1   **Special issue: Mixed species forests. Risks, resilience and management**

2   **Mixing has limited impacts on the foliar nutrition of European beech and Scots pine trees**

3   **across Europe**

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24

Abstract

26 Tree species-mixing has been suggested as one option to counteract the adverse effects of global change

27 on tree mineral nutrition, yet the effect of mixing on nutrient availability remains poorly documented.

28 We therefore analyzed the current foliar nutrient (N, P, K, Ca, Mg) quantities and *ilr* balances (isometric

29 log transformed ratios between elements or groups of elements) for 261 European beech and 248 Scots

30 pine trees from 15 sites, each consisting of one beech-pine mixed stand and the respective monocultures,

31 across a gradient of environmental conditions in Europe. We hypothesized an overall positive effect of

32 mixing on tree foliar nutrient content, and that this mixing effect would be stronger on nutrient-poor sites.

33 Using linear mixed models and multivariate linear regression models, we first tested for the effects of

34 species (beech/pine) and composition (pure/mixed) across all sites; we then investigated whether the

35 species-mixing effect was related to site fertility.

36 The nutrient composition of beech leaves and pine needles differed significantly for all *ilr* balances. For

37 both species, significant mixing effects were detected for some nutrients and *ilr* balances; those effects,

38 however, could not be consistently related to contrasted nutrient composition between species. For most

39 nutrients and *ilr* balances, the mixing effect was influenced by the site nutritional status, but the pattern

40 differed from expectation: absence or minor differences between monocultures and mixtures at the lower

41 end of the chemical fertility gradient, and maximum differences in rich soils.

42 The contrasting foliar nutrient composition of pine and beech trees and the site nutrient status only partly

43 explained the mixing effects on tree mineral nutrition. Our results claim for a better understanding of

44 nutrient-related mechanisms associated with complementarity and points towards the need to further

45 expand the existing frameworks to account for the multivariate nature of tree nutrition.

46

49

1. Introduction

Many recent studies underline that tree mineral nutrition is deteriorating in Europe under global environmental changes (Jonard et al. 2015, Peñuelas et al. 2013). For instance, negative trend in foliar P concentration were found for *Fagus sylvatica* (L.) and *Pinus sylvestris* (L.) along with negative Ca and Mg trends in the case of the first species and negative S trend for the latter one (Jonard et al. 2015). While concentrations of some elements are declining, N foliar concentrations tend to increase for several species (Jonard et al. 2015). Such opposite trends induce foliar imbalances which, in turn, may impact the ability of forests to provide ecosystem services such as biomass production (Oren and Schulze 1989). In that context, species-mixing could be an efficient management tool to improve stand nutrition through the potential positive effects of interspecies interactions on the availability, uptake or use efficiency of nutrients (Ammer 2019, Forrester and Bauhus 2016, Nickmans et al. 2015, Rothe and Binkley 2001).

Nutrient *availability* is influenced by many processes. Richards et al. (2010) reported several ways by which tree species mixture may improve nutrient supply, including enhanced mineralisation, reduced loss of nutrient through leaching and erosion, increased rate of N_2 fixation or weathering, and increased quality, quantity and decomposition rates of the leaf litter. Species-mixing effects on nutrient *uptake* are often associated with differences in physiological, morphological or phenological characteristics between the tree species involved in the mixture (Forrester and Bauhus 2016). An example is fine root overyielding in tree mixtures resulting from belowground niche differentiation for species with contrasting rooting traits

68　(Leuschner et al. 2001). Through improvement of soil resources, species-mixing could also induce a

69　reallocation of carbon to aboveground biomass, resulting in a higher *nutrient use efficiency* compared to

70　pure stands (Epron et al. 2013, Forrester et al. 2006). All the processes listed above have to be seen in the

71　light of the biogeochemical niche hypothesis (Urbina et al. 2017). The hypothesis states that, because of

72　differences in growth and nutrient use strategies, co-occuring species use mineral elements in different

73　proportions, which leads to species-specific stoichiometry and associated stoichiometric flexibility. Such

74　differences in biogeochemical niches between coexisting species allows for reduced competition and

75　nutrient use optimization at the community level (Urbina et al. 2017).

76　It is frequently thought that admixing tree species has beneficial impacts on tree nutrition. In temperate

77　forests, those effects have mostly been studied for mixed stands of coniferous and broadleaved trees

78　(Brown 1992, Rothe and Binkley 2001, Thelin et al. 2002). Such expected impacts result from differences

79　in biogeochemical cycle, nutrient demand and nutrient use between conifers and broadleaves. For

80　instance, nitrogen mineralization and nitrification fluxes are generally lower in coniferous than in

81　broadleaves stands, an effect commonly attributed to more acidic conditions, higher C:N ratio of organic

82　matter, and higher amounts of inhibiting and/or recalcitrant compounds under conifers (Augusto et al.

83　2015). The same mechanisms could also hold true for other nutrients such as P and Ca because their

84　availability is partly related to organic matter mineralization. Additional processes involve distinct impacts

85　of coniferous vs. broadleaved trees on atmospheric deposition and mineral weathering (Augusto et al.

86　2015). Differences in nutrient demand and nutrient use between coniferous and broadleaves species also

87　leave room for beneficial impacts of admixing these species. Such differences include a generally higher

88　nutrient concentration in litter and fine roots for broadleaves, and longer nutrient residence time for

89　conifers (Augusto et al 2015).

90　The impact of admixing coniferous and broadleaf species on tree nutrition has been highlighted in previous

91　studies. For instance, Brandtberg (2001) found that admixing birch species (*Betula pendula* Roth and

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92   *Betula pubescens* Ehrh.) and Norway spruce (*Picea abies* (L.) Karst) increased P and K concentrations of

93   spruce needles (but did not influence N, Ca, Mg or Mn concentrations). Thelin et al. (2002) reported higher

94   P and K concentration in Norway spruce needles when mixed with beech, birch or oak (*Quercus robur* L.

95   and *Quercus petraea* (Matt.) Liebl.). Brown (1992) found that interspecific differences in tissue

96   concentration of N and possibly P, were key factors responsible for contrasting types of mixture effects

97   (positive, compensatory and negative) on Norway spruce when mixed with Scots pine, black alder and

98   sessile oak, respectively. However, other studies failed to find any significant differences in foliar nutrient

99   concentrations between pure and mixed stands of conifers and broadleaved species (Heinsdorf 1997,

100   Magh et al. 2018, Neft and Stangl 1985, Rothe et al. 2003). An explanation for those inconsistent results is

101   the dependency of species-mixing effects to environmental conditions (Ratcliffe et al. 2017). Building on

102   the framework developed by Forrester and Bauhus (2016), effects of mixing on nutrition are expected to

103   increase along a gradient of decreasing nutrient availability provided that species interactions improve the

104   availability, uptake or resource use efficiency of limiting nutrients. In accordance with this pattern, Magh

105   et al. (2018) found that in the absence of any water limitation, there was a facilitative effect of silver-fir on

106   N nutrition of beech in N-limited sites, but an antagonistic effect in N-rich soils. However such a simple

107   framework might not be sufficient to explain the full range of patterns for nutrient related mixing effects

108   along environmental gradients. First, it does not account for non linearity of the relationship between

109   resource availability and mixing effects. For instance, while their focus was on the mixing effect on drought

110   exposure, de Streel et al. (2019) highlighted tipping points along a nearly similar environmental gradient.

111   Second, the above framework does not take into account simultaneous limiting factors. Sardans and

112   Peñuelas (2007), however, showed that species-mixing effects on foliar nutrition could be influenced by

113   site water status through its impact on nutrient uptake.

114   The assessment of tree nutrition is largely based on analyzing single foliar nutrient concentrations or

115   contents (i.e. amount of a nutrient in a given number of leaves/needles), as well as ratios between pairs

116   of elements (e.g. Jonard et al. 2015). This approach yields valuable information about mineral elements

117   that are essential to plant functioning and to their metabolism, and the reported values can easily be

118   compared with thresholds (Mellert and Göttlein 2012). More recently, a complementary method, where

119   nutrients are subjected to isometric log-ratio (*ilr*) transformation (see Materials and methods below), has

120   been proposed to better account for the interactions between nutrients while limiting biases related to

121   using raw compositional data (Egozcue et al. 2003). Since its development, this method was successfully

122   applied in different contexts. For instance, Parent et al. (2013b) used it to analyse the mineral plasticity of

123   cloudberry and Parent et al. (2013c), Modesto et al. (2014) and Hájek et al. (2014) used it to study the

124   nutrition of Mango trees, maize or mosses, respectively. Recently, Collin et al. (2016) used the *ilr* method

125   to study the nutritional characteristics of Sugar Maple (*Acer saccharum* Marsh.) and Red Maple (*Acer*

126   *rubrum* L.) under varying proportions of conifers, while Nowaki et al. (2017) used this approach to assess

127   the response of irrigated tomato crops to P fertilization.

128   The objective of this paper was to assess the impact of mixing broadleaves and conifers on tree nutrition.

129   Focusing on the widespread European beech - Scots pine mixture, we sampled the foliage of 261 beech

130   (*Fagus sylvatica* L.) and 248 pine (*Pinus sylvestris* L.) trees at 15 sites distributed over Europe, each of which

131   contained a triplet of plots including a mixture and the monocultures of each species. We used the *ilr*

132   approach to obtain a synthetic and non redundant characterization of the overall nutritional signature, in

133   complement to the classical nutrient content/concentration approach.

134   We hypothesized that differences in foliar nutritional characteristics between species would result in

135   significant mixing effects on tree nutrition. We further hypothesized that mixing effects would be

136   dependent on site nutritional status.

137   **2. Materials and methods**

138   **2.1.   Study area and site/stand characteristics**
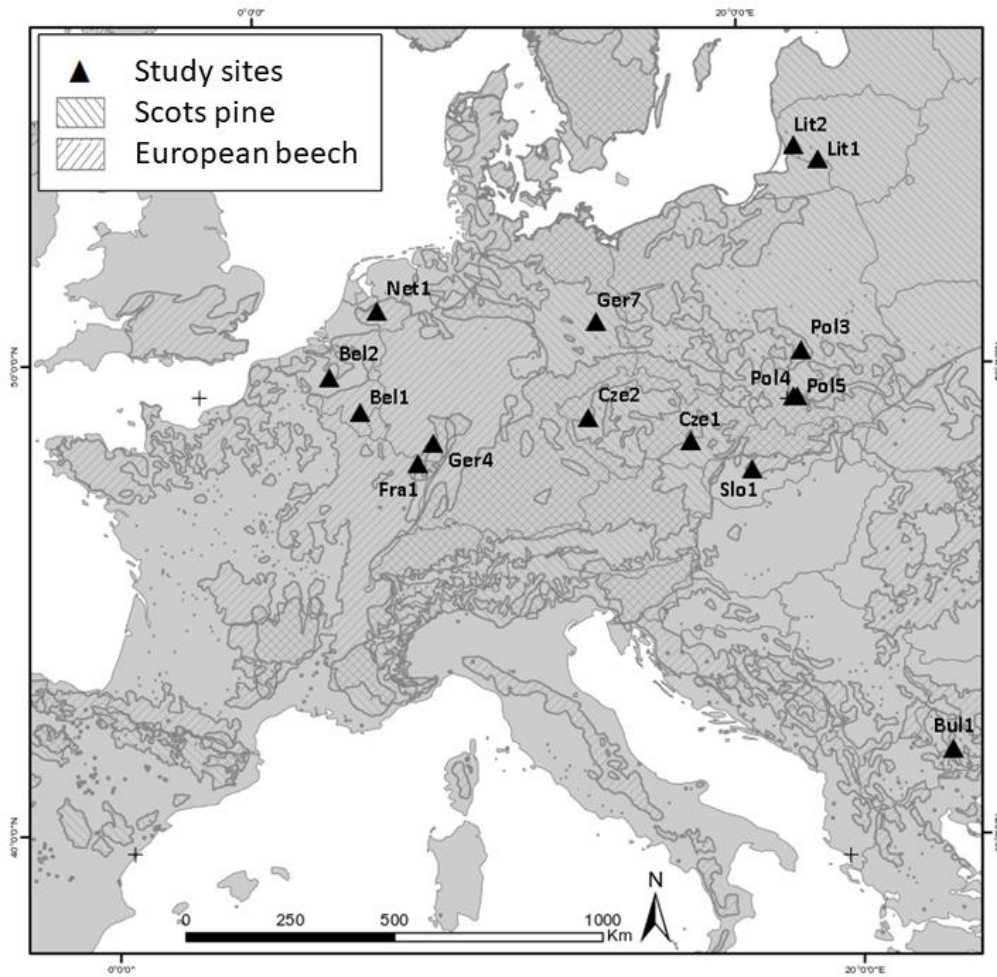
139

140   *Figure 1 Distribution of the 15 EuMIXFOR beech-pine triplets used in the present study and distribution of European beech and*

141   *Scots pine according to EUFORGEN (www.euforgen.org).*

142   This study is based on data from 15 sites each consisting of pure stands of pine and beech and a mixed

143   stand of both species (Figure 1), with all three of them located in similar conditions. The sites have been

144   established under the COST Action FP1206 EuMIXFOR (European Network on Mixed Forests). This network

145   covers a large gradient of environmental conditions and site nutrient status (Figure 2; Tables A1 and A2)

146   within the overlapping natural ranges of pine and beech. Elevations varies between 20 and 1190 m a.s.l;

7

147   mean annual precipitation sum (P) ranges from 520 to 1175 mm and mean annual temperature (T) from

148   6 to 10.5 °C. The stands are mostly even-aged and mono-layered. A summary of stand characteristics is

149   provided in Table A3. In the mixtures, the percentage of basal area represented by scots pine ranged from

150   25 % to 69 %; total basal area ranged from 16 to 79 m^2 ha^{-1} and stand age from 45 to 130 years. Stand size

151   ranged from 0.025 to 0.73 ha for pure beech stands, from 0.025 to 1.55 ha for pure pine stands and from

152   0.045 to 0.462 ha for mixed stands (Table A3). No silvicultural activities had been conducted in the stands

153   during the preceding decade. A standard protocol for tree data collection (diameters, heights of trees and

154   crown bases) and tree coring was applied. The full measurement protocol was described in details by Heym

155   et al. (2017).

156

157   **2.2.   Leaf collection, pretreatment and analysis**

158   Across the 15 sites-network, 509 trees (261 European beech and 248 Scots pine) were sampled. In each

159   site and plot (pure beech, pure pine, mixed beech-pine), 5 to 10 trees were sampled per species (i.e. beech

160   or pine in the pure stands, beech and pine in the mixed stand) among the dominant trees during the

161   summer 2016 (second part of the vegetation period, before autumnal colouring) for a total of 24 to 40

162   sampled trees per site. Current year leaves/needles were collected from several branches located in the

163   upper third of the crown, resulting in one sample per tree. Leaves/needles were dried at 40°C until

164   constant weight was achieved for the nutrient analysis. To determine the dry mass, a subsample was

165   additionally oven-dried at 70°C (50 leaves/500 needles). Foliar N concentrations were measured using the

166   dry combustion method with a Flash Analyzer (Thermo Finnigan Flash EA 1112 elemental analyser) and

167   the other elements (P, K, Ca and Mg) were determined by ICP spectrometry (Varian 720 E-S) after digestion

168   with HNO_3 in a microwave oven (Milestone UltraWAVE, Germany). All analyses were done on a per tree

169   basis.

170

2.3.   Tree nutrition assessment

For each tree, the foliar nutrient composition was characterized by single nutrient (N, P, K, Ca, Mg) concentrations and contents, as well as by isometric log transformed ratios (*ilr*) between elements or groups of elements (Table 1).

Nutrient concentrations and contents. Nutrient concentrations in current-year foliage were first used to rank each site by comparison to species-specific reference thresholds (Mellert and Göttlein 2012). Though such thresholds don't account for nutrient interactions (e.g. Marschner 2011), they are useful as baseline values to assess the nutrient status (e.g. Jonard et al. 2015). For all subsequent analyses, we used nutrient contents instead of nutrient concentrations to account for differences in leaf/needle dry mass resulting from contrasting growing conditions which would impact foliar nutrient concentrations through 'concentration' / 'dilution' effects (Binkley and Fisher 2013, Jarrell and Beverly 1981). The content of each individual nutrient in 50 leaves or 500 needles was calculated by multiplying its concentration by the corresponding leaf or needle dry mass.

Isometric log transformed ratios (ilr). The chemical composition of a plant tissue is a closed system in that all constituting nutrients are constrained to sum to 100%. Data corresponding to quantitative descriptions of the parts of a closed system are defined as "compositional data" (Collin et al. 2016). Among others, the components of such system are not independent. Indeed, if the value of one component increases, the value of at least one other component has to decrease in order to keep the sum constant. Therefore, specific compositional data analyses techniques such as the isometric log-ratio techniques have been developped. Despite its limited use for the assessment of tree foliar nutrition, the ilr transformation technique has been proved useful for analysing plant nutrient composition (Hájek et al. 2014, Modesto et

192   al. 2014, Parent et al. 2013b, Parent et al. 2013c). In addition to constraining the system to 100%, it also

193   accounts for the physiological interactions between nutrients (Collin et al. 2016, Nowaki et al. 2017).

194   The *ilr* transformation technique gives information about the relative amounts of elements or groups of

195   elements, allowing D-1 orthogonal (geometrically independent) balances to be produced for a D part

196   composition. D is the number of measured elements plus a filling value (Fv). The filling value corresponds

197   to the difference between the unit or scale of measurement (e.g. 100%) and the sum of all measured

198   elements (Parent et al. 2013). In this context, a balance, hereafter referred to as *ilr* balance, is defined as

199   the relationship between group of parts (groups composed of one or several nutrients) (Egozcue et al.

200   2005). A closure operation is applied to the resulting matrix of compositional data (Aitchison 1986), which

201   computes the constant sum of components as follows (Egozcue and Pawlowky-Glahn 2005):

202    $$S^D = C(c_1, c_2, \ldots, c_D) = \left[\frac{c_1 k}{\sum_{i=1}^{D} c_i}, \frac{c_2 k}{\sum_{i=1}^{D} c_i}, \ldots, \frac{c_D k}{\sum_{i=1}^{D} c_i} \right]$$ (Eq. 1)

203   Where S^D is a vector of D components adding up to a constant k (e.g. 100%), C is the closure operator, c_i is

204   the ith part of a composition of D components.

205   The *ilr* transformation is then applied to the closed data. This system of lineary independent ratios is called

206   Sequental Binary Partition (SBP) and describes the D-1 orthogonal balances between nutrients or groups

207   of nutrients (Parent et al. 2013). The SBP of a D-elements composition is a (D-1) x D matrix where columns

208   correspond to the parts of the composition and rows to the *ilr* balances. In the SBP, elements labelled "+1"

209   correspond to balances numerators, elements labelled "-1" correspond to balance denominators and

210   elements labelled "0" are not part of the balance in question. This SBP is defined *a priori*, for instance,

211   based on user knowledge. In our case, the SBP (Table 1) is based on prior knowledge of plant nutrition

212   (Collin et al. 2016, Marschner 2011, Parent et al. 2013). The first partition contrasts all measured elements

213   (N, P, K, Ca, Mg) to the filling value (Fv). The second partition contrasts Mg and Ca to K, P and N ([Mg, Ca|K,

214   P,N]). The sub-compositions are then divided into [K|P, N], [P|N] and [Mg|Ca]. [P|N] is representative of

215   the Redfield ratio which is an indicator of the balance between processes associated with the synthesis of

216   N-rich proteins and the synthesis of P-rich r-ARN respectively (Ågren 2004, Loladze and Elser 2011).

217   [Mg|Ca] is an indicator of the geographical position and soil mineralogy (Walworth and Summer 1988).

218   *Table 1 Sequential Binary Partition (SBP) of foliar nutrients of beech and pine trees based on prior knowledge of nutrient*

219   *interaction in higher plants and number of components in the (+) et (-) groups (n⁺ and n⁻, respectively).*

Balance	SBP parts						n^+	n^-
[-1 subset| +1 subset]	N	P	K	Ca	Mg	Fv		
[Fv|N, P, K, Ca, Mg]	1	1	1	1	1	-1	5	1
[Mg, Ca|K, P, N]	1	1	1	-1	-1	0	3	2
[K|P, N]	1	1	-1	0	0	0	2	1
[P|N]	1	-1	0	0	0	0	1	1
[Mg|Ca]	0	0	0	1	-1	0	1	1

220

221   Once the SBP was defined, we used the following equation to calculate the *ilr* balances (Egozcue and

222   Pawlowsky-Glahn 2005):

223    $$ilr_j = \sqrt{\frac{n_j^+ n_j^-}{n_j^+ + n_j^-}} \ln \frac{g(c_j^+)}{g(c_j^-)}$$ (Eq. 2)

224   *Ilr_j* corresponds to the jth isometric log-ratio, n_j^+ and n_j^- are the number of components in the (+) et (-)

225   groups, $g(c_j^+)$ and $g(c_j^-)$ are the geometric means of groups (+) and (-). $\sqrt{(n_j^+ n_j^-)/(n_j^+ + n_j^-)}$ corresponds

226   to the orthogonal coefficient of the jth balance defined in the SBP. *Ilr* balances are conventionally presented

227   in the form [components in denominator|components in numerator] because log ratios become more

228   negative as the denominator increases and hence, the balance leans to the left as in algebra where

229   negative numbers are located on the left side of the zero (Nowaki et al. 2017, Parent et al. 2013b). Thus,

230   an increase in *ilr* balance can be attributed to either a decrease in the left part of the balance or an increase

231   in the right part of the balance. For instance, the [P|N] balance is defined by $\sqrt{\frac{1}{2}} \, ln\frac{N}{P}$. As a consequence,

232   an increase in N or a decrease in P concentration will lead to increased *ilr* balance value and the [P|N]

233   partition leans to the right (Collin et al. 2016, Parent et al. 2013b).

234

235   **2.4.   Statistical analyses**

236   To test the differences in foliar *ilr* balances, foliar nutrient concentration and foliar nutrient content

237   between species, we conducted Student's t-Test. By informing about the biogeochemical niche of the

238   studied species, those analyses are used to interprete the species-mixing effects.

239   In order to test whether stand composition (pure vs. mixed), species identity (beech vs. pine) and their

240   interaction influenced the current *ilr* balance or the foliar nutrient content, we fitted linear mixed models

241   on each *ilr* balance or nutrient content, considering site and stand nested within site as random factors

242

243   $$Y_{hijs} = \beta \times E_{hjs} + a_s(0, \sigma_s^2) + a_{j|s}(0, \sigma_j^2) + \varepsilon(0, \sigma_\varepsilon^2)$$ (Eq. 3)

244

245   Y_{hijs} represents the *ilr* balance/content of interest for the ith individual (tree) in the jth stand (pure or mixed)

246   and the sth site for species h (pine or beech), β is the vector of the fixed effect parameters (species identity,

247   stand composition and their interaction), E is the matrix of the predictors of the fixed effects, a_s is the

248   random factor characterized by the inter-site variance σ^2_s, $a_{j|s}$ is the random factor characterized by the

249   inter-stand variance within a same site σ^2_j and ε is the error term of variance σ^2_ε. In addition, linear mixed

250   effect models using site as random factor were used to test the significance of the difference between

251   pure and mixed stands within each species for each *ilr* balance or nutrient content.

252

253 We then investigated the relationship between the species-mixing effect on tree nutrition, and the site

254 nutritional status. To do so, we computed the difference between the *ilr* balance/nutrient content per tree

255 and the corresponding average value per site, for each species separately, and used this index as the

256 response variable. Stand composition (pure vs. mixed), site nutritional status and their interaction were

257 used as explanatory variables in multivariate linear regression models (eq. 4):

258

259 $$Y_{ijs} - \overline{Y}_s = a + \beta \times E_{js} + \varepsilon(0, \sigma_\varepsilon^2) \hspace{3cm} \text{(Eq. 4)}$$

260

261 Y_{ijs} represents the *ilr* balance/content of interest for the ith individual (tree) in the jth stand (pure or mixed)

262 and the sth site for each species separately, and $\overline{Y}_s$ is the average value of the *ilr* balance/content of either

263 beech or pine trees across the pure and mixed stands in site *s*. α is the intercept, β is the vector of the

264 parameters, E is the predictor matrix (stand composition, site nutritional status and their interaction) and

265 ε is the error term of variance σ_ε^2. Considering that the *ilr* balance [Fv|Mg, Ca, K, P, N] contrasts five mineral

266 elements of major importance for tree growth and functioning against all other elements, we used its

267 average value per site and species as an index of site nutritional status for all *ilr* balance models. For the

268 foliar nutrient content models, we used the average value of the corresponding nutrient content per site

269 and species as the index of site nutritional status. In all cases, the variables used as indicators of site

270 nutritional status were centered. The contrasting average values of either *ilr* balances or nutrient contents

271 between sites resulted in empirical gradients of nutritional status.

272

273 Dependent variables of the mixed and multivariate linear models were normally distributed with the

274 exception of the nutrient content used in the mixed models which were therefore ln-transformed.

275 All statistical analyses were conducted using the R software, version 3.4.1 (R Core Team, 2019).

276   Compositional data analyses were conducted using the "composition" package (Van den Boogaart et al.

277   2019). The closure operation was conducted using the "acomp" function while the *ilr* transformation

278   operation was done on the closed data space with the "ilr" function. Mixed models were fitted with the

279   package "nlme" (Pinheiro et al. 2017).

280

281   **3.   Results**

282   **3.1.   Sites nutrient status**

283   The range of foliar nutrient concentrations within and among sites for beech and pine, along with the

284   corresponding optimum range (Mellert and Göttlein 2012), is presented in Figure 2. For both species and

285   most combinations of sites and nutrients, there was a high variability in foliar concentrations. Also, the

286   studied sites spanned a large range of tree nutrient status (Tables A1 and A2).

287   With the exception of K for pine, all nutrients were found to be deficient for at least one combination of

288   site and species (Table A2). While nutrition appeared to be non limiting for neither beech nor pine in two

289   sites (BUL1, CZE1), some sites showed deficiencies for more than one nutrient. For beech, BEL1 and FRA1

290   were deficient for the same three nutrients (P, Ca, Mg); for pine, two different nutrient deficiencies were

291   observed in FRA1 (P, Mg), LIT2 (N, Ca) and POL3 (N, P). At some sites, the same nutrients appeared to be

292   limiting for both beech and pine (Ca in BEL1; P and Mg in FRA1). Two sites (GER7 and LIT1) displayed no

293   deficiencies for beech while deficiencies were observed for pine (P and N limitation in GER7; N limitation

294   in LIT1).

14
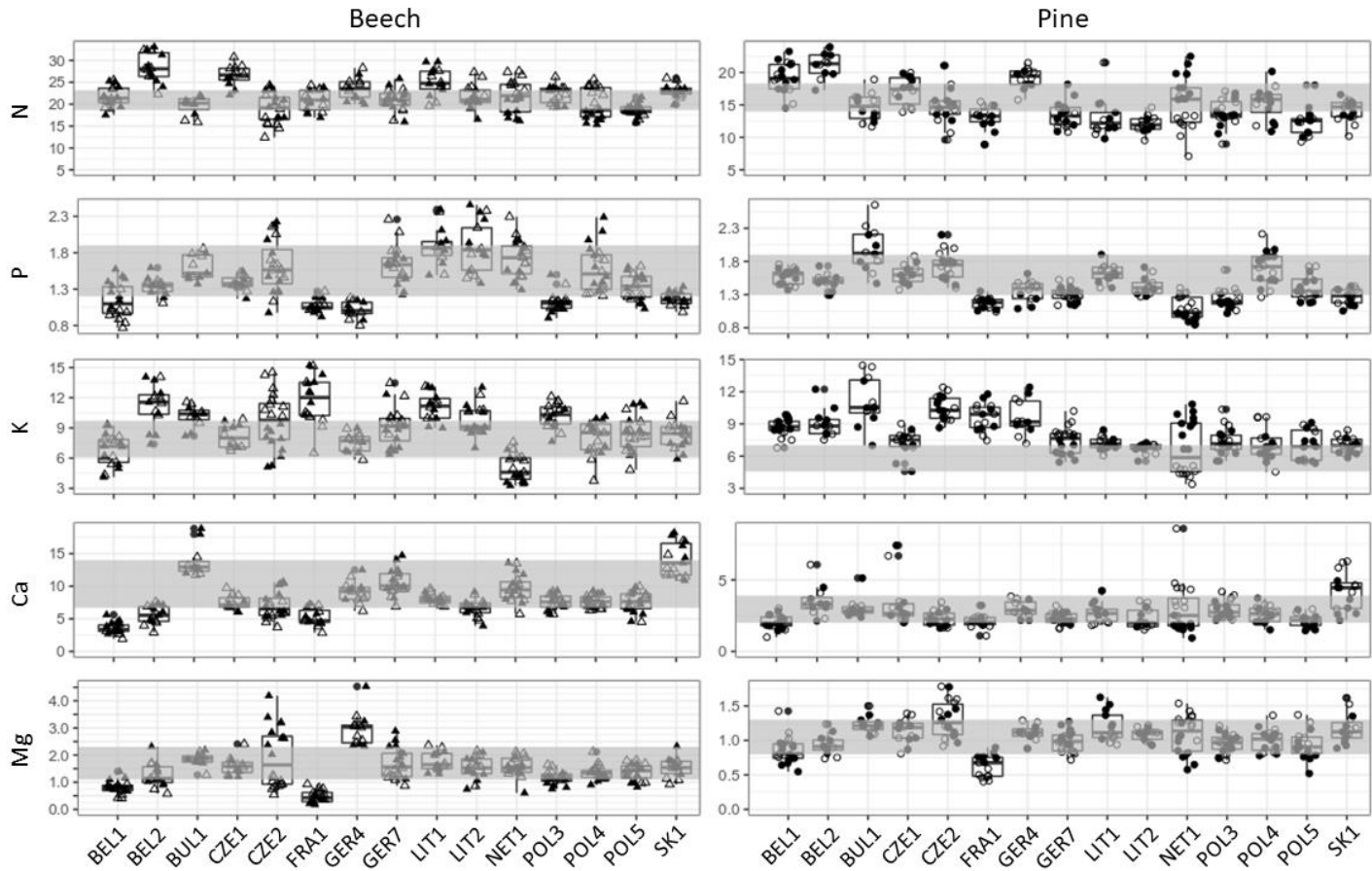
Figure 2 Foliar nutrient concentration (mg.g^{-1}) of N, P, K, Ca and Mg in beech leaves (left) and current-year pine needles (right) in the different sites. For each combination of site and species, box plots are used for concentrations across stands (pure, mixed). Individual tree nutrient concentrations are represented by triangles (beech) or dots (pine); filled and open symbols denote pure and mixed stands, respectively. The grey banner indicates the optimum range of foliar nutrient concentrations as defined by Mellert and Göttlein (2012). Y axis may differ between species.

3.2. Foliar nutrient composition of beech and pine

As shown in Table 2, beech leaves and current year pine needles differed significantly for all investigated *ilr* balances. The [Fv|Mg, Ca, K, P, N], [K|P, N], [P|N] and [Mg|Ca] balances were higher in beech leaves, while the [Mg, Ca|K, P, N] was higher in current year pine needles. While P concentrations were similar for both species, the concentrations of all other nutrients were higher in beech leaves compared to current

307 pine needles; the relative difference between species was most pronounced for Ca, followed by Mg and N

308 concentrations, and then K (Table 2).

309 *Table 2 Summary of the foliar nutrient composition of beech and current year pine needles. Mean ilr balances* (1) *and nutrient*

310 *concentrations (mg.g^{-1})* (2) *across all stands and sites (standard errors in parentheses). For each foliar variable (ilr balances or*

311 *nutrient concentrations) means within a column that are followed by the same letter do not differ at* P < 0.05 (Student's t-Tests).

(1)	[Fv|Mg, Ca, K, P, N]		[Mg, Ca|K, P, N]		[K|P, N]		[P|N]		[Mg|Ca]	
Beech	-4.83a	(±0.01)	0.77a	(±0.03)	-0.36a	(±0.02)	1.94a	(±0.01)	1.22a	(±0.02)
Pine	-5.17b	(±0.01)	1.34b	(±0.02)	-0.44b	(±0.01)	1.66b	(±0.01)	0.66b	(±0.01)

(2)	N		P		K		Ca		Mg	
Beech	21.9a	(±0.2)	1.4a	(±0.0)	9.0a	(±0.2)	8.1a	(±0.2)	1.5a	(±0.0)
Pine-	15.1b	(±0.2)	1.4a	(±0.0)	8.1b	(±0.1)	2.7b	(±0.1)	1.0b	(±0.0)

312

313

3.3. Stand composition effect on *ilr* balances and nutrient content

315 Species identity had a significant effect on all *ilr* balances (Figure 3).

316 For all investigated *ilr* balances, at least one species displayed a significant species-mixing effect (Figure 3).

317 For beech, [Mg, Ca|K, P, N] and [P|N] balances were higher in mixed stands than in monocultures while

318 the opposite was true for [K|P, N]. For pine, mixed stands displayed higher [Fv|Mg, Ca, K, P, N] and

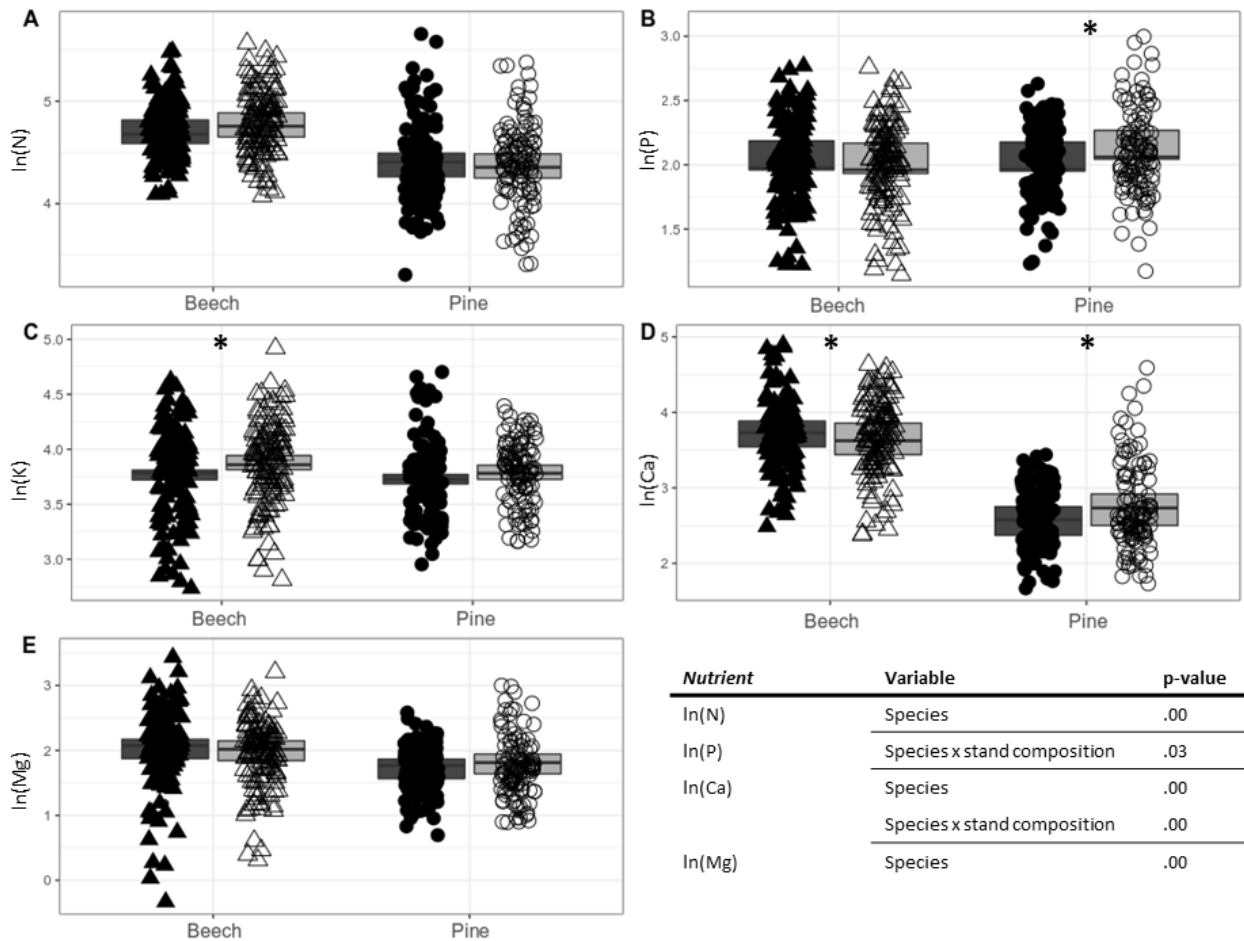319 [Mg|Ca] balances and lower [Mg, Ca|K, P, N] and [P|N] balances than monocultures.

320 For three *ilr* balances ([Mg, Ca|K, P, N], [Mg|Ca] and [P|N]), the effect of stand composition differed

321 between species (Figure 3; significant species × stand composition interaction), with two *ilr* balances

16

322   showing opposite effects of mixing: compared to monocultures the [Mg, Ca|K, P, N] and [P|N] balances in

323   mixed stands were higher for beech and lower for pine.
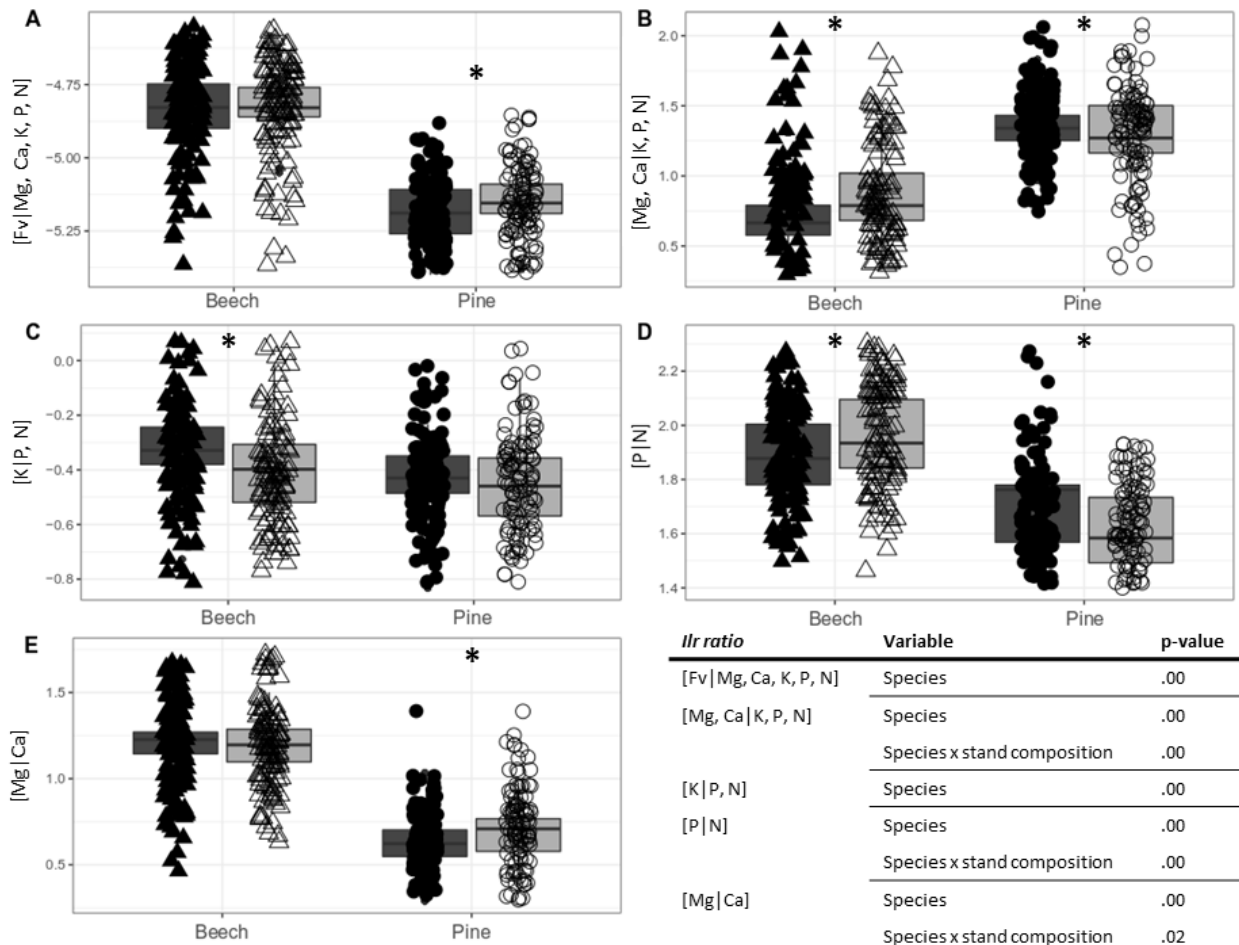
324

325   *Figure 3 Beech and pine foliar ilr balances (boxplots and individual (tree) observations) as a function of species identity*

326   *(beech/pine - current-year needles) and stand composition (pure/mixed) across all sites. The individual tree observations are*

327   *depicted by black filled triangles (beech) or dots (pine); filled and open symbols denote pure and mixed stands, respectively. Black*

328   *stars indicate significant differences between pure and mixed stands of a same species at P < 0.05 from linear mixed models*

329   *adjusted on each species and each ilr balance separately. The statistical significant effects of the mixed models (Eq. 3) are shown*

330   *in the enclosed table, while the detailed statistics for all ilr balance models are given in Table A4.*

331

332   Looking at nutrient contents (Figure 4), mixing increased P contents in pine and K contents in beech;

333   compared to the monocultures, Ca contents of the mixed stands were lower in beech and higher in pine.

334

Nutrient	Variable	p-value
ln(N)	Species	.00
ln(P)	Species x stand composition	.03
ln(Ca)	Species	.00
	Species x stand composition	.00
ln(Mg)	Species	.00

335

336   *Figure 4 Beech and pine foliar nutrient contents (boxplots and individual observations as a function of species identity*

337   *(beech/pine - current-year needles) and stand composition (pure/mixed) across all sites. The individual tree observations are*

338   *depicted by triangles (beech) or dots (pine); filled and open symbols denote pure and mixed stands, respectively. Black stars*

339   *indicate significant differences between pure and mixed stands of a same species at P < 0.05 from linear mixed models adjusted*

340   *on each species and each nutrient content separately. The statistically significant effects of the mixed models (Eq. 3) are shown in*

341   *the enclosed table, while the detailed statistics for all models are given in Table A5.*

342

343 **3.4.   Impact of site nutrient status on species mixing effect**

344 The effects of species-mixing effect on *ilr* balances along the gradient of site nutritional status for beech

345 and pine are displayed in Figure 5 and 6, respectively.

346 For beech, the [Mg|Ca] and [P|N] balances were significantly affected by the site nutrient level, the stand

347 composition, and their interaction. For both *ilr* balances, the difference between pure and mixed stands

348 was highest at the lower end of the gradient where mixed stands were associated with higher [P|N] and

349 lower [Mg|Ca] balances, compared to pure stands. For the [Mg,Ca|K,P,N] and [K|P,N] balances, only the

350 interaction of site and stand was significant, indicating that the site nutrient status had a different impact

351 in pure and in mixed stands. No significant effect was detected for the [Fv|elements] balance.

352 For pine, the [Fv|elements] balance was significantly higher in mixed than in pure stands, and the effect

353 was constant along the soil fertility gradient. The interaction of site and stand was significant for the

354 [K|P,N] and [Mg|Ca] balances, showing significantly different responses to site nutrient status of pure and

355 mixed stands.

356
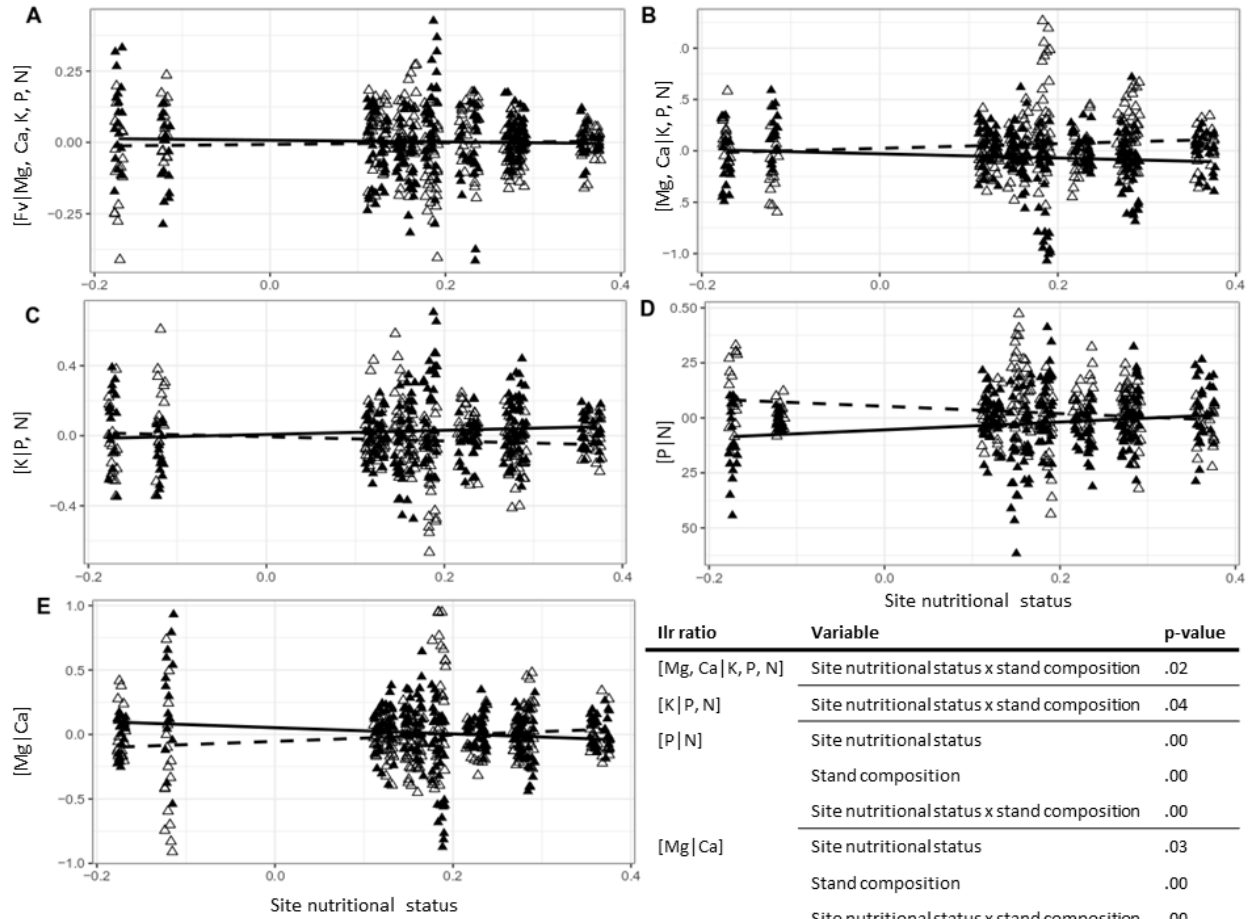
Figure 5 Difference between individual ilr balances of beech trees and the corresponding average value per site as a function of site nutritional status and stand composition. The ilr balance differences are represented by filled (pure stands) or open triangles (mixed stands). The lines represent the predictions for the pure (solid line) and mixed stands (dashed line), using Eq. 4. For all ilr balances, the site nutritional status is quantified by the site-average spe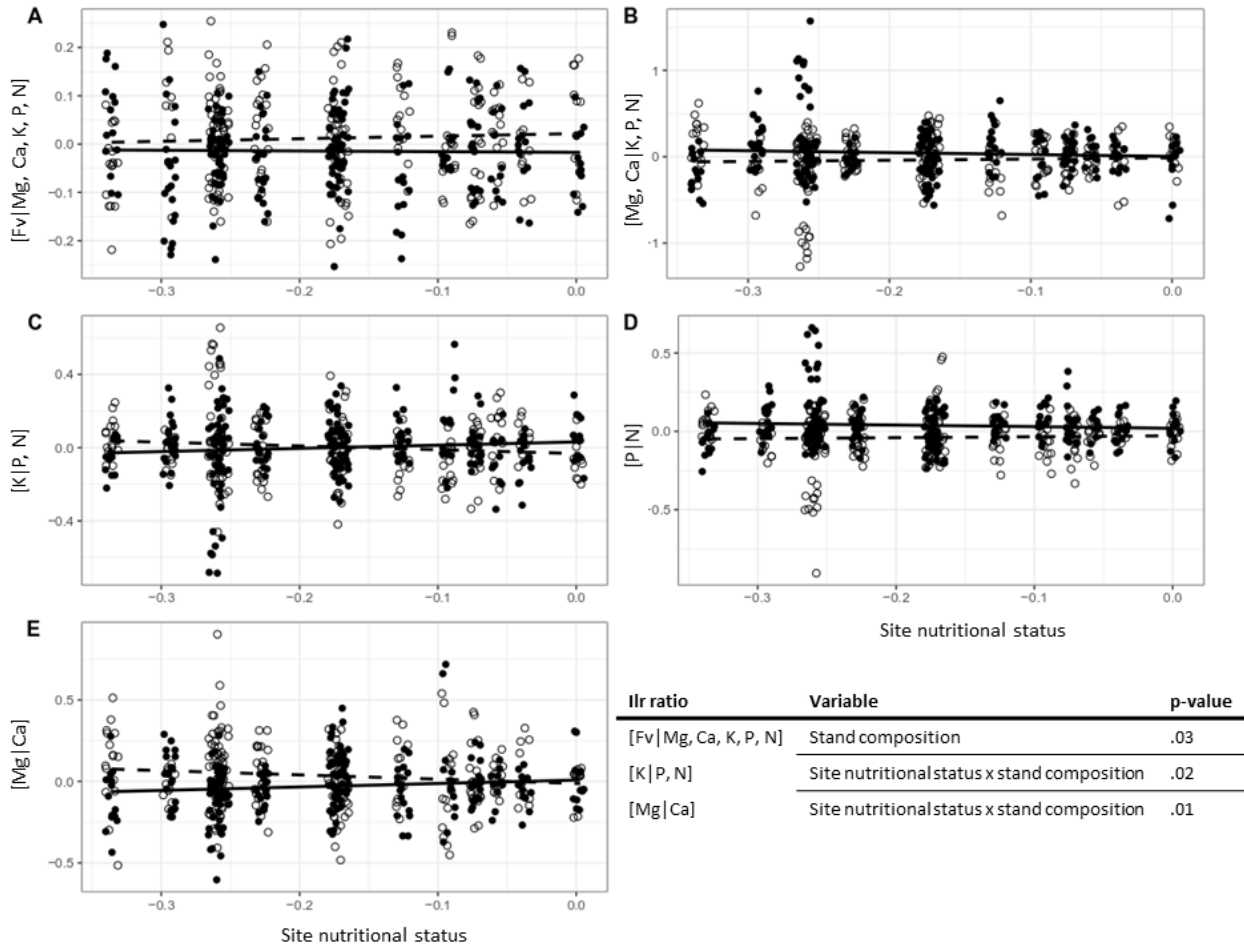cies-specific [Fv|Mg, Ca, K, P, N] ilr balances. Significant effects of the linear models (Eq. 4) are shown in the enclosed table, while the detailed statistics for all ilr balance models are given in Table A6. In the table, default value for stand composition is "pure".
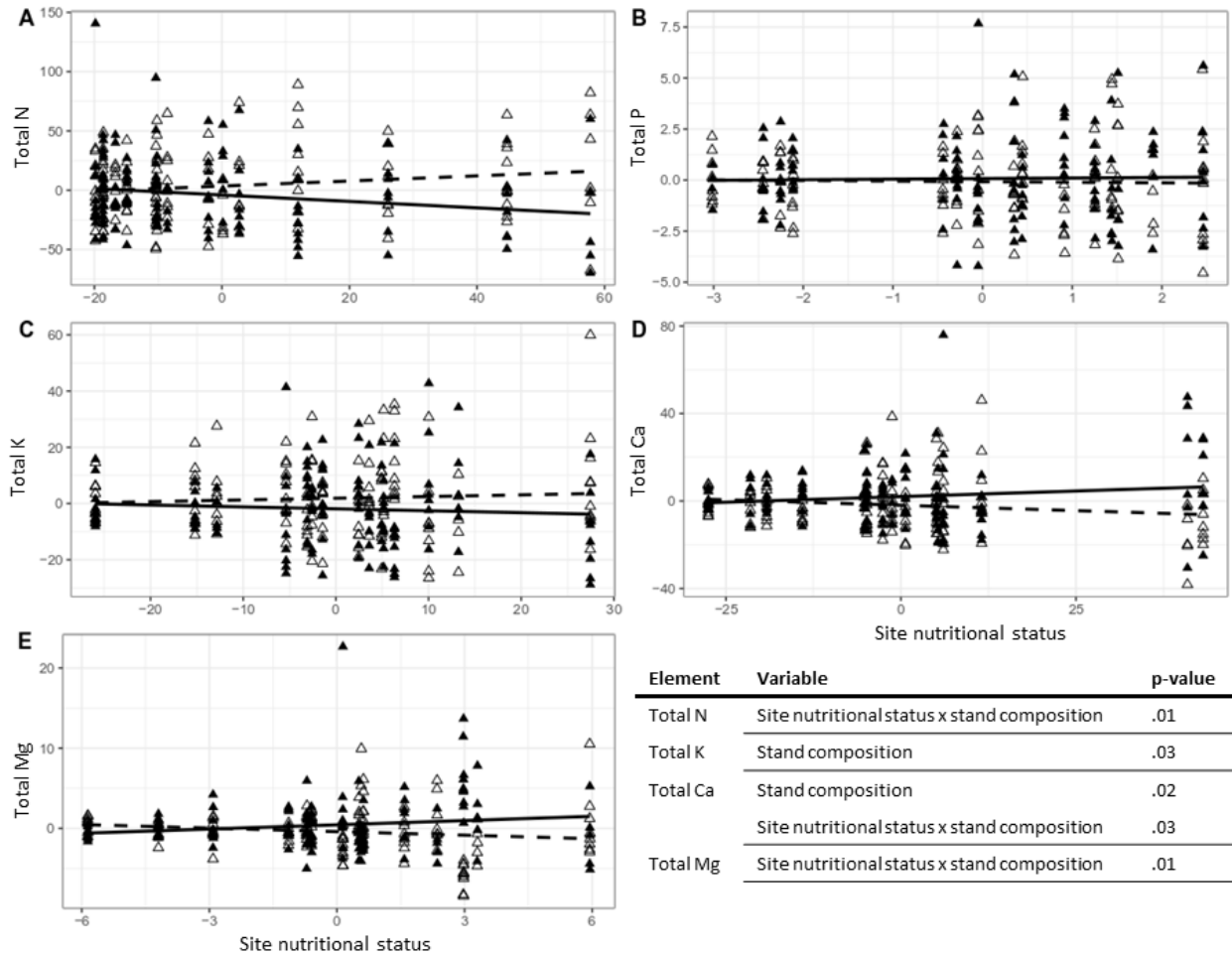
365

Figure 6 Difference between individual ilr balances of pine trees and the corresponding average value per site as a function of site

nutritional status and stand composition. The ilr balance differences are represented by filled (pure stands) or open dots (mixed

stands). The lines represent the predictions for the pure (solid line) and mixed stands (dashed line), using Eq. 4. For all ilr

balances, the site nutritional status is quantified by the site-average species-specific [Fv|Mg, Ca, K, P, N] ilr balance. Significant

effects of the linear models (Eq. 4) are shown in the enclosed table, while the detailed statistics for all ilr balance models are

given in Table A6. In the enclosed table, default value for stand composition is "pure".

Looking at the nutrient contents of beech leaves (Fig. 7), stand composition had a significant effect on both

K and Ca. The foliar K contents were higher in the mixed stands irrespective of site fertility; by contrast,

the effect of stand composition on Ca differed along the gradient, with lower Ca contents in mixtures on

nutrient-rich sites and almost similar contents for both stand types at the lower end of the gradient. For N

21

377   and Mg, the effect of site differed between pure and mixed stands, with the strongest difference among

378   stand types observed at the richer sites. There was no main stand composition effect, yet the associated

379   p-value was quite close to 0.05 (Table A7). P contents did not respond to either site nutrient status nor

380   stand composition. For pine nutrient contents (Fig. 8), P and Mg showed a similar pattern. There was an

381   overall significant site and stand composition effect, yet the effect of an increased site fertility was

382   negative in the pure stands and positive in the mixtures. For K and Ca, the effect of site also differed

383   between pure and mixed stands. For K, the interaction was associated with a significant site effect and to

384   opposite effects of stand composition at both extremities of the gradient (higher K content in mixed stand

385   at the lower end of the gradient and lower K content in mixed stand at the higher end of the gradient).

386   The Ca contents of pine needles were on average significantly higher in the mixed stands, yet the stand

387   composition effect was more pronounced as soil fertility increased. N contents in pine needles did not

388   change significantly with either stand composition nor site fertility.

389

Element	Variable	p-value
Total N	Site nutritional status x stand composition	.01
Total K	Stand composition	.03
Total Ca	Stand composition	.02
	Site nutritional status x stand composition	.03
Total Mg	Site nutritional status x stand composition	.01

390

Figure 7 Difference between individual foliar content of beech trees and the corresponding average value per site as a function of

site nutritional status and stand composition. The nutrient content differences are represented by filled (pure stands) or open
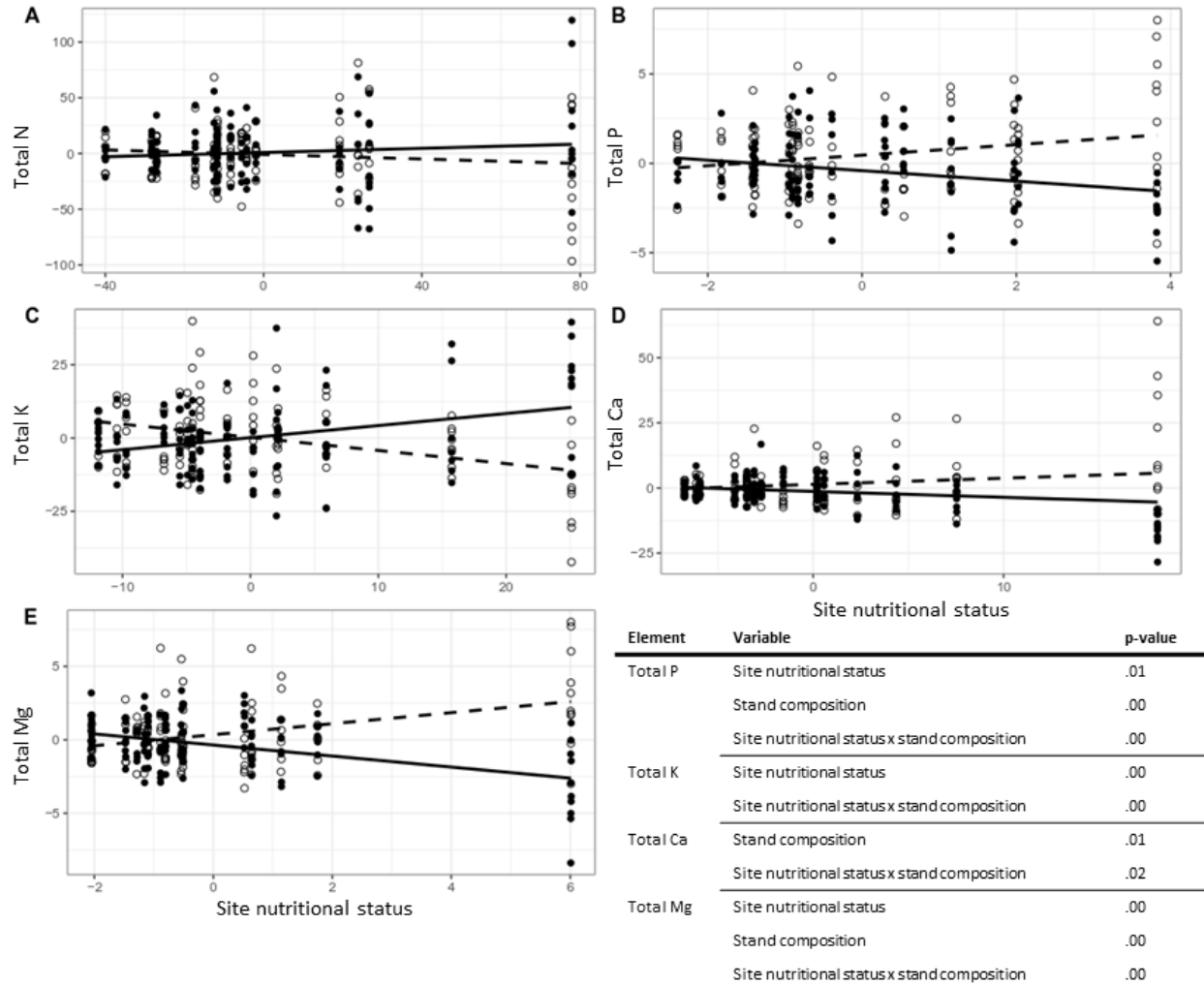
triangles (mixed stands). The lines represent the predictions for the pure (solid line) and mixed stands (dashed line), using Eq. 4.

For all elements, the site nutritional status is quantified by the site-average species-specific nutrient content. Significant effects of

the linear models (Eq. 4) are shown in the enclosed table, while the detailed statistics for all nutrient content models are given in

Table A7. In the enclosed table, default value for stand composition is "pure".

397

Element	Variable	p-value
Total P	Site nutritional status	.01
	Stand composition	.00
	Site nutritional status x stand composition	.00
Total K	Site nutritional status	.00
	Site nutritional status x stand composition	.00
Total Ca	Stand composition	.01
	Site nutritional status x stand composition	.02
Total Mg	Site nutritional status	.00
	Stand composition	.00
	Site nutritional status x stand composition	.00

398

399 *Figure 8 Difference between individual foliar content of pine trees and the corresponding average value per site as a function of*

400 *site nutritional status and stand composition. The nutrient content differences are represented by filled (pure stands) or open*

401 *dots (mixed stands). The lines represent the predictions for the pure (solid line) and mixed stands (dashed line), using Eq. 4. For*

402 *all elements, the site nutritional status is quantified by the site-average species-specific nutrient content. Significant effects of the*

403 *linear models (Eq. 4) are shown in the enclosed table, while the detailed statistics for all nutrient content models are given in*

404 *Table A7. In the enclosed table, default value for stand composition is "pure".*

405

406 **4. Discussion**

407 **4.1. Foliar *ilr* balances and individual nutrient concentrations in beech and pine**

408

24

409   Our results showed that beech leaves and current year pine needles have distinct foliar nutrient

410   signatures. To start with, it is essential to remember that as a result of the computation procedure and

411   notation (Eq. 2), the *ilr* balances behave differently than traditional raw elemental ratios. An increase in *ilr*

412   balance means that either the left part (the balance denominator) has decreased, and/or the right part

413   (the balance numerator) has increased. In this respect, the higher [P|N] and [Mg|Ca] balances in beech

414   leaves compared to pine needles are consistent with the corresponding average nutrient concentrations

415   (Table 2). The increased [K|P,N] in beech leaves can mainly be attributed to the much higher N

416   concentrations in beech leaves compared to pine needles, given the limited difference in K concentrations

417   and the similarity of P concentrations. The significantly higher [Mg,Ca|K,P,N] balance in pine needles can

418   primarily be attributed to the strongly reduced Ca concentrations of pine needles compared to beech

419   leaves, and to a lesser extent, to the lower Mg concentrations of pine needles. Finally, the higher [Fv|Mg,

420   Ca, K, P, N] value in beech leaves is consistent with the higher concentrations of most measured nutrients

421   in beech leaves (Table 2), and their significantly lower Fv value (data not shown); the latter is composed

422   of unmeasured macro- and micro-elements. We are not able to point out those elements, which are

423   involved in this difference.

424   The differences in average nutrient concentrations between beech and pine irrespective of stand type

425   (pure/mixture) were in close agreement with literature data (González de Andrés et al. 2019, Mellert and

426   Göttlein, 2012), confirming the distinctly higher N, Ca and Mg concentrations in beech leaves compared

427   to current year pine needles (Liu et al. 2006, Sardans et al. 2015). According to Sardans et al. (2015) such

428   distinct foliar signatures probably result from long-term differentiation of metabolic and physiological

429   functions and morphology leading to species-specific optimal elemental composition according to the

430   biogeochemical niche hypothesis.

431   The contrasted elemental composition and stoichiometries thus indicates differences in nutritional

432   characteristics between species. Because distinct characteristics between co-occurring species are

433   necessary to entail complementarity, such differences could lead to reduced competition (Sardans et al.

434   2015) and improved nutrition in mixed stands.

435

436   **4.2.**   **Overall mixing effect on *ilr* balances and nutrient content**

437   When significant, the stand composition effect resulted in increased nutrient contents in the mixed stands

438   compared to the monocultures in all (P and Ca in pine, K in beech) but one case (Ca in beech) (Figs. 3 & 4).

439   The positive effect of beech on pine Ca and the corresponding negative effect of pine on beech Ca could

440   be related to the strong difference in nutrient Ca concentration between beech leaves and pine needles

441   (Table 2), resulting in changed nutrient inputs through litterfall. Differences in litterfall between species

442   have been shown to influence soil nutrient availability (Fassnacht and Gowerr 1999; Polyakova and Billor

443   2007; Vesterdal 1998). However, such a difference in nutrient composition between leaves and needles

444   could not explain the other synergistic effects. Indeed, pine P content and K beech content increased in

445   mixed stands compared to pure stands even though foliar P concentrations are similar for both species

446   and K foliar concentrations are lower in pine than in beech. In addition, no stand composition effect could

447   be detected for some nutrients e.g., for nitrogen contents in pine needles, despite the much higher N

448   concentrations in beech leaves compared to pine. Previous studies have found contrasting results on the

449   impact of species mixing on tree foliar nutrition depending on the element considered. Some studies are

450   consistent with our results. For instance, Brandtberg (2001) and Thelin et al. (2002) found P and K

451   concentrations in foliage of conifers to increase if broadleaves were present. Berger et al. (2009) found a

452   tendency to higher Ca concentrations in mixed stands compared to pure stands for coniferous species and

453   lower concentrations for beech stands. On the other hand, our findings differed from some other studies.

454   For instance, Magh et al. (2018) hypothesized broadleaves N acquisition to be lower in mixed stands due

455   to a negative impact of the more recalcitrant conifer litter on the mineralization rates of organic matter.

456   However, we did not find lower amounts of N in beech foliage in mixed stands compared to pure stands.

457   One possible explanation is that soil N availability is also strongly influenced by other environmental

458   drivers than litter quality (Anderson and Domsch 1993, Brown 1992). Such simultaneous influence of

459   several factors and their interaction on species-mixing effects on foliar are not well studied yet.

460   Altogether, our results suggest that differences in foliar nutrient concentrations between associated

461   species and the corresponding change in nutrient inputs, are not strong enough to be used as predictors

462   of overall species-mixing effects. Obviously, additional factors should be considered to explain the large

463   variability in species-mixing effects between sites. This is in line with the results from Rothe et al. (2003).

464   In their study, foliar nutrient pools were not always good predictors of nutrient availability. Several other

465   mechanisms such as the effect of species mixing on litter production through increased canopy packing

466   (Barbeito et al. 2017; Jucker et al. 2015; Pretzsch 2014; Scherer-Lorenzen et al. 2007) or species mixing

467   effect on rates of litter decomposition (Joly et al. 2016; Jonard et al. 2008) can be expected to also come

468   into play although we were not able to properly assess their relevance for and contribution to our findings.

469   More specifically, beech is expected to have a positive impact on pine nutrition through improvement of

470   the humus layer, the upper mineral soil and exploitation of nutrients from deeper soil horizons (Chodzicki

471   1934, Pretzsch et al. 2015). The higher nutrient content, lower C:N ratio and lower amount of recalcitrant

472   compound in beech litter compared to pine (Rumberger et al. 2004, Zhong and Makeschin 2004) could

473   lead to reduced soil acidity and richer humus which will in turn improve nutrient availability in mixed

474   stands compared to pure pine stands (Collin et al. 2016, González de Andrés et al. 2017). In addition, the

475   distinct potential rooting pattern of both species (heart-shaped fine root profile for beech vs. peak of fine

476   root biomass in the most superficial soil layers for pine; Prévosto and Curt 2004) can lead to higher

477   combined root occupancy in mixture and thus, more complete belowground exploitation at least in some

478   sites (González de Andrés 2017).

479   In addition to the observed effect on selected nutrient contents, mixing beech and pine changed nutrient

480   balances. Two *ilr* balances significantly differed between pure and mixed stands for both pine and beech

481   trees: [Mg,Ca|K,P,N], [P|N], yet in an opposite direction (Fig. 3). The three other *ilr* balances were also

482   affected by stand composition, but only for one species. To our knowledge, few studies analyzed the

483   influence of species mixing on tree foliar nutrition using elements balances. Thelin et al. (2002) found the

484   "classical" ratio P:N to be higher in Norway spruce needles when mixed with deciduous species (beech,

485   birch or oak), which is coherent with our finding. The absence of a species mixing effect on beech [Mg|Ca]

486   is coherent with the findings of Colin et al. (2016) who used isometric log-ratios to study the nutritional

487   characteristics of sugar maple and red maple in different contexts (pure stands vs. mixed with conifers;

488   increasing soil acidity levels). They explained the absence of effects by a simultaneous decrease in Mg and

489   Ca with increased conifer proportions. In contrast to our results, however, Collin et al. (2016) also found a

490   decrease in the [Fv|Mg, Ca, K, P, N] balance with increasing proportions of conifers, which they attributed

491   to the effect of increased soil acidity on Al and Mn availability (higher ionic activity in more acidic

492   conditions); as those elements were not measured in the leaves, they were expected to increase

493   correspondingly the Fv value. The dependency of those mechanisms on local factors, like the forest

494   management strategy, the acidifying potential of the species involved or the initial soil acidity could explain

495   contrasting results between studies. Importantly, the change in *ilr* balance suggests that mixing can modify

496   tree nutrition by altering some key nutrient balances, well beyond changing specific individual nutrients.

497   This suspected complementarity with regard to mineral nutrition could be, at least in part, the cause of

498   the increased productivity highlighted on the same network of pine and beech by Pretzsch et al. (2015) as

499   proposed by Burkhart and Tham (1992), Frivold and Kolström (1999), and Kelty (1992). However, our data

500   set does not allow to properly explore the relationships between tree/stand productivity and nutrition,

501   due to the limited number of sites compared to the potentially high number of processes involved (cf. e.g.

502   Forrester and Bauhus, 2016 for a comprehensive review on those processes).

503

4.3. Mixing effect as a function of site nutrient status

505 Regarding the impacts of mixing on nutrient contents, there was a close agreement between the results

506 of the overall analysis (Eq. 3) and the analysis where the nutrient gradient was explicitly accounted for (Eq.

507 4); the only exception to this agreement was Mg under pine for which the stand composition effect was

508 not significant in the overall analyses (p-value = 0.13). By contrast, we found more discrepancies between

509 the two approaches for the *ilr* balances. A possible explanation could be related to the index we used to

510 characterize the gradient of nutrient availability for the latter approach ([Fv|Mg, Ca, K, P, N] for all *ilr*

511 balances). We deliberatively selected Fv|[Mg, Ca, K, P, N] over the traditional site index (SI) in our case as

512 the latter integrates all site factors, and not only nutrients (Brandl et al. 2014). The use of other indicators

513 such as foliar nutrient remobilization (Achat et al. 2018), soil pH or soil exchangeable cation pools in future

514 studies should help improve our understanding of the dependency of species-mixing effect on site

515 chemical fertility. It is also important to note that, for beech, the sites were not homogenously distributed

516 along the [Fv|Mg, Ca, K, P, N] gradient (Fig. 5); the two sites at the lower end of the gradient could have

517 had a strong influence on the slope for some models (see the [P|N] balance for instance). One way to cope

518 with this would be to select alternative indicators of soil fertility (see above), and/or to increase the

519 sampling to fill-in the current gaps along the gradient.

520 Focusing on the contents, we expected the mixing effect to increase with a decrease in nutrient availability

521 according to the framework proposed by Forrester and Bauhus (2016). However, in all cases where we

522 found a significant overall stand composition effect (K and Ca for beech, Fig. 7; P, Ca, Mg for pine, Fig. 8),

523 we actually observed the opposite, with only minor or no difference between monocultures and mixtures

524 on the poorest sites, and maximum differences on richer sites.

525   The limited stand composition effect or even the lack of any significant mixing effect in low-nutrient sites

526   could first be explained by the co-occurrence of several nutrient limiting factors. Indeed, the uptake of a

527   nutrient is not only determined by its availability but also by the availability of other nutrients and their

528   interactions, and by environmental conditions such as soil pH or water availability (Marschner 2011;

529   Wilkinson 2000). Simultaneous limitations of distinct nutrients have been observed for both beech and

530   pine stands at several locations (for instance, site FRA1 displayed P and Mg deficiencies for both species

531   and BEL1 displayed Ca deficiency for both species and Mg deficiency for beech) and could originate from

532   the coexistence of several factors limiting nutrient availability. It might be advocated that even in the case

533   where the availability of one limiting nutrient has increased as a result of mixing, this would not translate

534   into an increased foliar content as far as another constraint limiting nutrient availability (e.g. limited water

535   availability) would still be present.

536   A second hypothesis to explain our results is related to all those situations where species interactions may

537   not improve the availability of the limiting nutrient. This could be due to the intrinsic pool of traits present,

538   where inter-specific differences in physiology, phenology or morphology do not improve nutrient

539   availability. Indeed, traits of the species involved in the mixture are of primary importance to determine

540   whether this mixture benefits from improvement of resources availability, uptake or use-efficiency

541   (Ammer 2019). We expected from an association of species with such distinct foliar traits as beech and

542   pine (for instance in term of leaf life span), to induce differences in foliar nutrient composition compared

543   to monocultures. However, such differences in traits may not systematically influence the availability,

544   uptake or resource-use efficiency of nutrients and thus, the foliar nutrient composition. Another possible

545   explanation is the existence of factors limiting the expression of any potential complementarity

546   interaction. Jucker et al. (2014) highlighted such a mechanism in Mediterranean mixed forests. They found

547   that species mixing improved stand growth through complementary light use but that this effect was

548   severely reduced if water resources became limiting. Considering the interspecific differences in rooting

549   patterns between beech and pine (Prévosto and Curt 2004) as a candidate process to increase nutrient

550   availability in mixed species stands compared to monocultures (Forrester and Bauhus 2016), any

551   constraint that would limit the potential development of the rooting system (e.g. anoxic conditions;

552   Kozlowski 1986) would alter this potential spatial stratification.

553   The largest differences in foliar nutrient contents between monocultures and mixed-species stands were

554   observed on the richer sites, with synergistic effects in all cases except for Ca in beech (see previous

555   section). The improved tree nutrient composition of the mixtures at the higher end of the fertility gradient

556   was unexpected, as those situations reflect normal ranges or even a surplus of the nutrient in question.

557   However, an adequate supply of one given nutrient does not exclude potential limitations of others,

558   whereas their availability could in turn limit the uptake of the target nutrient. If mixing removes this

559   constraint, this could explain why a synergistic effect of mixture occurred. An additional explanation is an

560   increased competition in pure stands on the nutrient rich sites due to increased growth, not compensated

561   for by the higher nutrient availability. Bravo-Oviedo et al. (2006) for instance, found that faster tree growth

562   in richer sites lead to increased competition and higher individual tree mortality. In this context, species-

563   mixing could reduce resource competition, leading to an increased species-mixing effect on richer sites.

564

565   **5. Conclusion**

566   Considering the impact of mixing on single nutrient contents, we mostly found either no effect or

567   synergies, with only one case where nutrient contents were lower in mixed stands (antagonism for Ca in

568   beech). Our results showed that the contrasting foliar nutrient compositions of pine and beech trees alone

569   were unable to explain the mixing effects on tree nutrition, which suggests that other mechanisms than a

570   simple change of nutrient inputs through litterfall are at play. Far beyond changing the foliar contents of

571   single individual nutrients, mixing was found to significantly alter key nutrient balances in both beech

572   leaves and current year pine needles.

573   Our analysis also revealed unexpected patterns of complementarity as a function of nutrient availability,

574   with only minor or no differences between monocultures and mixtures at the lower end of the nutritional

575   gradient, and maximum differences on nutrient rich soils. This clearly points to further expanding the

576   existing frameworks to account for the multivariate nature of tree nutrition. In this paper, we made a first

577   attempt in that direction by working on both contents and *ilr* balances. Further work is needed to

578   disentangle the underlying processes, using a combination of field work and modeling approaches. This

579   claims for the establishment of controlled standardized experiments across Europe to test for specific

580   mechanisms.

581

593

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