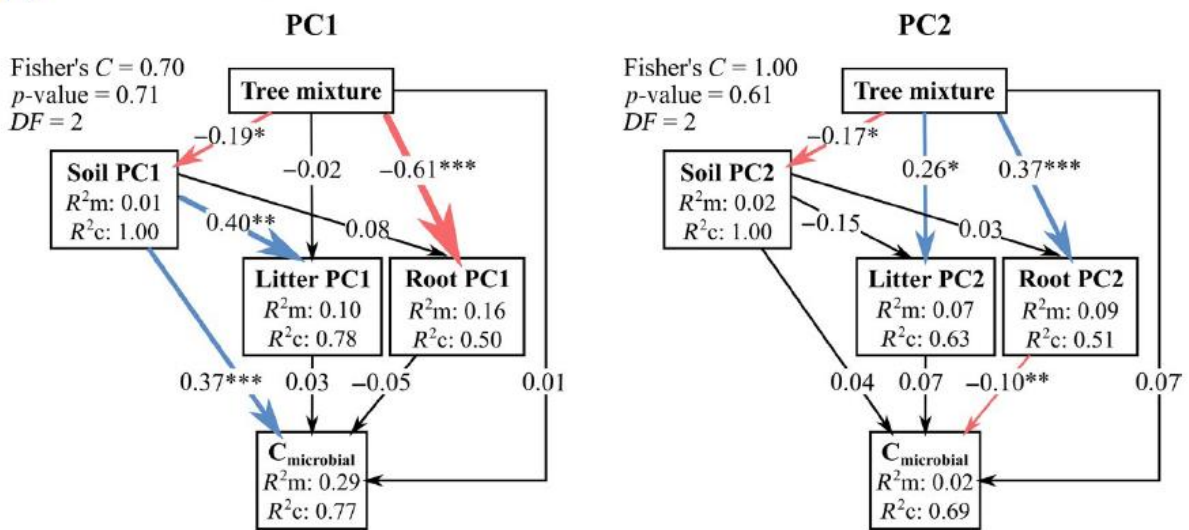
982 Data presented by site and by forest stand type can be found in Figure S4.



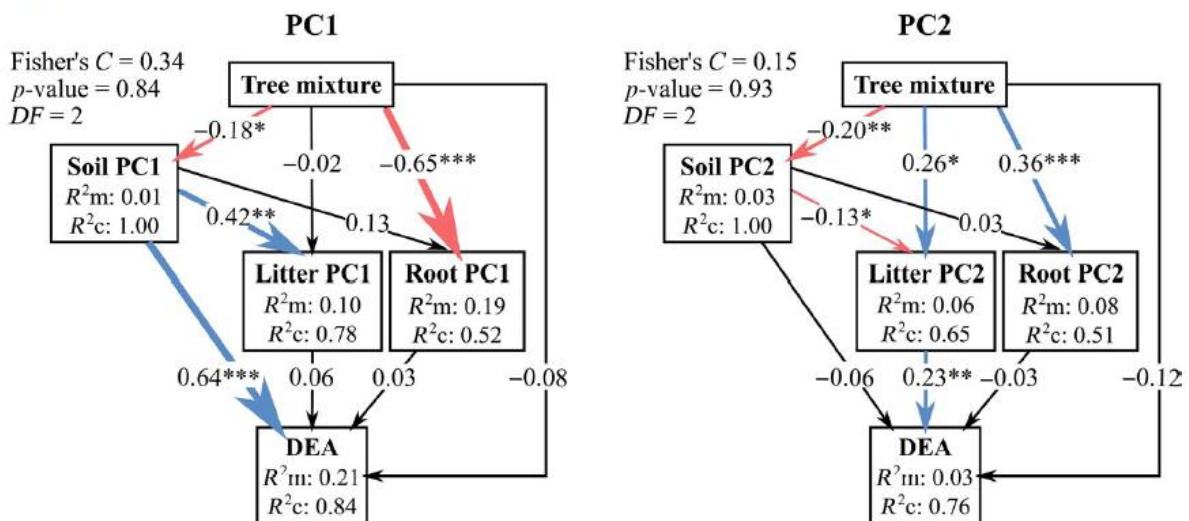
983

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:



993

994 Figure 4. Structural equation models (SEM) quantifying the relative importance of the

995 directional causal relationships between 3-species tree mixtures (Tree mixture), forest floor

996 litter characteristics (Litter PC1 and PC2), absorptive root traits (Root PC1 and PC2), and soil

997 parameters (Soil PC1 and PC2) on soil microbial functioning: **a.** microbial biomass ($C_{microbial}$;

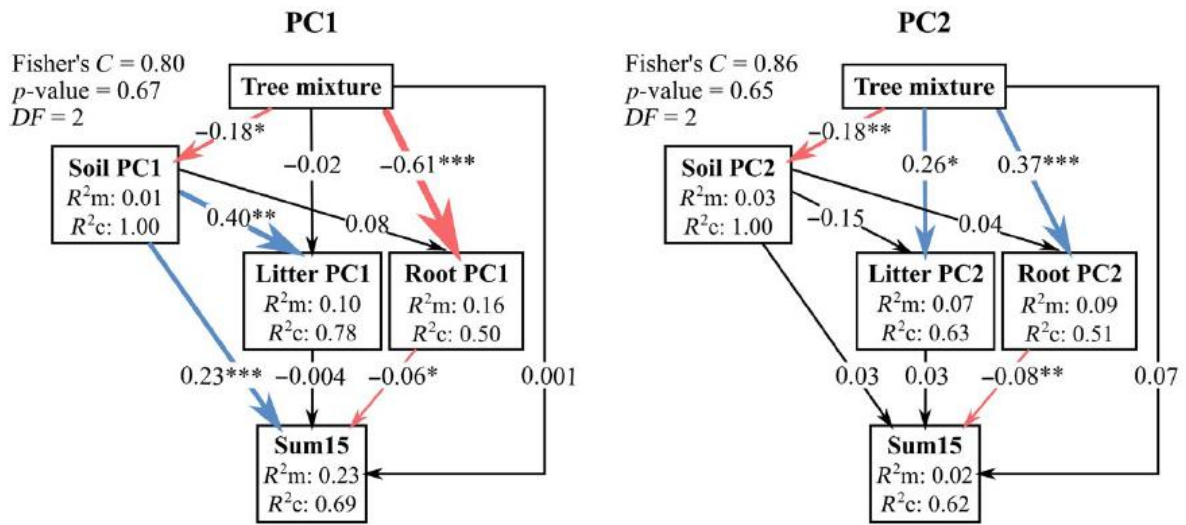
998 $\mu\text{g } C_{microbial} \text{ g}^{-1} \text{ dry soil}$) and **b.** potential denitrification enzyme activity (DEA; $\mu\text{g N-N}_2\text{O g}^{-1}$

999 dry soil h^{-1}). Positive relationships are indicated by blue arrows, negative relationships by red

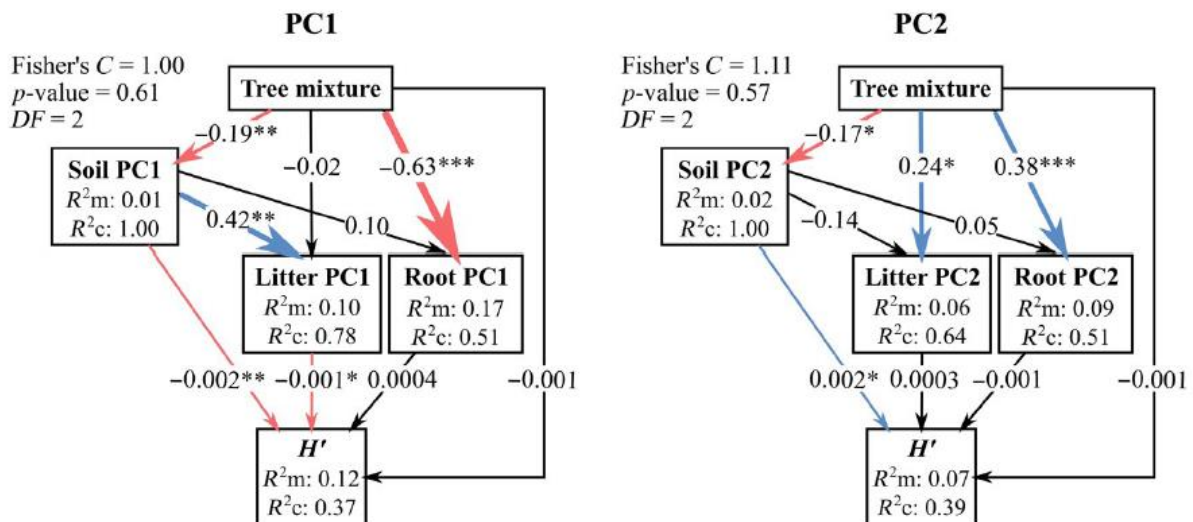
1000 arrows, and non-significant relationships by black arrows. Estimate values are positioned on

1001 the corresponding arrow, and p -values are coded as such: $p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$.

(C) Sum15:



(D) Shannon diversity:



1002

1003 Figure 4 Continued. Structural equation models (SEM) quantifying the relative importance of
 1004 the directional causal relationships between 3-species tree mixtures (Tree mixture), forest floor
 1005 litter characteristics (Litter PC1 and PC2), absorptive root traits (Root PC1 and PC2), and soil
 1006 parameters (Soil PC1 and PC2) on soil microbial functioning: **c.** sum of the microbial catabolic
 1007 respiration induced by 15 different C-sources (Sum15, $\mu\text{g C-CO}_2 \text{ g}^{-1} \text{ dry soil h}^{-1}$) and **d.**
 1008 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