Mixing has limited impacts on the foliar nutrition of European beech and Scots pine trees across Europe

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ABSTRACT

Tree species-mixing has been suggested as one option to counteract the adverse effects of global change on tree mineral nutrition, yet the effect of mixing on nutrient availability remains poorly documented.

We therefore analyzed the current foliar nutrient (N, P, K, Ca, Mg) quantities and *ilr* balances (isometric log transformed ratios between elements or groups of elements) for 261 European beech and 248 Scots pine trees from 15 sites, each consisting of one beech-pine mixed stand and the respective monocultures, across a gradient of environmental conditions in Europe. We hypothesized an overall positive effect of mixing on tree foliar nutrient content, and that this mixing effect would be stronger on nutrient-poor sites. Using linear mixed models and multivariate linear regression models, we first tested for the effects of species (beech/pine) and composition (pure/mixed) across all sites; we then investigated whether the species-mixing effect was related to site fertility.

The nutrient composition of beech leaves and pine needles differed significantly for all *ilr* balances. For both species, significant mixing effects were detected for some nutrients and *ilr* balances; those effects, however, could not be consistently related to contrasted nutrient composition between species. For most nutrients and *ilr* balances, the mixing effect was influenced by the site nutritional status, but the pattern differed from expectation: absence or minor differences between monocultures and mixtures at the lower end of the chemical fertility gradient, and maximum differences in rich soils.

The contrasting foliar nutrient composition of pine and beech trees and the site nutrient status only partly explained the mixing effects on tree mineral nutrition. Our results claim for a better understanding of nutrient-related mechanisms associated with complementarity and points towards the need to further expand the existing frameworks to account for the multivariate nature of tree nutrition.

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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 (Leuschner et al. 2001). Through improvement of soil resources, species-mixing could also induce a re-allocation of carbon to aboveground biomass, resulting in a higher *nutrient use efficiency* compared to pure stands (Epron et al. 2013, Forrester et al. 2006). All the processes listed above have to be seen in the light of the biogeochemical niche hypothesis (Urbina et al. 2017). The hypothesis states that, because of differences in growth and nutrient use strategies, co-occuring species use mineral elements in different proportions, which leads to species-specific stoichiometry and associated stoichiometric flexibility. Such differences in biogeochemical niches between coexisting species allows for reduced competition and nutrient use optimization at the community level (Urbina et al. 2017).

It is frequently thought that admixing tree species has beneficial impacts on tree nutrition. In temperate forests, those effects have mostly been studied for mixed stands of coniferous and broadleaved trees (Brown 1992, Rothe and Binkley 2001, Thelin et al. 2002). Such expected impacts result from differences in biogeochemical cycle, nutrient demand and nutrient use between conifers and broadleaves. For instance, nitrogen mineralization and nitrification fluxes are generally lower in coniferous than in broadleaves stands, an effect commonly attributed to more acidic conditions, higher C:N ratio of organic matter, and higher amounts of inhibiting and/or recalcitrant compounds under conifers (Augusto et al. 2015). The same mechanisms could also hold true for other nutrients such as P and Ca because their availability is partly related to organic matter mineralization. Additional processes involve distinct impacts of coniferous vs. broadleaved trees on atmospheric deposition and mineral weathering (Augusto et al. 2015). Differences in nutrient demand and nutrient use between coniferous and broadleaves species also leave room for beneficial impacts of admixing these species. Such differences include a generally higher nutrient concentration in litter and fine roots for broadleaves, and longer nutrient residence time for conifers (Augusto et al 2015).

The impact of admixing coniferous and broadleaf species on tree nutrition has been highlighted in previous studies. For instance, Brandtberg (2001) found that admixing birch species (*Betula pendula*

Roth and *Betula pubescens* Ehrh.) and Norway spruce (*Picea abies* (L.) Karst) increased P and K concentrations of spruce needles (but did not influence N, Ca, Mg or Mn concentrations). Thelin et al. (2002) reported higher P and K concentration in Norway spruce needles when mixed with beech, birch or oak (*Quercus robur* L. and *Quercus petraea* (Matt.) Liebl.). Brown (1992) found that interspecific differences in tissue concentration of N and possibly P, were key factors responsible for contrasting types of mixture effects (positive, compensatory and negative) on Norway spruce when mixed with Scots pine, black alder and sessile oak, respectively. However, other studies failed to find any significant differences in foliar nutrient concentrations between pure and mixed stands of conifers and broadleaved species (Heinsdorf 1997, Magh et al. 2018, Neft and Stangl 1985, Rothe et al. 2003). An explanation for those inconsistent results is the dependency of species-mixing effects to environmental conditions (Ratcliffe et al. 2017). Building on the framework developed by Forrester and Bauhus (2016), effects of mixing on nutrition are expected to increase along a gradient of decreasing nutrient availability provided that species interactions improve the availability, uptake or resource use efficiency of limiting nutrients. In accordance with this pattern, Magh et al. (2018) found that in the absence of any water limitation, there was a facilitative effect of silver-fir on N nutrition of beech in N-limited sites, but an antagonistic effect in N-rich soils. However such a simple framework might not be sufficient to explain the full range of patterns for nutrient related mixing effects along environmental gradients. First, it does not account for nonlinearity of the relationship between resource availability and mixing effects. For instance, while their focus was on the mixing effect on drought exposure, de Streel et al. (2019) highlighted tipping points along a nearly similar environmental gradient. Second, the above framework does not take into account simultaneous limiting factors. Sardans and Peñuelas (2007), however, showed that species-mixing effects on foliar nutrition could be influenced by site water status through its impact on nutrient uptake.

The assessment of tree nutrition is largely based on analyzing single foliar nutrient concentrations or contents (i.e. amount of a nutrient in a given number of leaves/needles), as well as ratios between pairs of elements (e.g. Jonard et al. 2015). This approach yields valuable information about mineral elements that are essential to plant functioning and to their metabolism, and the reported values can easily be compared with thresholds (Mellert and Göttlein 2012). More recently, a complementary method, where nutrients are subjected to isometric log-ratio (*ilr*) transformation (see Materials and methods below), has been proposed to better account for the interactions between nutrients while limiting biases related to using raw compositional data (Egozcue et al. 2003). Since its development, this method was successfully applied in different contexts. For instance, Parent et al. (2013b) used it to analyse the mineral plasticity of cloudberry and Parent et al. (2013c), Modesto et al. (2014) and Hájek et al. (2014) used it to study the nutrition of Mango trees, maize or mosses, respectively. Recently, Collin et al. (2016) used the *ilr* method to study the nutritional characteristics of Sugar Maple (*Acer saccharum* Marsh.) and Red Maple (*Acer rubrum* L.) under varying proportions of conifers, while Nowaki et al. (2017) used this approach to assess the response of irrigated tomato crops to P fertilization. Although the relationship between foliar nutrient ratios and tree growth remains unclear (Binkley and Fisher, 2019), critical ratios have been shown to influence a number of important processes such as masting (Fernández-Martínez et al., 2019) or herbivory (Lind and Barbosa 2012), and are often related to forest dieback (Hevia et al. 2019).

The objective of this paper was to assess the impact of mixing broadleaves and conifers on tree nutrition. Focusing on the widespread European beech - Scots pine mixture, we sampled the foliage of 261

beech (*Fagus sylvatica* L.) and 248 pine (*Pinus sylvestris* L.) trees at 15 sites distributed over Europe, each of which contained a triplet of plots including a mixture and the monocultures of each species. We used the *ilr* approach to obtain a synthetic and non redundant characterization of the overall nutritional signature, in complement to the classical nutrient content/concentration approach.

We hypothesized that differences in foliar nutritional characteristics between species would result in significant mixing effects on tree nutrition. We further hypothesized that mixing effects would be dependent on site nutritional status.

2. Materials and methods

2.1. Study area and site/stand characteristics

This study is based on data from 15 sites each consisting of pure

stands of pine and beech and a mixed stand of both species (Fig. 1), with all three of them located in similar conditions. The sites have been established under the COST Action FP1206 EuMIXFOR (European Network on Mixed Forests). This network covers a large gradient of environmental conditions and site nutrient status (Fig. 2; Tables A1 and A2) within the overlapping natural ranges of pine and beech. Elevations varies between 20 and 1190 m a.s.l; mean annual precipitation sum (P) ranges from 520 to 1175 mm and mean annual temperature (T) from 6 to 10.5 °C. The stands are mostly even-aged and mono-layered. A summary of stand characteristics is provided in Table A3. In the mixtures, the percentage of basal area represented by scots pine ranged from 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 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 0.045 to 0.462 ha for mixed stands (Table A3). No silvicultural activities had been conducted in the stands during the preceding
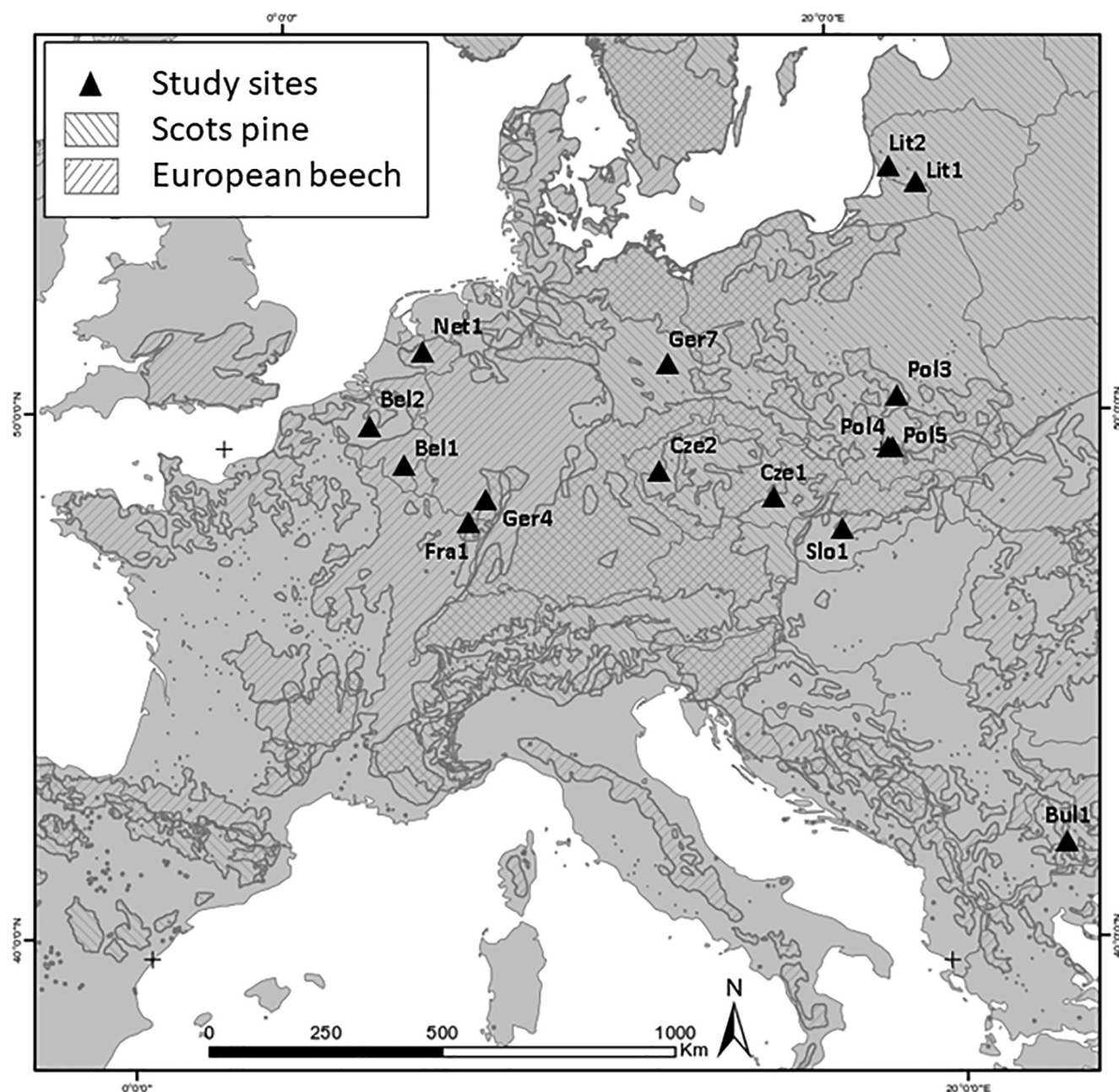

Fig. 1. Distribution of the 15 EuMIXFOR beech-pine triplets used in the present study and distribution of European beech and Scots pine according to EUFORGEN (www.euforgen.org).
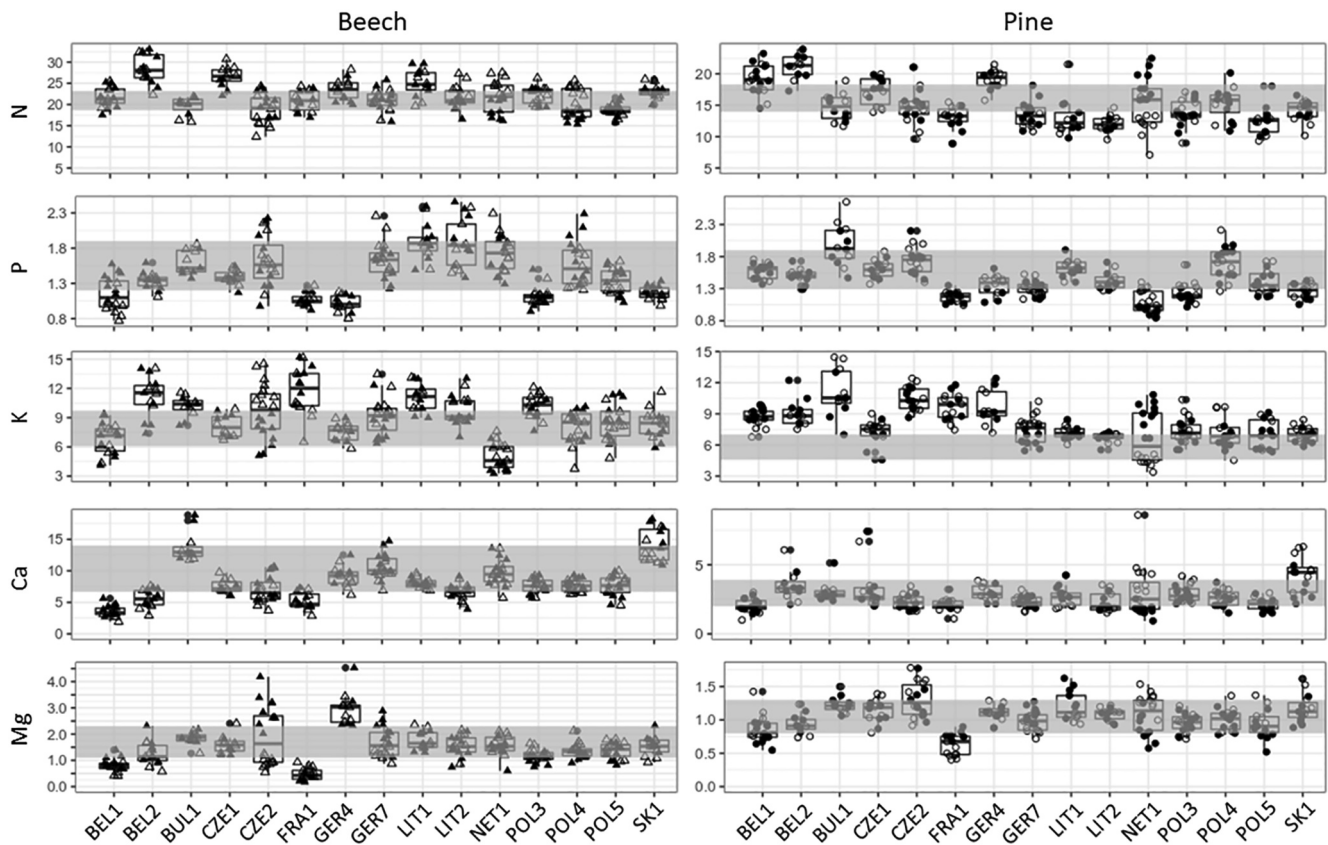
Fig. 2. Foliar nutrient concentration (mg.g^{-1}) of N, P, K, Ca and Mg in beech leaves (left) and current-year pine needles (right) in the different sites. For each combination of site and species, box plots are used for concentrations across stands (pure, mixed). Individual tree nutrient concentrations 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.

decade. A standard protocol for tree data collection (diameters, heights of trees and crown bases) and tree coring was applied. The full measurement protocol was described in details by Heym et al. (2017).

2.2. Leaf collection, pretreatment and analysis

Across the 15 sites-network, 509 trees (261 European beech and 248 Scots pine) were sampled. In each site and plot (pure beech, pure pine, mixed beech-pine), 5–10 trees were sampled per species (i.e. beech or pine in the pure stands, beech and pine in the mixed stand) among the dominant trees during the summer 2016 (second part of the vegetation period, before autumnal colouring) for a total of 24–40 sampled trees per site. Current year leaves/needles were collected from several branches located in the upper third of the crown, resulting in one sample per tree. Leaves/needles were dried at 40 °C until constant weight was achieved for the nutrient analysis. To determine the dry mass, a subsample was additionally oven-dried at 70 °C (50 leaves/500 needles). Foliar N concentrations were measured using the dry combustion method with a Flash Analyzer (Thermo Finnigan Flash EA 1112 elemental analyser) and the other elements (P, K, Ca and Mg) were determined by ICP spectrometry (Varian 720 E-S) after digestion with HNO_3 in a microwave oven (Milestone UltraWAVE, Germany). All analyses were done on a per tree basis.

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

Table 1
Sequential Binary Partition (SBP) of foliar nutrients of beech and pine trees based on prior knowledge of nutrient interaction in higher plants and number of components in the (+) et (−) groups (n$^+$ and n$^-$, respectively).

Balance [−1 subset| +1 subset]	SBP parts						n$^+$	n$^-$
	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

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 developed. 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 al. 2014, Parent et al., 2013b, 2013c). In addition to constraining the system to 100%, it also accounts for the physiological interactions between nutrients (Collin et al. 2016, Nowaki et al. 2017).

The *ilr* transformation technique gives information about the relative amounts of elements or groups of elements, allowing D-1 orthogonal (geometrically independent) balances to be produced for a D part composition. D is the number of measured elements plus a filling value (Fv). The filling value corresponds to the difference between the unit or scale of measurement (e.g. 100%) and the sum of all measured elements (Parent et al., 2013a). In this context, a balance, hereafter referred to as *ilr* balance, is defined as the relationship between groups composed of one or several nutrients (Egozcue and Pawlowsky-Glahn 2005). A closure operation is applied to the resulting matrix of compositional data (Aitchison 1986), which computes the constant sum of components as follows (Egozcue and Pawlowsky-Glahn 2005):

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

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 the ith part of a composition of D components.

The *ilr* transformation is then applied to the closed data. This system of lineary independent ratios is called Sequential Binary Partition (SBP) and describes the D-1 orthogonal balances between nutrients or groups of nutrients (Parent et al., 2013a). The SBP of a D-elements composition is a (D-1) × D matrix where columns correspond to the parts of the composition and rows to the *ilr* balances. In the SBP, elements labelled "+1" correspond to balances numerators, elements labelled "−1" correspond to balance denominators and elements labelled "0" are not part of the balance in question. This SBP is defined *a priori*, for instance, based on user knowledge. In our case, the SBP (Table 1) is based on prior knowledge of plant nutrition (Collin et al. 2016, Marschner 2011, Parent et al., 2013a). The first partition contrasts all measured elements (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, P,N]). The sub-compositions are then divided into [K|P, N], [P|N] and [Mg|Ca]. [P|N] is representative of the Redfield ratio which is an indicator of the balance between processes associated with the synthesis of N-rich proteins and the synthesis of P-rich r-ARN respectively (Ågren 2004, Loladze and Elser 2011). [Mg|Ca] is an indicator of the geographical position and soil mineralogy (Walworth and Sumner, 1988).

Once the SBP was defined, we used the following equation to calculate the *ilr* balances (Egozcue and Pawlowsky-Glahn 2005):

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

Ilr_j corresponds to the jth isometric log-ratio, n_j^+ and n_j^- are the number of components in the (+) et (−) 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 to the orthogonal coefficient of the jth balance defined in the SBP. *Ilr* balances are conventionally presented in the form [components in denominator|components in numerator] because log ratios become more negative as the denominator increases and hence, the balance leans to the left as in algebra where negative numbers are located on the left side of the zero (Nowaki et al. 2017, Parent et al. 2013b). Thus, an increase in *ilr* balance can be attributed to either a decrease in the

left part of the balance or an increase 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, an increase in N or a decrease in P concentration will lead to increased *ilr* balance value and the [P|N] partition leans to the right (Collin et al. 2016, Parent et al. 2013b).

2.4. Statistical analyses

To test the differences in foliar *ilr* balances, foliar nutrient concentration and foliar nutrient content between species, we conducted Student's t-Test. By informing about the biogeochemical niche of the studied species, those analyses are used to interpret the species-mixing effects.

In order to test whether stand composition (pure vs. mixed), species identity (beech vs. pine) and their interaction influenced the current *ilr* balance or the foliar nutrient content, we fitted linear mixed models on each *ilr* balance or nutrient content, considering site and stand nested within site as random factors

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

Y_{hijs} represents the *ilr* balance/content of interest for the ith individual (tree) in the jth stand (pure or mixed) and the sth site for species *h* (pine or beech), β is the vector of the fixed effect parameters (species identity, stand composition and their interaction), E is the matrix of the predictors of the fixed effects, a_s is the random factor characterized by the inter-site variance σ_s^2, $a_{j|s}$ is the random factor characterized by the inter-stand variance within a same site σ_j^2 and ε is the error term of variance σ_ε^2. In addition, linear mixed effect models using site as random factor were used to test the significance of the difference between pure and mixed stands within each species for each *ilr* balance or nutrient content.

We then investigated the relationship between the species-mixing effect on tree nutrition, and the site nutritional status. To do so, we computed the difference between the *ilr* balance/nutrient content per tree and the corresponding average value per site, for each species separately, and used this index as the response variable. Stand composition (pure vs. mixed), site nutritional status and their interaction were used as explanatory variables in multivariate linear regression models (Eq. (4)):

$$Y_{ijs} - \bar{Y}_s = a + \beta \times E_{js} + \varepsilon(0, \sigma_\varepsilon^2) \quad (4)$$

Y_{ijs} represents the *ilr* balance/content of interest for the ith individual (tree) in the jth stand (pure or mixed) and the sth site for each species separately, and $\bar{Y}_s$ is the average value of the *ilr* balance/content of either beech or pine trees across the pure and mixed stands in site *s*. α is the intercept, β is the vector of the parameters, E is the predictor matrix (stand composition, site nutritional status and their interaction) and ε is the error term of variance σ_ε^2. Considering that the *ilr* balance [Fv|Mg, Ca, K, P, N] contrasts five mineral elements of major importance for tree growth and functioning against all other elements, we used its average value per site and species as an index of site nutritional status for all *ilr* balance models. For the foliar nutrient content models, we used the average value of the corresponding nutrient content per site and species as the index of site nutritional status. In all cases, the variables used as indicators of site nutritional status were centered. The contrasting average values of either *ilr* balances or nutrient contents between sites resulted in empirical gradients of nutritional status.

Dependent variables of the mixed and multivariate linear models were normally distributed with the exception of the nutrient content used in the mixed models which were therefore ln-transformed.

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

Compositional data analyses were conducted using the "composition" package (Van den Boogaart et al. 2019). The closure operation was conducted using the "acomp" function while the *ilr* transformation

operation was done on the closed data space with the "ilr" function. Mixed models were fitted with the package "nlme" (Pinheiro et al. 2017).

3. Results

3.1. Site nutrient status

The range of foliar nutrient concentrations within and among sites for beech and pine, along with the corresponding optimum range (Mellert and Göttlein 2012), is presented in Fig. 2. For both species and most combinations of sites and nutrients, there was a high variability in foliar concentrations. Also, the studied sites spanned a large range of tree nutrient status (Tables A1 and A2).

With the exception of K for pine, all nutrients were found to be deficient for at least one combination of site and species (Table A2). While nutrition appeared to be non limiting for neither beech nor pine in two sites (BUL1, CZE1), some sites showed deficiencies for more than one nutrient. For beech, BEL1 and FRA1 were deficient for the same three nutrients (P, Ca, Mg); for pine, two different nutrient deficiencies were observed in FRA1 (P, Mg), LIT2 (N, Ca) and POL3 (N, P). At some sites, the same nutrients appeared to be limiting for both beech and pine (Ca in BEL1; P and Mg in FRA1). Two sites (GER7 and LIT1) displayed no deficiencies for beech while deficiencies were observed for pine (P and N limitation in GER7; N limitation in LIT1).

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 pine needles; the relative difference between species was most pronounced for Ca, followed by Mg and N concentrations, and then K (Table 2).

3.3. Stand composition effect on ilr balances and nutrient content

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

For all investigated *ilr* balances, at least one species displayed a significant species-mixing effect (Fig. 3). For beech, [Mg, Ca|K, P, N] and [P|N] balances were higher in mixed stands than in monocultures while the opposite was true for [K|P, N]. For pine, mixed stands displayed higher [Fv|Mg, Ca, K, P, N] and [Mg|Ca] balances and lower [Mg, Ca|K, P, N] and [P|N] balances than monocultures.

For three *ilr* balances ([Mg, Ca|K, P, N], [Mg|Ca] and [P|N]), the effect of stand composition differed between species (Fig. 3; significant species × stand composition interaction), with two *ilr* balances showing opposite effects of mixing: compared to monocultures the [Mg, Ca|K, P, N] and [P|N] balances in mixed stands were higher for beech and lower for pine.

Looking at nutrient contents (Fig. 4), mixing increased P contents in pine and K contents in beech; compared to the monocultures, Ca contents of the mixed stands were lower in beech and higher in pine.

3.4. Impact of site nutrient status on species mixing effect

The effects of species-mixing effect on *ilr* balances along the gradient of site nutritional status for beech and pine are displayed in Figs. 5 and 6, respectively.

For beech, the [Mg|Ca] and [P|N] balances were significantly affected by the site nutrient level, the stand composition, and their interaction. For both *ilr* balances, the difference between pure and mixed stands was highest at the lower end of the gradient where mixed stands were associated with higher [P|N] and lower [Mg|Ca] balances, compared to pure stands. For the [Mg,Ca|K,P,N] and [K|P,N] balances, only the interaction of site and stand was significant, indicating that the site nutrient status had a different impact in pure and in mixed stands. No significant effect was detected for the [Fv|elements] balance.

For pine, the [Fv|elements] balance was significantly higher in mixed than in pure stands, and the effect was constant along the soil fertility gradient. The interaction of site and stand was significant for the [K|P,N] and [Mg|Ca] balances, showing significantly different responses to site nutrient status of pure and mixed stands.

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 and Mg, the effect of site differed between pure and mixed stands, with the strongest difference among stand types observed at the richer sites. There was no main stand composition effect, yet the associated p-value was quite close to 0.05 (Table A7). P contents did not respond to either site nutrient status nor stand composition. For pine nutrient contents (Fig. 8), P and Mg showed a similar pattern. There was an overall significant site and stand composition effect, yet the effect of an increased site fertility was negative in the pure stands and positive in the mixtures. For K and Ca, the effect of site also differed between pure and mixed stands. For K, the interaction was associated with a significant site effect and to opposite effects of stand composition at both extremities of the gradient (higher K content in mixed stand at the lower end of the gradient and lower K content in mixed stand at the higher end of the gradient). The Ca contents of pine needles were on average significantly higher in the mixed stands, yet the stand composition effect was more pronounced as soil fertility increased. N contents in pine needles did not change significantly with either stand composition nor site fertility.
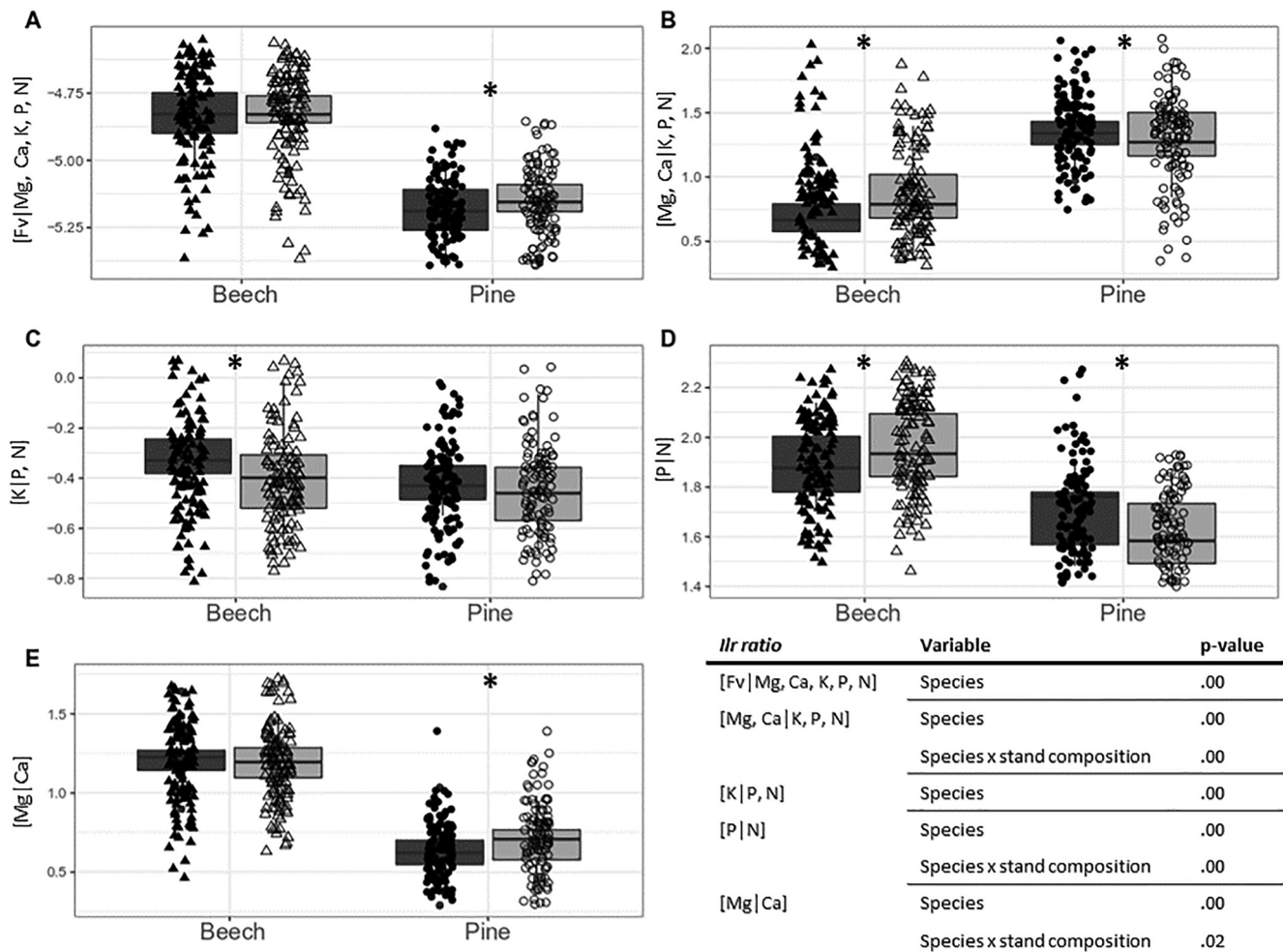
Table 2

Summary of the foliar nutrient composition of beech and current year pine needles. Mean ilr balances (1) and nutrient concentrations (mg.g^{-1}) (2) across all stands and sites (standard errors in parentheses). For each foliar variable (ilr balances or 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)

Fig. 3. Beech and pine foliar ilr balances (boxplots and individual (tree) observations) as a function of species identity (beech/pine - current-year needles) and stand composition (pure/mixed) across all sites. The individual tree observations are depicted by black filled triangles (beech) or dots (pine); filled and open symbols denote pure and mixed stands, respectively. Black stars indicate significant differences between pure and mixed stands of a same species at P < 0.05 from linear mixed models adjusted on each species and each ilr balance separately. The statistical significant effects of the mixed models (Eq. (3)) are shown in the enclosed table, while the detailed statistics for all ilr balance models are given in Table A4.

4. Discussion

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

Our results showed that beech leaves and current year pine needles have distinct foliar nutrient signatures. To start with, it is essential to remember that as a result of the computation procedure and notation (Eq. (2)), the *ilr* balances behave differently than traditional raw elemental ratios. An increase in *ilr* balance means that either the left part (the balance denominator) has decreased, and/or the right part (the balance numerator) has increased. In this respect, the higher [P|N] and [Mg|Ca] balances in beech leaves compared to pine needles are consistent with the corresponding average nutrient concentrations (Table 2). The increased [K|P,N] in beech leaves can mainly be attributed to the much higher N concentrations in beech leaves compared to pine needles, given the limited difference in K concentrations and the similarity of P concentrations. The significantly higher [Mg,Ca|K,P,N] balance in pine needles can primarily be attributed to the strongly reduced Ca concentrations of pine needles compared to beech leaves, and to a lesser extent, to the lower Mg concentrations of pine needles. Finally, the higher [Fv|Mg, Ca, K, P, N] value in beech leaves is consistent with the higher concentrations of most measured nutrients in beech leaves (Table 2), and their significantly lower Fv value (data not

shown); the latter is composed of unmeasured macro- and micro-elements. We are not able to point out those elements, which are involved in this difference.

The differences in average nutrient concentrations between beech and pine irrespective of stand type (pure/mixture) were in close agreement with literature data (González de Andrés et al. 2019, Mellert and Göttlein, 2012), confirming the distinctly higher N, Ca and Mg concentrations in beech leaves compared to current year pine needles (Liu et al. 2006, Sardans et al. 2015). According to Sardans et al. (2015) such distinct foliar signatures probably result from long-term differentiation of metabolic and physiological functions and morphology leading to species-specific optimal elemental composition according to the biogeochemical niche hypothesis.

The contrasted elemental composition and stoichiometries thus indicates differences in nutritional characteristics between species. Because distinct characteristics between co-occurring species are necessary to entail complementarity, such differences could lead to reduced competition (Sardans et al. 2015) and improved nutrition in mixed stands.

4.2. Overall mixing effect on ilr balances and nutrient content

When significant, the stand composition effect resulted in increased nutrient contents in the mixed stands compared to the monocultures in
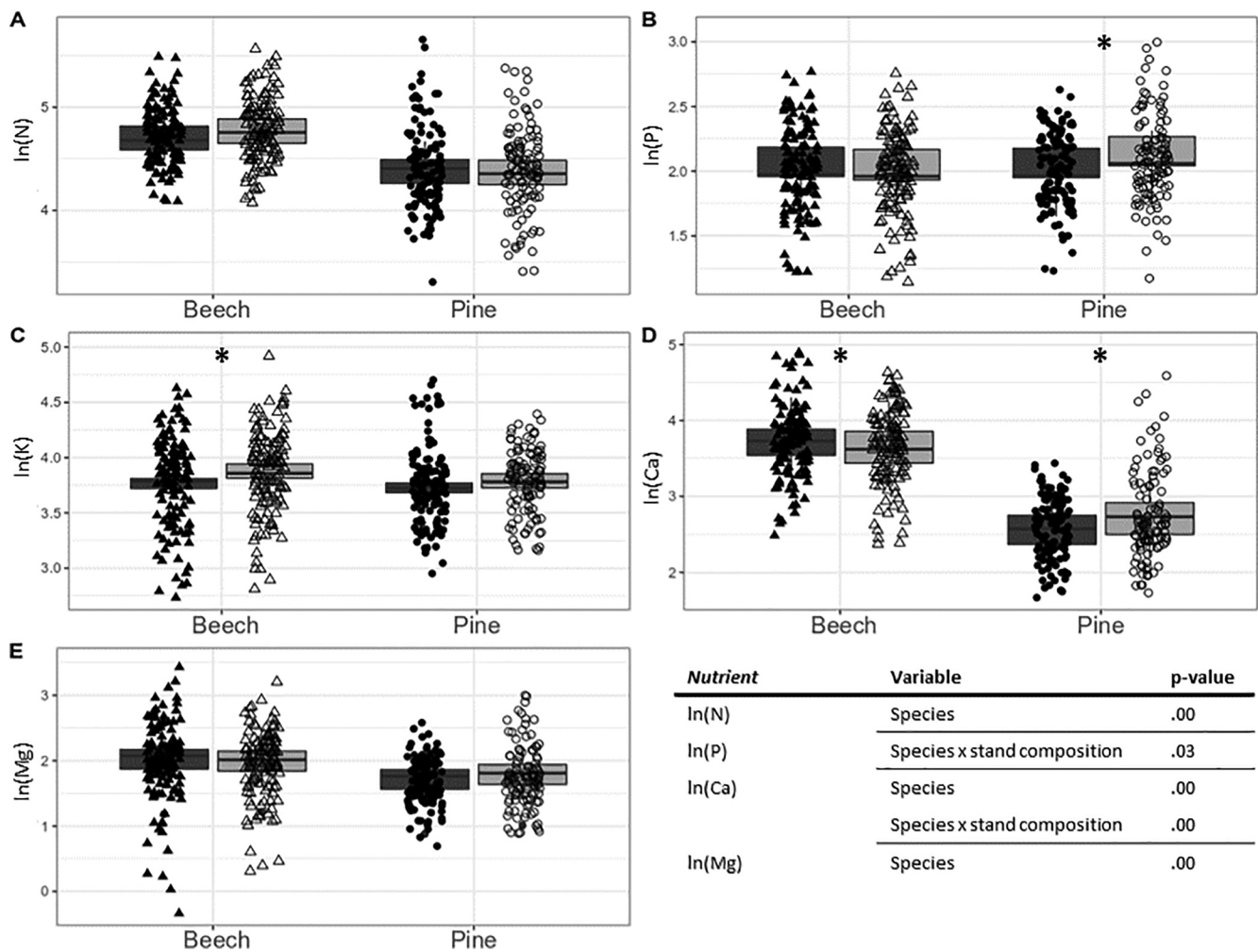
Nutrient	Variable	p-value
ln(N)	Species	.00
ln(P)	Species x stand composition	.03
ln(Ca)	Species	.00
	Species x stand composition	.00
ln(Mg)	Species	.00

Fig. 4. Beech and pine foliar nutrient contents (boxplots and individual observations as a function of species identity (beech/pine - current-year needles) and stand composition (pure/mixed) across all sites. The individual tree observations are depicted by triangles (beech) or dots (pine); filled and open symbols denote pure and mixed stands, respectively. Black stars indicate significant differences between pure and mixed stands of a same species at P < 0.05 from linear mixed models adjusted on each species and each nutrient content separately. The statistically significant effects of the mixed models (Eq. (3)) are shown in the enclosed table, while the detailed statistics for all models are given in Table A5.

all (P and Ca in pine, K in beech) but one case (Ca in beech) (Figs. 3 & 4). The positive effect of beech on pine Ca and the corresponding negative effect of pine on beech Ca could be related to the strong difference in nutrient Ca concentration between beech leaves and pine needles (Table 2), resulting in changed nutrient inputs through litterfall. Differences in litterfall between species have been shown to influence soil nutrient availability (Fassnacht and Gowerr, 1999; Polyakova and Billor, 2007; Vesterdal, 1998). However, such a difference in nutrient composition between leaves and needles could not explain the other synergistic effects. Indeed, pine P content and K beech content increased in mixed stands compared to pure stands even though foliar P concentrations are similar for both species and K foliar concentrations are lower in pine than in beech. In addition, no stand composition effect could be detected for some nutrients e.g., for nitrogen contents in pine needles, despite the much higher N concentrations in beech leaves compared to pine. Previous studies have found contrasting results on the impact of species mixing on tree foliar nutrition depending on the element considered. Some studies are consistent with our results. For instance, Brandtberg (2001) and Thelin et al. (2002) found P and K concentrations in foliage of conifers to increase if broadleaves were present. Berger et al. (2009) found a tendency to higher Ca concentrations in mixed stands compared to pure stands for coniferous species and lower concentrations for beech stands. On the other hand,

our findings differed from some other studies. For instance, Magh et al. (2018) hypothesized broadleaves N acquisition to be lower in mixed stands due to a negative impact of the more recalcitrant conifer litter on the mineralization rates of organic matter. However, we did not find lower amounts of N in beech foliage in mixed stands compared to pure stands. One possible explanation is that soil N availability is also strongly influenced by other environmental drivers than litter quality (Anderson and Domsch 1993, Brown 1992). Such simultaneous influence of several factors and their interaction on species-mixing effects on foliar are not well studied yet.

Altogether, our results suggest that differences in foliar nutrient concentrations between associated species and the corresponding change in nutrient inputs, are not strong enough to be used as predictors of overall species-mixing effects. Obviously, additional factors should be considered to explain the large variability in species-mixing effects between sites. This is in line with the results from Rothe et al. (2003). In their study, foliar nutrient pools were not always good predictors of nutrient availability. Several other mechanisms such as the effect of species mixing on litter production through increased canopy packing (Barbeito et al., 2017; Jucker et al., 2015; Pretzsch, 2014; Scherer-Lorenzen et al., 2007) or species mixing effect on rates of litter decomposition (Joly et al., 2016; Jonard et al., 2008) can be expected to also come into play although we were not able to properly assess their
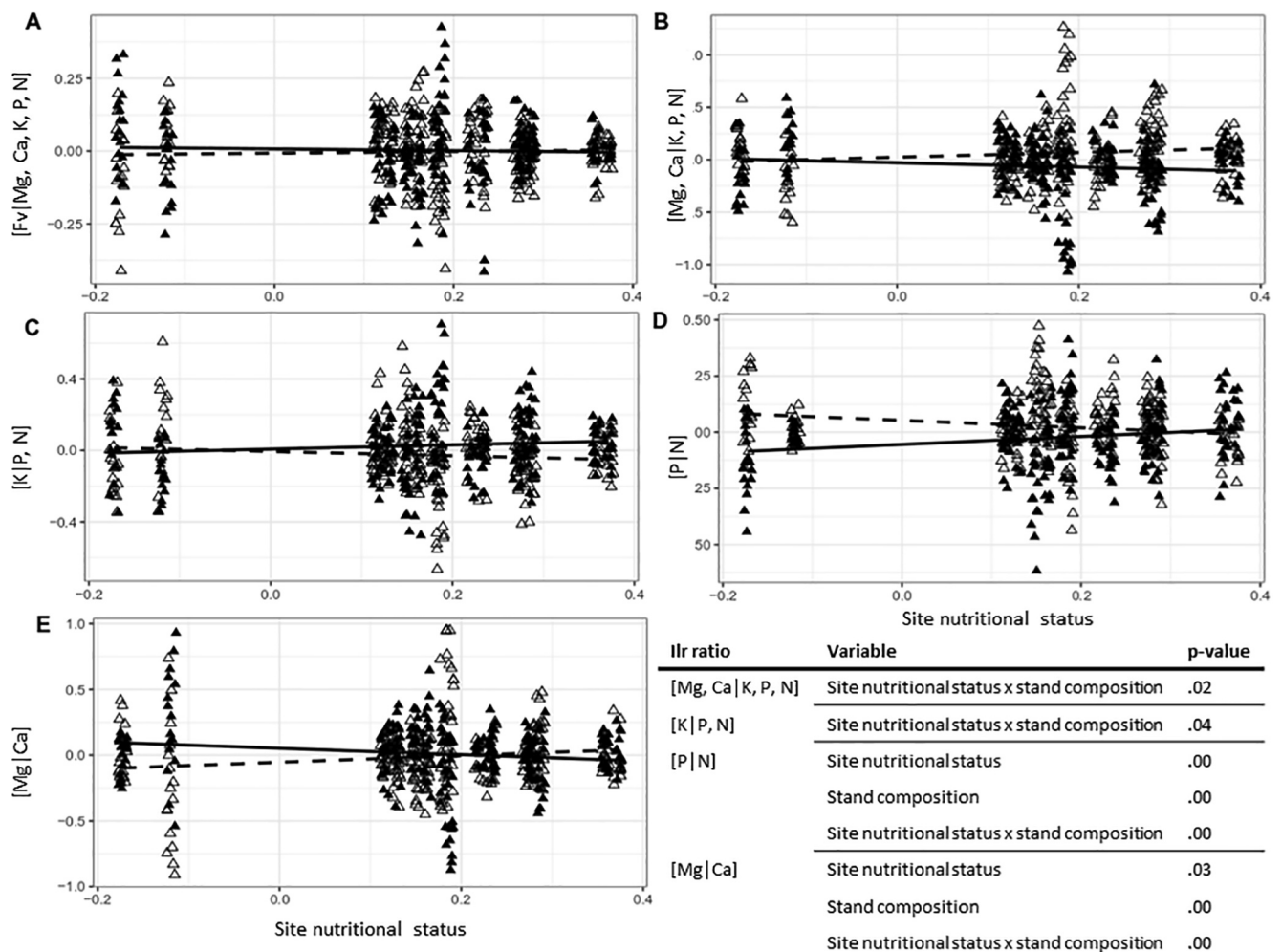
Fig. 5. Difference between individual ilr balances of beech trees and the corresponding average value per site as a function of site nutritional status and stand composition. The ilr balance differences are represented by filled (pure stands) or open triangles (mixed stands). The lines represent the 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 species-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".

relevance for and contribution to our findings. More specifically, beech is expected to have a positive impact on pine nutrition through improvement of the humus layer, the upper mineral soil and exploitation of nutrients from deeper soil horizons (Chodzicki 1934, Pretzsch et al. 2015). The higher nutrient content, lower C:N ratio and lower amount of recalcitrant compound in beech litter compared to pine (Rumberger et al. 2004, Zhong and Makeschin 2004) could lead to reduced soil acidity and richer humus which will in turn improve nutrient availability in mixed stands compared to pure pine stands (Collin et al. 2016, González de Andrés et al. 2017). In addition, the distinct potential rooting pattern of both species (heart-shaped fine root profile for beech vs. peak of fine root biomass in the most superficial soil layers for pine; Prévosto and Curt 2004) can lead to higher combined root occupancy in mixture and thus, more complete belowground exploitation at least in some sites (González de Andrés et al. 2017).

In addition to the observed effect on selected nutrient contents, mixing beech and pine changed nutrient balances. Two *ilr* balances significantly differed between pure and mixed stands for both pine and beech trees: [Mg,Ca|K,P,N], [P|N], yet in an opposite direction (Fig. 3). The three other *ilr* balances were also affected by stand composition, but only for one species. To our knowledge, few studies analyzed the influence of species mixing on tree foliar nutrition using elements balances. Thelin et al. (2002) found the "classical" ratio P:N to be higher in Norway spruce needles when mixed with deciduous species

(beech, birch or oak), which is coherent with our finding. The absence of a species mixing effect on beech [Mg|Ca] is coherent with the findings of Collin et al. (2016) who used isometric log-ratios to study the nutritional characteristics of sugar maple and red maple in different contexts (pure stands vs. mixed with conifers; increasing soil acidity levels). They explained the absence of effects by a simultaneous decrease in Mg and Ca with increased conifer proportions. In contrast to our results, however, Collin et al. (2016) also found a decrease in the [Fv|Mg, Ca, K, P, N] balance with increasing proportions of conifers, which they attributed to the effect of increased soil acidity on Al and Mn availability (higher ionic activity in more acidic conditions); as those elements were not measured in the leaves, they were expected to increase correspondingly the Fv value. The dependency of those mechanisms on local factors, like the forest management strategy, the acidifying potential of the species involved or the initial soil acidity could explain contrasting results between studies. Importantly, the change in *ilr* balance suggests that mixing can modify tree nutrition by altering some key nutrient balances, well beyond changing specific individual nutrients. This suspected complementarity with regard to mineral nutrition could be, at least in part, the cause of the increased productivity highlighted on the same network of pine and beech by Pretzsch et al. (2015) as proposed by Burkhart and Tham (1992), Frivold and Kolström (1999), and Kelty (1992). However, our data set does not allow to properly explore the relationships between tree/stand
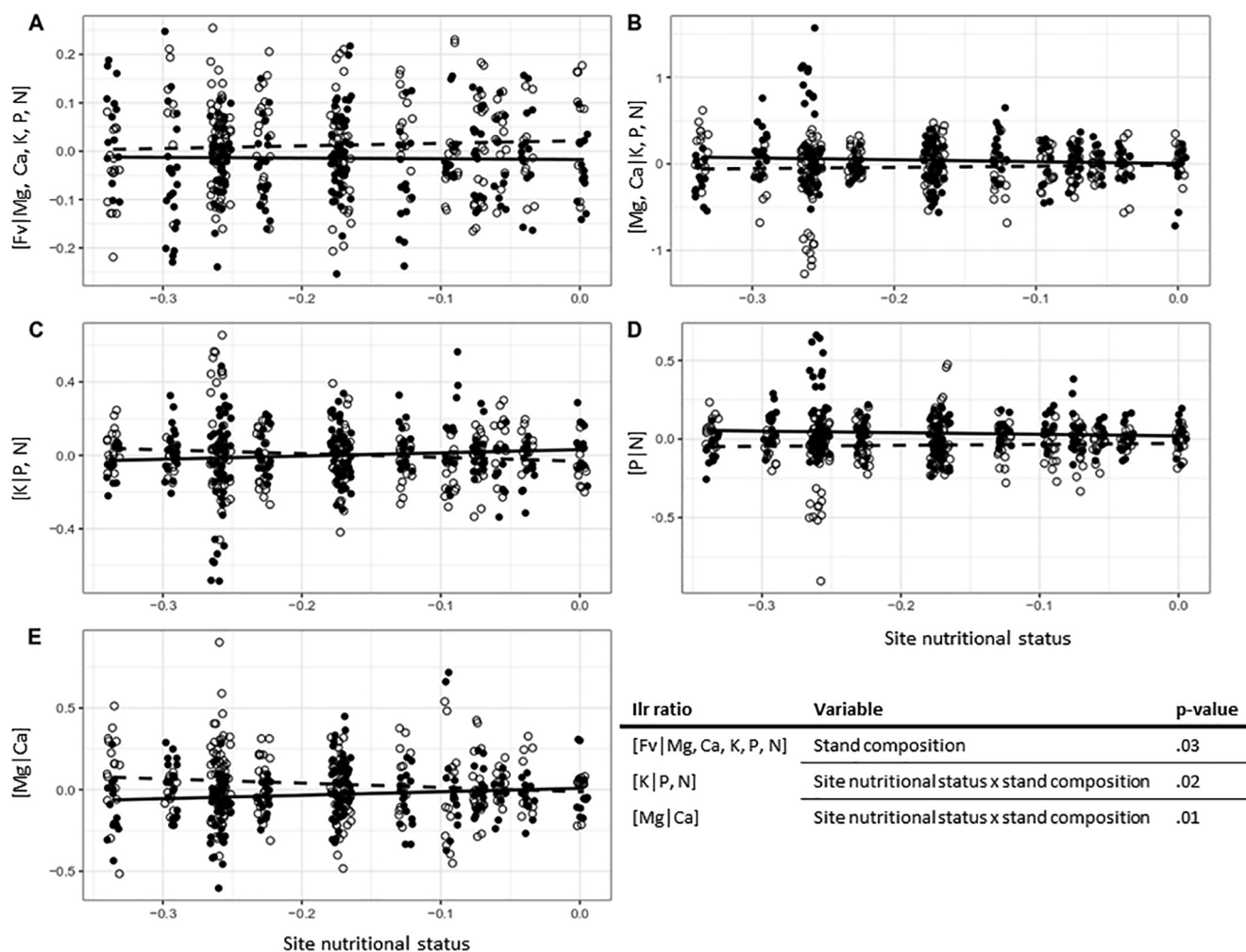
Ilr ratio	Variable	p-value
[Fv│Mg, Ca, K, P, N]	Stand composition	.03
[K│P, N]	Site nutritional status x stand composition	.02
[Mg│Ca]	Site nutritional status x stand composition	.01

Fig. 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".

productivity and nutrition, due to the limited number of sites compared to the potentially high number of processes involved (cf. e.g. Forrester and Bauhus, 2016 for a comprehensive review on those processes).

4.3. Mixing effect as a function of site nutrient status

Regarding the impacts of mixing on nutrient contents, there was a close agreement between the results of the overall analysis (Eq. (3)) and the analysis where the nutrient gradient was explicitly accounted for (Eq. (4)); the only exception to this agreement was Mg under pine for which the stand composition effect was not significant in the overall analyses (p-value = 0.13). By contrast, we found more discrepancies between the two approaches for the *ilr* balances. A possible explanation could be related to the index we used to characterize the gradient of nutrient availability for the latter approach ([Fv│Mg, Ca, K, P, N] for all *ilr* balances). We deliberately selected Fv│[Mg, Ca, K, P, N] over the traditional site index (SI) in our case as the latter integrates all site factors, and not only nutrients (Brandl et al. 2014). The use of other indicators such as foliar nutrient remobilization (Achat et al. 2018), soil pH or soil exchangeable cation pools in future studies should help improve our understanding of the dependency of species-mixing effect on site chemical fertility. It is also important to note that, for beech, the sites were not homogenously distributed along the [Fv│Mg, Ca, K, P, N]

gradient (Fig. 5); the two sites at the lower end of the gradient could have had a strong influence on the slope for some models (see the [P│N] balance for instance). One way to cope with this would be to select alternative indicators of soil fertility (see above), and/or to increase the sampling to fill-in the current gaps along the gradient.

Focusing on the contents, we expected the mixing effect to increase with a decrease in nutrient availability according to the framework proposed by Forrester and Bauhus (2016). However, in all cases where we found a significant overall stand composition effect (K and Ca for beech, Fig. 7; P, Ca, Mg for pine, Fig. 8), we actually observed the opposite, with only minor or no difference between monocultures and mixtures on the poorest sites, and maximum differences on richer sites.

The limited stand composition effect or even the lack of any significant mixing effect in low-nutrient sites could first be explained by the co-occurrence of several nutrient limiting factors. Indeed, the uptake of a nutrient is not only determined by its availability but also by the availability of other nutrients and their interactions, and by environmental conditions such as soil pH or water availability (Marschner, 2011; Wilkinson, 2000). Simultaneous limitations of distinct nutrients have been observed for both beech and pine stands at several locations (for instance, site FRA1 displayed P and Mg deficiencies for both species and BEL1 displayed Ca deficiency for both species and Mg deficiency for beech) and could originate from the
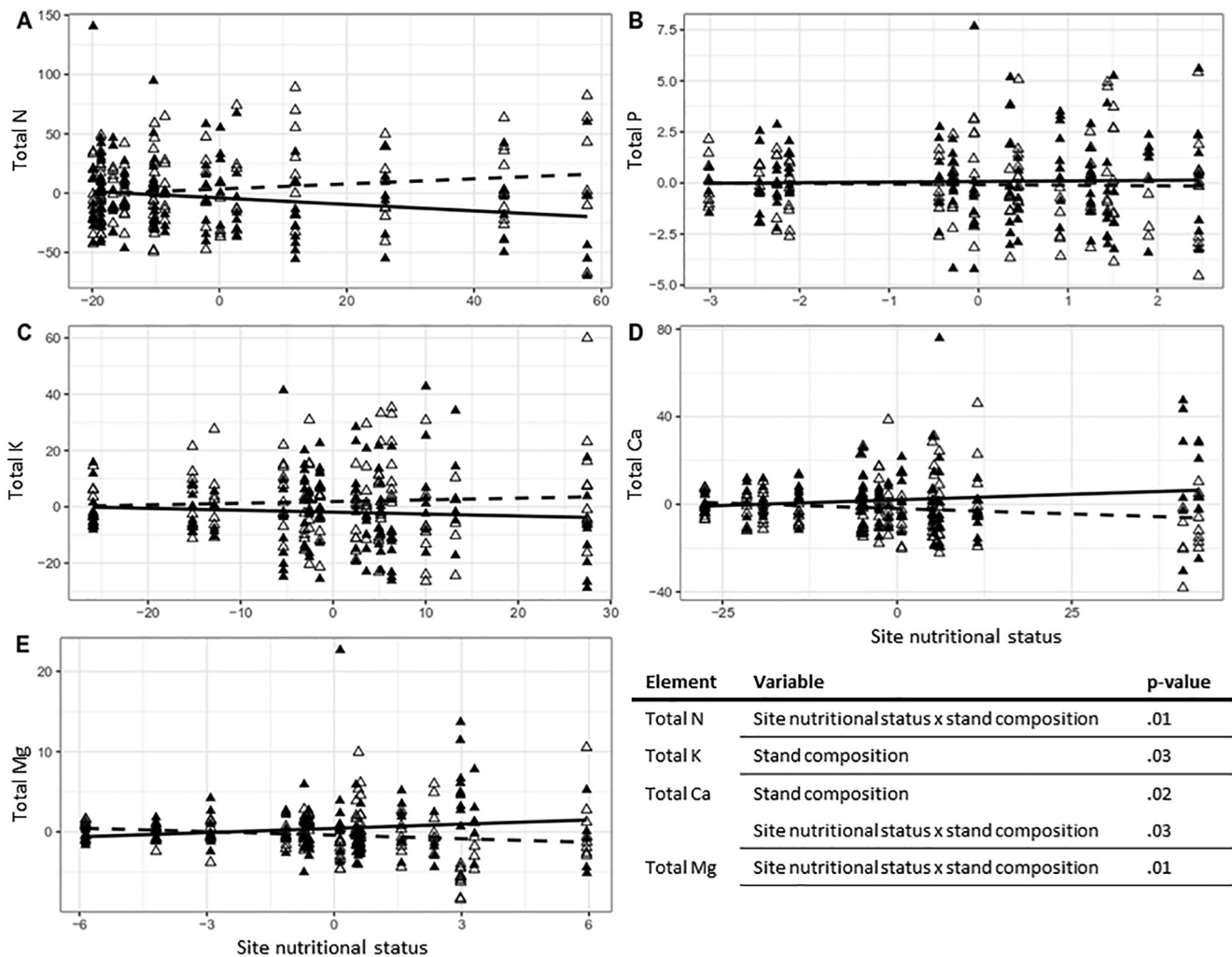
Element	Variable	p-value
Total N	Site nutritional status x stand composition	.01
Total K	Stand composition	.03
Total Ca	Stand composition	.02
	Site nutritional status x stand composition	.03
Total Mg	Site nutritional status x stand composition	.01

Fig. 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".

coexistence of several factors limiting nutrient availability. It might be advocated that even in the case where the availability of one limiting nutrient has increased as a result of mixing, this would not translate into an increased foliar content as far as another constraint limiting nutrient availability (e.g. limited water availability) would still be present.

A second hypothesis to explain our results is related to all those situations where species interactions may not improve the availability of the limiting nutrient. This could be due to the intrinsic pool of traits present, where inter-specific differences in physiology, phenology or morphology do not improve nutrient availability. Indeed, traits of the species involved in the mixture are of primary importance to determine whether this mixture benefits from improvement of resources availability, uptake or use-efficiency (Ammer 2019). We expected from an association of species with such distinct foliar traits as beech and pine (for instance in term of leaf life span), to induce differences in foliar nutrient composition compared to monocultures. However, such differences in traits may not systematically influence the availability, uptake or resource-use efficiency of nutrients and thus, the foliar nutrient composition. Another possible explanation is the existence of factors limiting the expression of any potential complementary interaction. Jucker et al. (2014) highlighted such a mechanism in Mediterranean mixed forests. They found that species mixing improved

stand growth through complementary light use but that this effect was severely reduced if water resources became limiting. Considering the interspecific differences in rooting patterns between beech and pine (Prévosto and Curt 2004) as a candidate process to increase nutrient availability in mixed species stands compared to monocultures (Forrester and Bauhus 2016), any constraint that would limit the potential development of the rooting system (e.g. anoxic conditions; Kozlowski 1986) would alter this potential spatial stratification.

The largest differences in foliar nutrient contents between monocultures and mixed-species stands were observed on the richer sites, with synergistic effects in all cases except for Ca in beech (see previous section). The improved tree nutrient composition of the mixtures at the higher end of the fertility gradient was unexpected, as those situations reflect normal ranges or even a surplus of the nutrient in question. However, an adequate supply of one given nutrient does not exclude potential limitations of others, whereas their availability could in turn limit the uptake of the target nutrient. If mixing removes this constraint, this could explain why a synergistic effect of mixture occurred. An additional explanation is an increased competition in pure stands on the nutrient rich sites due to increased growth, not compensated for by the higher nutrient availability. Bravo-Oviedo et al. (2006) for instance, found that faster tree growth in richer sites lead to increased competition and higher individual tree mortality. In this context, species-
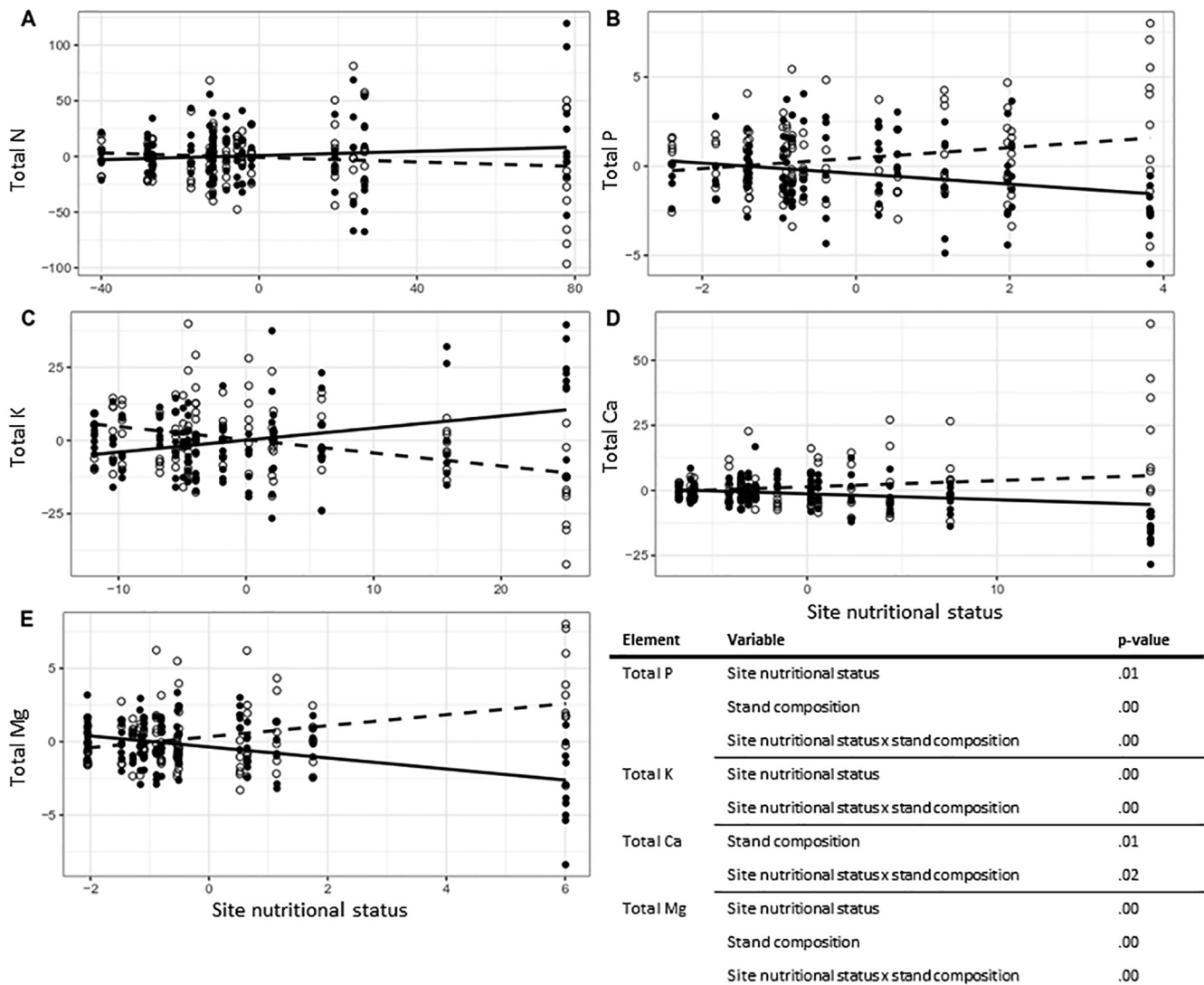
Element	Variable	p-value
Total P	Site nutritional status	.01
	Stand composition	.00
	Site nutritional status x stand composition	.00
Total K	Site nutritional status	.00
	Site nutritional status x stand composition	.00
Total Ca	Stand composition	.01
	Site nutritional status x stand composition	.02
Total Mg	Site nutritional status	.00
	Stand composition	.00
	Site nutritional status x stand composition	.00

Fig. 8. Difference between individual foliar content of pine 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 dots (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".

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

5. Conclusion

Considering the impact of mixing on single nutrient contents, we mostly found either no effect or synergies, with only one case where nutrient contents were lower in mixed stands (antagonism for Ca in beech). Our results showed that the contrasting foliar nutrient compositions of pine and beech trees alone were unable to explain the mixing effects on tree nutrition, which suggests that other mechanisms than a simple change of nutrient inputs through litterfall are at play. Far beyond changing the foliar contents of single individual nutrients, mixing was found to significantly alter key nutrient balances in both beech leaves and current year pine needles.

Our analysis also revealed unexpected patterns of complementarity as a function of nutrient availability, with only minor or no differences between monocultures and mixtures at the lower end of the nutritional gradient, and maximum differences on nutrient rich soils. This clearly points to further expanding the existing frameworks to account for the multivariate nature of tree nutrition. In this paper, we made a first attempt in that direction by working on both contents and *ilr* balances. Further work is needed to disentangle the underlying processes, using a combination of field work and modeling approaches. This claims for the establishment of controlled standardized experiments across Europe to test for specific mechanisms.

CRediT authorship contribution statement

Géraud de Streel: Conceptualization, Methodology, Validation, Investigation, Writing - original draft, Formal analysis, Data curation, Visualization. **Christian Ammer:** Investigation, Resources, Writing - review & editing. **Peter Annighöfer:** Investigation, Resources, Writing - review & editing. **Ignacio Barbeito:** Investigation, Resources, Writing - review & editing. **Kamil Bielak:** Investigation, Resources, Writing - review & editing. **Andrés Bravo-Oviedo:** Investigation, Resources, Writing - review & editing. **Gediminas Brazaitis:** Investigation, Resources, Writing - review & editing. **Włodzimierz Buraczyk:** Investigation, Resources, Writing - review & editing. **Catherine Collet:** Investigation, Resources, Writing - review & editing. **Vaclav Hurt:**

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foreco.2020.118551.

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