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Title: Effect of a tree mixture and water availability on soil nutrients and extracellular enzyme activities along the soil profile in an experimental forest

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Keywords: biodiversity, drought, nutrients, precipitation, soil extracellular enzyme activity

Highlights:

- Synergistic effect of tree diversity on C and N enzymes in the mid-soil
- Irrigation increased N and P enzyme activities in the upper soil layers
- No interaction effect between tree species composition and water availability
- Strong positive correlation between the available P and the C_{enz}:P_{enz} ratio

Abstract

An increasing number of studies demonstrate that tree species biodiversity can affect primary productivity and nutrient cycling in forests due to several factors, such as complementarity, facilitation or selection effects. For instance, resource partitioning in soils has been found to allow a more optimized nutrient uptake in mixed species plots compared with monocultures. However, how these effects will be modified by climate change – such as water availability – is not as well understood, especially in deep soil layers. Therefore, we specifically asked how water availability may influence the effect of tree mixtures on soil microorganism activity by measuring extracellular enzyme activities (EEAs) and available nutrients along the soil profile (down to 90 cm) in a 10-year-old plantation in southwestern France, which manipulates tree species composition (*Pinus pinaster* and *Betula pendula*, in monocultures and in mixed plots) and irrigation. Our results showed that EEAs directly depend on tree species composition and water conditions in interaction with soil depth; we found a positive effect of mixing birch and pine on carbon (C)- and nitrogen (N)-related EEAs at an intermediate soil depth (15–30 cm soil layer), while the effect of increasing water availability increased phosphorus (P)-related EEAs mostly in the upper soil layers (0 to 30 cm). However, we found no significant interactive effect between tree diversity and water availability on EEAs, underlying that the negative effect of lower water availability cannot be offset by the positive effect of mixing tree species. Differences in EEAs and available nutrients along the soil profile highlight the importance to look at different soil depths to better understand how nutrient cycling may be affected by increasing tree diversity and changes in water availability.

1. Introduction

Forest biodiversity provides a multitude of ecosystem goods and services (Gamfeldt et al., 2013; Jactel et al., 2017; Liang et al., 2016). However, forests in many areas are threatened by more intense and frequent drought periods (IPCC, 2014; Schwalm et al., 2012), with important negative consequences such as on nutrient cycling (He and Dijkstra, 2014; Schlesinger et al., 2015) or even on tree survival (Hartmann et al., 2018). A well-explored option to mitigate these negative effects is to increase tree stand diversity (Lebourgeois et al., 2013; Pretzsch et al., 2013). Indeed, mixed-species forests generally have been found to be more productive than pure stands (Baeten et al., 2019; Cardinale et al., 2007; Liang et al., 2016; Zhang et al., 2012) and are more stable to climatic fluctuations due to overyielding, species asynchrony and favorable species interactions (Jucker et al., 2014a; Kardol et al., 2018; Morin et al., 2014). However, recent studies have shown that the positive effect of tree diversity on forest productivity might be annihilated under drought conditions (Jactel et al., 2018). In addition, not all tree species mixtures are more resistant to water stress (Grossiord, 2019). Although many studies on biodiversity effects focused on aboveground plant productivity, only a few addressed belowground productivity and soil functioning (Alberti et al., 2017; Brassard et al., 2013; Ma and Chen, 2016). Furthermore, we know little about how limiting water conditions may affect biodiversity benefits and if their interactive effects may affect soil microbial activity and biogeochemical cycling in mixed forests.

An important step of carbon (C), nitrogen (N), and phosphorus (P) cycling is the decomposition of organic matter that derives from leaf and root litter (Hättenschwiler et al., 2005; Zechmeister-Boltenstern et al., 2015). Among the main mechanisms involved in decomposition, one process is based on extracellular enzymes

(Sinsabaugh et al., 2002). Soil microorganisms release extracellular enzymes to acquire energy and resources by degrading neighboring soil organic matter (Burns, 1982; Burns et al., 2013). Studying soil extracellular enzyme activities (EEAs) can thus give information on potential belowground microbial mineralization processes and how microbial communities drive C, N, and P cycling in soils (Allison et al., 2007; Fanin et al., 2016a; Henry, 2013). Soil EEAs are measured to assess microbial responses to global changes, including precipitation changes in varying ecosystems (Henry, 2013). EEAs have been found to be negatively affected by water stress (Brockett et al., 2012; Schlesinger et al., 2015), including in forests (Sardans and Peñuelas, 2005). A recent global meta-analysis concluded that drought led to a significant decrease in urease and phenol oxidase activity, while an increase in precipitation significantly increased N-related enzyme activities (Xiao et al., 2018). However, these effects may vary with soil depth due to water runoff dynamics and infiltration from topsoil to deeper soils. Topsoil is thus expected to be more positively affected by an increase in precipitation due to its increase in soil moisture, a major factor determining EEAs in soils (Brockett et al., 2012), whereas deeper soils may be less affected by precipitation, have less varying moisture and thus less effect on EEAs (Fanin et al., 2019).

Tree species may also have important effects on EEAs due to their varying litter qualities (Weand et al., 2010). Recently, Alberti et al. (2017) found a positive synergistic effect of tree diversity on EEAs, due to the influence of varying litter traits on soil C and N mineralization. These results suggest that an increase in tree species richness may influence biogeochemical cycling due to stimulation of microbial activity (Hacker et al., 2015; Steinauer et al., 2014). Because tree species diversity may positively influence microbial biomass and activity (Chen et al., 2019; Lucas-

Borja et al., 2012; Thakur et al., 2015; West et al., 2006), and that both microbial biomass and substrate pools decrease with depth (Agnelli et al., 2004; Fierer et al., 2003), there is a need to study the effect of tree diversity on EEAs along the soil profile (Herold et al., 2014; Loeppmann et al., 2016; Stone et al., 2014). The diversity effects may also directly depend on root biomass along the litter-soil continuum. Mixing tree species with contrasting root systems may lead to niche partitioning along the soil profile (Brassard et al., 2013), which can benefit exploration of the total soil volume (Schmid and Kazda, 2002) and total nutrient uptake (Richards et al., 2010). In addition, facilitation mechanisms between associated species, such as hydraulic lift (Zapater et al., 2011), may be especially advantageous in mixed stands during drought events. Thus, investigating how tree diversity may offset the negative effects of drought along the soil profile would provide new insights into how biodiversity effects on nutrient cycling may change in a future climate.

In this study, our primary objective was to investigate the interactive effect between tree diversity and water availability on available nutrients and EEAs along the soil profile. In practice, we sampled soils at five depths in an experimental plantation where both tree diversity and water availability (through irrigation) were manipulated and tested three hypotheses. First, we tested the hypothesis (H_1) that EEAs would be higher in a two-species mixture than in the corresponding pure plots since mixing different litter qualities may lead to more synergistic litter decomposition (Chapman et al., 2013; Cornwell et al., 2008). Specifically, we hypothesized that the effect of mixing trees on EEAs should be higher in the topsoil layer, where the effect of tree species identity is highest (Augusto et al., 2002) and where the organic matter quantity is highest and richest in nutrients (Jobbágy and Jackson, 2001; Melillo et al., 1989). Secondly, we hypothesized (H_2) an increase in water supply would have a positive effect on EEAs by alleviating the water constraint (Sardans and Peñuelas, 2005; Xiao et al., 2018). We anticipate that the effect of increasing water availability should be highest in the topsoil, mainly because microbial communities at the soil surface are often more subjected to water stress than those living in deeper soil horizons (Fanin et al., 2019). Finally, we hypothesized (H_3) that the positive effect of mixing two tree species on EEAs would be strongest in the plots experiencing the dry summers. This is because mixing tree species with contrasting root exploration strategies may favor drought resistance through facilitation mechanisms (Bello et al., 2019).

2. Materials and methods

2.1. Experimental site and sample collection

We conducted this study at the ORPHEE experiment in southwestern France, 40 km southwest of Bordeaux (44°44.35' N, 00°47.9' W, altitude of 60 m asl) (Castagneyrol et al., 2013). It is part of the international Tree Diversity Network (TreeDivNet) and is located in the largest artificial forest in occidental Europe (Landes de Gascogne). The mean annual temperature is 12.5°C and the mean average precipitation is 870 mm (1993-2018), but most precipitation occurs from the fall to the spring, leading to very dry summers, which we consider representative of water stress. The former vegetation was a mature *Pinus pinaster* stand located on a flat area. The tree plantation in ORPHEE occurred in 2008, after a clear-cut of the pine forest, with a single plough and fertilizer application of 60 kg of P_2O_5 and 60 kg of K_2O per hectare prior to plantation. The predominant soil type is a podzol characterized by a coarse texture (95% sand) and low soil fertility (Augusto et al., 2010), particularly in phosphorus (Trichet et al., 2009). The water table is relatively shallow in wintertime, generally ranging from 40 to 80 cm below the soil surface. A total of 256 plots were planted representing five species (*Betula pendula* Roth., *Pinus pinaster* Soland. in Aït., *Quercus ilex* L., *Quercus pyrenaica* Willd., *Quercus robur* L.) in all possible combinations from one (monocultures) to five species mixtures. Each plot has 100 trees within a 20 m by 20 m area. Trees are planted in a substitutive (same tree density irrespective of the composition) alternate (trees from one species have neighbors from all other associated species) pattern. The plots are separated by 3 m from one another, and randomly located within eight blocks: four control (dry) blocks and four irrigated blocks. The irrigation was started in 2015, and occurs seasonally from May to October to alleviate summer stress. The start and end dates of irrigation differ slightly between years depending on meteorological and technical constraints. The water is taken directly from the groundwater, which has low concentrations of nitrogen and phosphorus, but higher concentrations of other elements, such as calcium and magnesium (Jolivet et al., 2007). A 2 m tall sprinkler installed in the center of each experimental plot sprays every day the equivalent of 3 mm of precipitation. On average for the three summers preceding our sampling, control blocks received 318 mm of water from early May to late September, while irrigated blocks received 777 mm of water during the same period. When using both temperature and precipitation (+ irrigation) to determine arid periods in an ombrothermic diagram (Walter and Lieth, 1967), control blocks are subjected to more stress than irrigated blocks (Fig. S1.).

Among all of the plots, we chose to work with three species composition treatments: pure silver birch (*Betula pendula* Roth.) plots, pure maritime pine (*Pinus pinaster* Soland. in Aït.) plots, and the mixed birch-pine plots, which we used as a proxy to study tree diversity effects. These two species were selected because, contrary to the three other tree species, they grew fast despite the poorness of the sandy soil, which enabled the building of fairly dense forest stands. In 2018, the average tree height was about 8 m for the two species. The understory was mostly composed of Bracken, Molinia, Gorse, and ericaceous shrubs, with a few minority species (Besom heath, Buckthorn, Bramble, Honeysuckle). As a whole, the understory layer represented less than 1 % of the total aboveground biomass and the diversity was similar across all treatments, with on average six to seven vascular species per plot.

We sampled plots in all eight blocks (4 irrigated, 4 dry) from March 19th to 21st 2018. In each plot, we collected samples at four different locations, each surrounded by four trees, i.e. four pines, four birches, or two pines and two birches. The forest floor (layer of litter above the soil mineral layers) was collected using a quadrant (10 cm × 20 cm). Samples were dried at 40°C until weight stabilization and ground. At each sampling location, we also collected a soil core down to 0.9 m depth in two steps. The top 0–15 cm of soil was collected manually with a soil corer (8 cm width). The bottom 15–90 cm of soil was collected with a mechanical drill, attached onto an auger (4 cm width). Afterwards, the soil cores were divided into five layers: 0–5, 5–15, 15–30, 30–60, and 60–90 cm. Soil samples were immediately placed in coolers and transported to the nearby lab, where each sample was sieved to 2 mm and homogenized. The roots were cleaned and stored in 10% alcohol until further analyses. One soil subsample was kept at 4°C for immediate extraction of available nutrients, and a second subsample was stored at -20°C for enzyme assays. Soil moisture was assessed for each sample by comparing the fresh to the dry soil weights after at least 72 h at 50°C. There were thus a total of 480 samples collected for nutrients, enzymes, soil moisture and root analyses (3 species composition × 2 water levels × 4 blocks × 5 depths × 4 replicates, Fig. S2). Composites of the four cores were made for each soil layer per plot, and these samples were then air-dried for chemical analysis (3 species compositions × 2 water levels × 4 blocks × 5 depths = 120 samples).

In addition to the soil samples, we collected litterfall sampled from litter traps that were installed in all plots in three blocks per water treatment at the beginning of the 2017 fall season. Two circular traps were installed in each plot: the traps were plastic cylinders with a height of around 20 cm, a metal net on the inferior opening, and a surface area of 0.716 m^2. We collected the litter in November 2017, made one composite per plot, dried the samples at 40°C until weight stabilization and then ground them up (3 species compositions × 2 water levels × 3 blocks = 18 samples). The C:N ratios were determined after measuring total C and N by dry combustion (NF ISO 10694 and 13878).

2.2. Available nutrients

After sample collection, Available ammonium (NH_4^+), nitrate (NO_3^-), and phosphate (PO_4^{-2}) were measured in one aliquot of all the soil samples, corresponding to 480 analyses (Fig. S2). In brief, 15 mL of ultrapure water was added to a glass vial with a 3 g aliquot of the soil sample, which was then shaken for 16 h at 4°C. Water extraction was favored over KCl extraction due to potential interference of the extractant with available phosphate in sandy podzols (Achat, 2009), and because it has a similar extraction efficiency for mineral N as in saline solutions (Gallet-Budynek, personal communication). The solution was placed vertically at ambient temperature for an hour to enable sedimentation, after which the supernatant was transferred into a tube through a Whatman filter of 8µm mesh size. The solution was left at ambient temperature for 3 h, and then filtered a second time with a syringe equipped with a 5 µm mesh paper. The final solution extractant was poured into scintillation tubes to measure NO_3^-, NH_4^+ and PO_4^- by colorimetry (San++, Automated Wet Chemistry Analyzer, Breda, Netherlands). The total available mineral N was expressed as the sum of mineral N from NO_3^- and NH_4^+ (µg N g^{-1} dry soil) and available mineral P as PO_4^{-2} (µg P g^{-1} dry soil).

2.3. Enzyme assays

In the same samples previously described for available nutrients, on the other aliquot stored frozen at -20°C, we measured the potential activity of seven hydrolytic soil enzymes. We measured enzymes that catalyze the degradation of organic carbon (β-1,4-glucosidase [BGLU]), 1,4-β-D-cellobiohydrolase [CBH], α-1,4-glucosidase [AGLU], and β-xylosidase [XYL]), nitrogen (β-1,4-N-acetyl-glucosaminidase [NAG], L-leucine aminopeptidase [LAP]), and phosphorus (acid phosphatase [AP]) (Bell et al., 2013; Fanin et al., 2018). Briefly, 2.75 g of the thawed soil sample was homogenized in 91 ml of 50 mM sodium acetate buffer in a blender for 1 min. Soil slurries of two technical replicates were then added to a 96-deepwell (800 µl) microplate using an eight-channel electronic pipette (Eppendorf Xplorer Plus, Hamburg, Germany). Additional quench control replicates of the soil slurry, 4-methylumbelliferone or 7-amino-4-methylcoumarin standard curves (200 µl of respectively 0-100 µM and 0-10 µM concentrations) and controls without substrate addition (soil + 200 µl water), were included with each sample. Soil slurries were incubated with fluorometric substrates for 3 h at 25 °C. After the incubation period, plates were centrifuged for 3 min at 3000 rpm, after which 250 µl of supernatant was transferred from each well into a black flat-bottomed 96-well plate. The fluorescence was measured by a microplate reader (Synergy H1 microplate reader, Biotek, Winooski, USA) using an excitation wavelength at 365 nm and emission wavelength at 450 nm. From the fluorescent values and standard calibration curves, each enzyme activity was calculated and converted to nmol g^{-1} dry soil h^{-1}, and then converted to mmol kg^{-1} to express the enzymes as quantities. We then summed the BGLU, CBH, AGLU, and XYL quantities for total C-related enzymes (hereafter referred to as C_{enz}), and the NAG and LAP quantities for the total N-related enzymes (in the following referred to as N_{enz}). We refer to the acid phosphatase (AP) quantity as P_{enz}. The ratios of total enzymes were subsequently calculated, which we refer to as C_{enz}:N_{enz} , C_{enz}:P_{enz}, and N_{enz}: P_{enz}. This is a tool that has been frequently used to measure ecological stoichiometry and microbial limitations (Fanin et al., 2016a; Sinsabaugh et al., 2008; Stone et al., 2014). However, differences between potential enzyme activities and enzyme responses to nutrient additions have recently been found to be ambiguous, so some care must be taken when interpreting enzyme ratios as growth-limiting factors (Rosinger et al., 2019).

2.4. Root biomass

Roots were extracted from the same soil samples in which available nutrients and enzymes were analyzed. Root extraction consisted of sieving the collected samples and then watering the remaining roots to clean them and remove the soil. The live roots were sorted and separated into the different species. A representative portion of each root sample was oven-dried for 4 days at 40°C and weighed. For each soil sample, the root biomass data of both the tree species (pine and birch) and the understory species were used in our statistical analyses to determine their effect on the potential enzyme activities in a redundancy analysis (see section 2.6).

2.5. Soil chemical analyses

The subsequent soil analyses were done at the plot level by making composites of the four soil cores at each of the five depths along the soil profile (3 species compositions × 2 water levels × 4 blocks × 5 depths = 120 samples). The soil pH was determined in a 1:10 soil:water suspension. The C:N ratios were determined after measuring total C and N by dry combustion (NF [i.e., French standard] ISO [i.e., international standard] 10694 and 13878; AFNOR, 1999). The particle size distribution was analyzed for each block (one composite sample per block and per soil layer), using the fractionation method without decarbonatation (for five fractions, i.e., clay, fine silt, coarse silt, fine sand, and coarse sand) according to French standards (norm NF X 31-107; AFNOR, 1999).

2.6. Statistical analyses

All of the statistical analyses were done using R software (version 3.5.1). First, extreme values were removed following the outlier labelling rule with a conservative tuning parameter of $g = 2.2$ (Hoaglin and Iglewicz, 1987). On average, 1.25% of data were removed. Weighted means, using soil weight per layer, were used when presenting values per soil column.

Mixed models were used to assess the effect of the following fixed effect factors: tree species composition (pure birch, pure pine, mixed birch-pine), water availability (irrigation, control), soil depth (five depths as levelled factors) and their interaction, on the enzyme activities and available nutrients. Block was included as a random factor to account for the spatial structure of our experimental design. Plots were then nested within the block random factor to enable a comparison of treatments within each block separately. Finally, the sampling replicate number was nested within the plot to account for the non-independency of soil horizons within a soil core of a certain plot. Soil moisture was added as a co-variable in the mixed model. We then applied contrasts and performed post-hoc tests Tukey HSD post-hoc tests, with $\alpha = 0.05$ used as the level to define significance.

We calculated non-additive effects of mixing birch and pine trees by comparing observed values in the mixed-species plots to expected values, calculated as the mean enzyme quantities of the component species in the monocultures (Barantal et al., 2014). We used a Welch two sample t-test to test whether the observed enzyme quantities differed from the expected enzyme quantities for control and irrigated treatments. The percentage increase in enzyme quantities (the relative mixture effect) was calculated by ((observed - expected)/expected) * 100, according to Wardle *et al.* (1997). Then, we used a redundancy analysis (RDA) to elucidate the relationships between individual enzymes and their respective biochemical variables (i.e. available nutrients, root biomass, and soil characteristics), included as the so-called environmental variables. We also included enzyme ratios as supplementary variables in the RDA. Finally, we used Mantel tests with Spearman's rank correlations (n = 999 permutations) to quantify the relationship between enzyme activities and environmental variables.

3. Results

3.1. Soil chemical properties

Soil texture did not vary between irrigated and control blocks with on average 95% sand, 2% silt, and 3% clay (Table S1). Soil pH increased down the soil profile in all plots (Table S2), and varied according the interaction between soil depth and plot species composition ($p = 0.028$). Additionally, soil moisture varied between depths in all plots ($p < 0.001$, Table S2), with a decreasing trend from the top to the bottom soil. Averaged across all species treatments, soil moisture was significantly higher in the irrigated blocks than in the control blocks for the 0–5 cm ($p < 0.05$) and the 30–60 cm layers ($p < 0.01$) (Table S2), despite that the soil sampling was done in March, a period when the irrigation was switched off.

The forest floor C:N ratio was significantly higher in irrigated than in control blocks ($p = 0.02$, Table 1). The forest floor C:N in the pure birch was significantly lower than that in the mixed species plots ($p = 0.002$) and the pure pine plots ($p < 0.001$, Table 1). Irrigation increased the litterfall C:N ratio in the three different species compositions (Table 1).

3.2. Enzyme activities and available nutrients

There was a significant effect of the interaction between the plot species compositions and soil depth for the C-related EEAs ($p < 0.0001$), N-related EEAs ($p = 0.01$), and P-related EEA ($p = 0.02$) (Table 2). In both pure birch and pure pine plots, C- and N-related EEAs decreased steadily from the 0–5 cm to the 60–90 cm layer (Fig 1A). However, the pattern was different in mixed birch and pine plots: there was a sharp increase in EEAs from the 5–15 cm layer to the 15–30 cm layer. At this 15–30 cm mid soil layer, C-related EEAs were significantly higher than those in pure plots, with an increase on average of 77 % (Fig. 1A), and N-related EEAs were significantly higher (+ 49 %) than in pure pine plots ($p = 0.037$) (Fig. 1B). This resulted in synergistic, non-additive effects at this intermediate soil layer (Fig. 2): the effect of mixing birch and pine was however restricted to this soil depth, and was independent of irrigation with a similar increase in both control and irrigated blocks for C- and N-related EEAs. In contrast, the P-related enzyme activity decreased with depth similarly in all species compositions, but was 24 % lower in the mixed plot at the 30–60 cm layer compared with the monocultures (Fig. 1C). There were more C- and N-related EEAs in pure birch than pure pine plots at the 0–5 cm layer in control blocks, but no overall significant differences were observed along the soil profile between the monocultures for each of the C-, N- and P-related EEAs.

Our results also showed an overall positive effect of irrigation on N-related EEAs (Table 2): averaged among species treatments along the soil profile, irrigation increased N-related EEAs by 28 % compared with control blocks (Fig. 3A). From the 0–5 cm to the 5–15 cm soil layer, there was a sharp decrease of N-related EEAs in both control and irrigated blocks. These values remained steady down to the 15–30 cm layer, after which there was another steady decline down to the 60–90 cm layer. For P-related EEAs, the overall enzyme pattern was similar to N-related EEAs, with a steady decline down to the 60–90 cm layer. However, the significant effect of irrigation on the P-related EEA depended on soil depth (Table 2): AP activity in irrigated blocks tended to be higher only in the first 30 cm, with an increase on average of 20 % compared with control blocks (Fig. 3B).

Although total N varied in the top 0–5 cm soil layer from an average of 1.06 mg N g^{-1} in control blocks to 1.26 mg N g^{-1} in irrigated blocks (data not shown), we found that available N was relatively low in both water treatments (Fig. 4). N availability was interactively affected by soil depth and irrigation ($p < 0.0001$): adding water decreased available N by 40 % in the first soil horizons layers (0–15 cm) while it increased available N by 55% in depth (30–90 cm) (Fig. 4A). Available P also differed along soil depth (Table 2). It increased from 0–5 to 5–15 cm in all plots and then steadily decreased until the 60–90 cm layer (Fig. 4B).

3.3. Environmental effects

Mantel tests showed that the seven individual hydrolytic enzymes were significantly correlated with the available nutrients and the belowground root traits ($\rho = 0.569$, $p < 0.001$). Biplots from the RDA distinguished the C-related EEAs (XYL, BGLU, AGL, CBH) from the N-related EEAs (NAG, LAP) and P-related EEA (AP) along the second axis, which weakly represented the variance of the system (Fig. 5). Available N was negatively related to the N-related EEAs along the first axis. The total tree species' fine root biomass within a plot and soil moisture, were also negatively related to the available N. The C-related EEAs fitted closely with the available P, which was unrelated to the AP enzyme. Both the $C_{enz}:P_{enz}$ and $C_{enz}:N_{enz}$ ratios (supplementary variables in green, Fig. 5) were closely related to the available P. The understory species fine root biomass within a plot species composition was generally unrelated to the individual enzyme activities.

As a whole, we found no correlation between the available N and the $C_{enz}:N_{enz}$ ratio (adj. $R^2 = 0.003$, $p = 0.12$, Fig. 6A). However, we found a highly significant positive correlation between the available P and the $C_{enz}:P_{enz}$ enzyme ratios (adj. $R^2 = 0.24$, $p < 0.0001$, Fig. 6B). This relationship was consistent across the three species compositions and water availability treatments.

4. Discussion

By using a 10-year-old plantation which manipulates tree species composition and irrigation in southwestern France, we investigated the effect of mixing birch and pine, water availability, and their interaction on seven hydrolytic extracellular enzyme activities (EEAs) and available nutrients along the soil profile. Our results are partly in agreement with our two first hypotheses, thereby demonstrating that EEAs directly depend on tree species composition and water conditions. In contrast to our last hypothesis, we did not find any effect of the interaction between water availability and species mixing on EEAs, underlying that the negative effect of lower water availability cannot be offset by the positive effect of mixing tree species.

4.1. Effect of mixing tree species

In agreement with our first hypothesis, we found that mixing birch and pine had a positive effect on C- and N-related EEAs in interaction with soil depth (Table 2). However, contrary to our expectations that this effect should be more pronounced in the topsoil (Alberti et al., 2017), we observed a strong synergistic increase in C-related and N-related EEAs in the 15–30 cm soil layer compared to both birch and pine monocultures (Fig. 1, Fig. 2). While the litter quality was significantly higher in birch than in pine plots (lower C:N ratio, Table 1), the EEAs in the pure birch plots were only slightly higher than those in the pure pine plots (Fig. 1). Although we expected an influence of tree species identity (Purahong et al., 2016) and litter quality on microbial decomposition and EEAs (Sinsabaugh et al., 2002), the little influence of the tree species on EEAs has been found in other studies. For instance, Herold *et al.* (2014) found no differences between coniferous and deciduous central European forests on six of our measured EEAs. Likewise, Weand *et al.* (2010) described no tree species effect on C-, N- and P-related enzymes in five single-species plots in an American Northeastern hardwood forest. This was also true for the N- and P-related EEAs studied in five tree species in a temperate secondary forest in Northeastern China (Diao et al., 2020). Therefore, it is unlikely that the increase in EEAs at the mid-soil layer of the mixed birch-pine plot was related to the different and complementary foliar litter quality of the two species. Instead, we interpret our results as a consequence of the higher nutrient demand of mixed stands.

Indeed, higher tree productivity in mixed stands may stimulate soil microbial biomass through an increase in root exudate quantity and diversity (Steinauer et al., 2016), or favor C allocation to ectomycorrhizal fungi to get N from organic molecules to sustain tree biomass production (Fernandez and Kennedy, 2016; Lindahl and Tunlid, 2015). Alternatively, niche partitioning in root systems or increase in root biomass along the soil profile may have increased nutrient uptake (Jose et al., 2006), which may exacerbate, in turn, nutrient limitation of microorganism communities (Kuzyakov and Xu, 2013). In both cases, this implies that there is likely a strong demand by microorganisms to degrade the soil organic matter (Fatemi et al., 2016) at this middle soil layer in mixed stands, and indicates that the potential effects of tree diversity on EEAs are not only limited to the superficial soil layers.

Contrary to C- and N-related EEAs, we found that there was a decrease in the P-related EEA in the 30–60 cm soil layer in the mixed-species plots compared to both birch and pine monocultures (Fig. 1C). This may be due to a shift in the nutrient needs of the microorganisms (Mooshammer et al., 2014) or because the changes in tree species diversity may lead to a shift in the structure of microbial communities (Liu et al., 2018; Pei et al., 2016; Schweitzer et al., 2008). For instance, changes in fungi to bacteria ratios may alter the stoichiometric requirements at the community scale (Fanin et al., 2013), which could explain the reduced and different effect of tree diversity on N- and P-related EEAs. On the other hand, this may be caused by differential root uptake and competition for the available nutrients between microorganisms and both tree and understory roots (Kuzyakov and Xu, 2013; Richardson and Simpson, 2011). This hypothesis is supported by the tendency of available N and P to decrease and increase respectively in mixed plots (Fig. S3), although the overall effect of tree species for the entire soil column was non-significant (Table 2). Taken together, these findings suggest that there is a shift in microbial C:N:P stoichiometry from monocultures to the two-species mixture along the soil profile.

4.2. Effect of water availability

In accordance with our second hypothesis, we found an increase in both N- and P-related EEAs with irrigation. Overall, EEAs were lower in the control blocks (Table S3), which were subject to the long dry summers of southwestern France (Fig. S1). Although we sampled in early spring, i.e. several months after the irrigation had been switched off, we still found differences in soil moisture between control and irrigated blocks (Table 1). The legacy of sub-optimal water conditions for the past eight years in the control blocks may have led to a decrease in both microbial biomass (Brockett et al., 2012) and tree and understory root biomass (Table S2; Brunner et al., 2015). The lower EEAs in these dry control blocks could thus be due to fewer microorganisms and roots, leading to less root exudation and enzyme stimulation (Zhang et al., 2019). This interpretation is supported by our RDA results, where tree fine root biomass points in the same direction as soil moisture (Fig. 5), demonstrating a positive covariation between these variables. The drought episodes experienced by control blocks and not by irrigated blocks, at various periods during the three summers (Fig. S1), may have caused a significant change in the composition of root exudates, both during these drought periods and after rewetting (Gargallo-Garriga et al., 2018). Alternating periods of drying and rewetting, and changes in root exudation, can also have an influence on microbial hotspots and thus microbial abundance and activity (Kuzyakov and Blagodatskaya, 2015), which could help explain lower EEAs in control blocks.

In line with our expectations, the effect of irrigation was higher in the topsoil for the P-related EEA (Fig. 3). This is probably because microbial communities benefit more of increasing humidity in soil layers that are more subjected to drought and where the substrate is relatively nutrient rich (Fanin et al., 2019). However, this effect was relatively constant throughout the soil column for N-related EEAs. This result is in line with the idea that there is a shift in the requirements of microbial communities at the community scale along the soil profile (Fanin et al., 2013; Mooshammer et al., 2014), and may explain the variability in nutrient availability from the top to the bottom soil layers (Fig. 4). Indeed, irrigation had a negative effect on available N in the topsoil. This is probably due to important N uptake by trees and the understory, coupled with a leaching of soluble mineral N-forms (NH_4^+ and NO_3^-) into the deeper soil layers, where irrigation had a positive effect on available N (Table 2, Fig. 4A). In contrast, the available P was generally low and unaffected by the different factors (Table 2, Fig. 4B). The lack of significant increase in available P within the 0–30 cm soil layer may be due to a strong and rapid absorption by tree roots in the topmost layer. High understory root biomass in deeper soils (Table S2) and a general decrease in P desorption along the soil profile (Achat et al., 2013), may explain the decrease in available P along the soil profile. The differing effects of water availability on available nutrients point towards a strong influence of organic matter quality and associated microorganism communities to explain the EEAs' responses.

Furthermore, because C-related enzymes were not as affected as other EEAs by irrigation, the result reinforces the idea that changes in organic matter quality are also important drivers of EEAs under low water availability. The lower litterfall and forest floor C:N ratios in dry blocks compared to irrigated blocks support this hypothesis (Table 1). Indeed, higher tree growth rates when water conditions are favourable reduce available N input to soils, with further consequences on N limitations in our study system. Along the entire soil profile, we found less available N in the non-irrigated blocks (Table S3). Taken together, these results highlight that changes in precipitation regimes may have important consequences on EEAs through direct effects of water availability and indirect effects through tree growth on organic matter quality and nutrient availability.

4.3. Effect of the interaction between mixing tree species and water availability

Although we hypothesized that mixing birch and pine could mitigate the effects of lower water availability on nutrient availability and EEAs, we did not find such a compensatory effect (Table 2). Studies at larger scales have shown that biodiversity may become more beneficial as water limitations increase (Lebourgeois et al., 2013; Pretzsch et al., 2013; Ratcliffe et al., 2017), but some other studies contradict this statement. For example, Jucker *et al.* (2014b) found that the magnitude of the biodiversity effect on aboveground biomass production decreases in strength during drought years, and Jactel *et al.* (2018) found high overyielding due to mixing tree species at wet sites at the stand level. Additionally, we suggest that the lack of a significant interaction effect between mixing birch and pine trees and water availability might be due to the spatial segregation of the individual effects of these two factors. While mixing the two tree species tended to increase EEAs in the mid-soil layer, irrigation tended to increase EEAs and decrease available N in the top soil. Moreover, we collected our samples in March, when the control blocks were not under water stress and the irrigation treatment had not yet begun for the year (Table 1).

Finally, the lack of effect due to the species mixing may be simply the consequence of the lack of differences between the tree species themselves. Indeed, we did not see different patterns of the effect of water availability on EEAs between the two monocultures. This might be surprising given that *Pinus pinaster* is a fairly more drought tolerant species compared to *Betula pendula* (Choat et al., 2012). However, the tolerance difference is quantitatively modest (sapwood hydraulic vulnerability P50: *Pinus pinaster* = -2.8 to -3.7 Mpa, Betula pendula = -2.2 to -2.4 Mpa (Choat et al., 2012; Delzon, unpublished data)), and both species' roots similarly explored the entire soil profile in the dry control and irrigation treatments (Table S2), which could explain the small effects of species identity and, in turn, of mixing species. Additional analyses including more tree species and more sampling dates are needed to further evaluate the relationship between EEAs and tree diversity, as well as intra annual temporal variation of EEAs and biodiversity effects (Cardinale et al., 2012).

4.4. Environmental effects and larger implications for nutrient cycling

The RDA gives us new insight into how environmental variables may have an effect on different enzyme activities (Fig. 5). Available N was negatively correlated with the tree species' fine root biomass along the first axis, likely due to rapid nutrient absorption by roots, which led to N-depletion in the topsoil where roots thrive (Giehl and Wirén, 2014). N-related EEAs may have been stimulated by this low N availability in order to maintain high growth rates (Kaye and Hart, 1997), or by tree root exudation (Renella et al., 2006; Zhang et al., 2019). Indeed, N-related EEAs were also positively correlated to tree species' fine root biomass (Fig. 5). To a lesser extent, the P-related enzyme was negatively correlated to available P, likely due to similar feedback mechanisms.

Interestingly, we also found that C-related EEAs were strongly related to available P. This implies that when P availability is high, microorganisms are strongly limited by C, inciting them to produce more C-related enzymes to get access to the energy produced by decomposing organic matter (Fanin et al., 2017, 2016b). This hypothesis was supported by the significant positive correlation between available P and the C_{enz}:P_{enz} ratio across all species and water availability treatments (Fig. 6B), which we did not find between the available N and the C_{enz}:N_{enz} ratio (Fig. 6A). This is likely because alleviating P limitation generates a new constraint for microorganisms and thus the necessity to acquire C from organic molecules in these P-poor podzol soils (Achat et al., 2009; Augusto et al., 2017). This relationship was true regardless of species composition, water availability, or soil depth, suggesting that P limitation plays a more important role in controlling C-related enzymes. Thus, potential C sequestration is likely to be limited by P availability in these nutrient-poor forests.

4.5. Conclusions

This study revealed that water supply and mixing pine and birch trees could have important long-term effects on soil extracellular enzyme activities (EEAs). First, from a management perspective, our results suggest that overall C sequestration in our system is likely to be constrained by P availability, regardless of water availability. Second, we found C-N-P decoupling effects as a result of tree species mixing or improved water availability, which may alter microorganism stoichiometry and belowground C, N, P fluxes. Finally, the lack of interactive effects between mixing birch and pine, and water supply, suggests that the negative effect of lower water availability on microbial activity cannot be offset by the positive effect of mixing these two species. The next step will be to sample at different seasons to investigate the possible temporal variations of EEAs and to measure other belowground processes such as protein depolymerisation and mineralization, in order to assess the dynamics of interactive effects between water availability and tree diversity over seasons and disentangle underlying mechanisms.

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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Figures

Figure Captions

Fig. 1. Distribution of the extracellular enzyme activities (EEAs) along the soil profile in pure birch (circles, *yellow*), mixed birch + pine (squares, *blue*) and pure pine plots (diamonds, *green*); A) C-related EEAs (sum of XYL, AGLU, BGLU and CBH); B) N-related EEAs (sum of NAG and LAP); and C) the P-related EEA (AP). The water availability treatments were pooled for each species composition treatment. Data points represent means at each depth interval of the four replicates per plot, in each of the eight blocks (n = 32) and error bars represent standard errors of the mean.

Fig. 2. Synergistic non-additive effect of mixing birch and pine in the 15–30 cm soil layer on extracellular enzyme activities (EEAs). Bars represent observed means for the four replicates in each species compositions in the four blocks per water treatment (n = 16) and error bars represent standard errors of the mean for pure birch (*yellow*), observed mixed birch + pine (B + P, *light blue*) and pure pine plots (*green*). The expected additive effect of mixing two tree species (*dark blue*), calculated as the average value between observed pure plots, is superposed onto the observed values of mixing the two tree species. Significant values come from Welch two sample t-tests between the expected and observed values for the mixed species plot. A) C-related EEAs (sum of XYL, AGLU, BGLU, and CBH); B) N-related EEAs (sum of NAG and LAP).

Fig. 3. Distribution of the extracellular enzyme activities (EEAs) along the soil profile in dry control blocks (circles, *red*) and irrigated blocks (squares, *blue*) blocks; A) N-related EEAs (sum of NAG and LAP); and B) the P-related EEA (AP). The tree species composition

treatments were pooled for each water availability treatment. Data points represent means at each depth interval of the four replicates in each of the three plots (species compositions) in the four blocks per water treatment (n = 48) and error bars represent standard errors of the mean.

Fig. 4. Distribution of the available nutrients along the soil profile in dry control blocks (circles, *red*) and irrigated blocks (squares, *blue*); A) available N (µg N g^{-1}); and B) available P (µg P g^{-1}). The tree species composition treatments were pooled for each water availability treatment. Data points represent means at each depth interval of the four replicates in each of the three plots (species compositions) in the four blocks per water treatment (n = 48) and error bars represent standard errors of the mean.

Fig. 5. Redundancy analysis (RDA) ordination biplot of the seven hydrolytic extracellular enzyme activities (circles: C-related = *red*, N-related = *yellow*, P-related = *purple*), and of the environmental parameters (available P, available N, tree fine root biomass, understory fine root biomass, and soil moisture; arrows, *green*). The continuous supplementary variables were not included in the biplot calculation but are represented on the RDA (C_{enz}:N_{enz}, C_{enz}:P_{enz} and N_{enz}:P_{enz} ratios; circles, *green*), (n = 457).

Fig. 6. Relationship between available nutrients and enzyme ratios in the pure birch (circles, *yellow*), mixed birch and pine (squares, *blue*) and pure pine plots (diamonds, *green*), (n = 464). A) Non-correlation between available nitrogen and the ratio between the C-related enzymes the and the N-related enzymes (C_{enz}:N_{enz}); B) Positive correlation between the available phosphorus and the ratio between the C-related enzymes and the P-related enzyme (C_{enz}:P_{enz}). The water availability treatments were pooled for each species composition treatment.

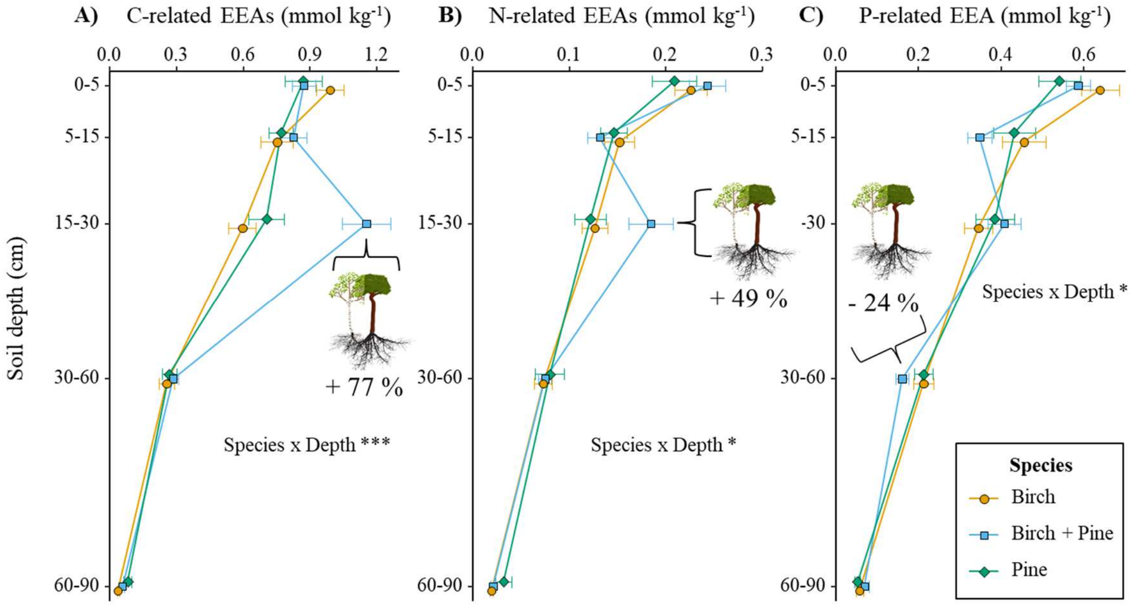

Figure 1

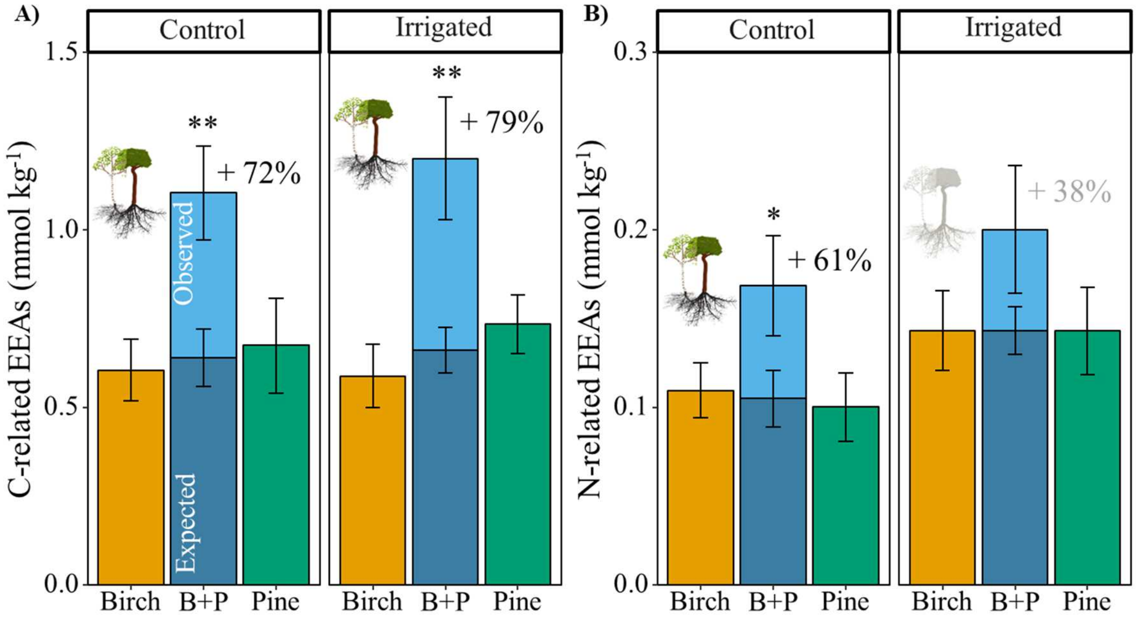

Figure 2

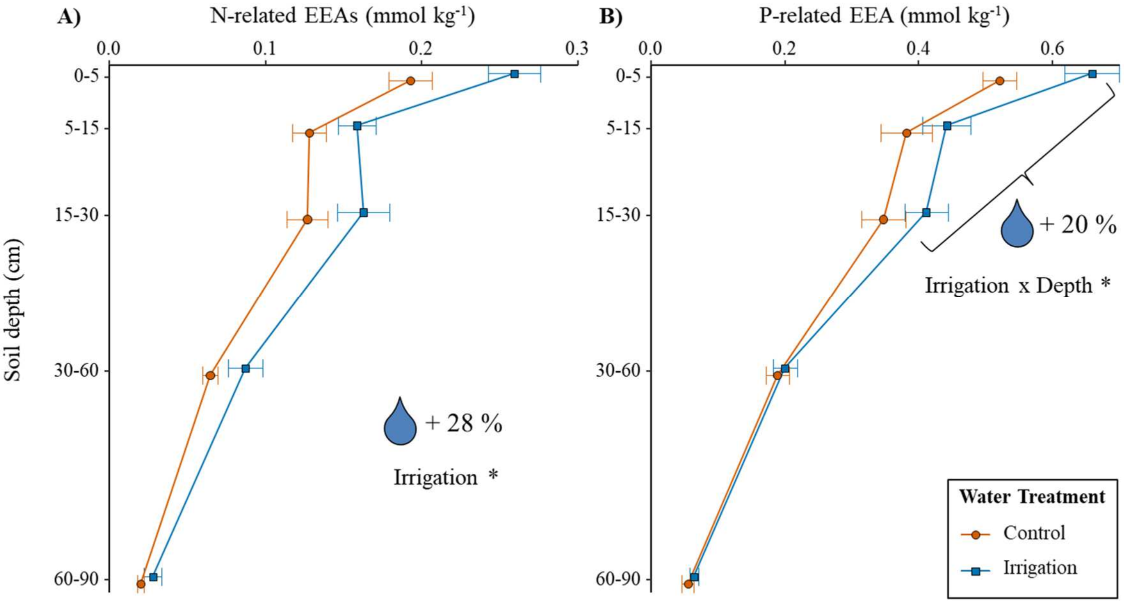

Figure 3

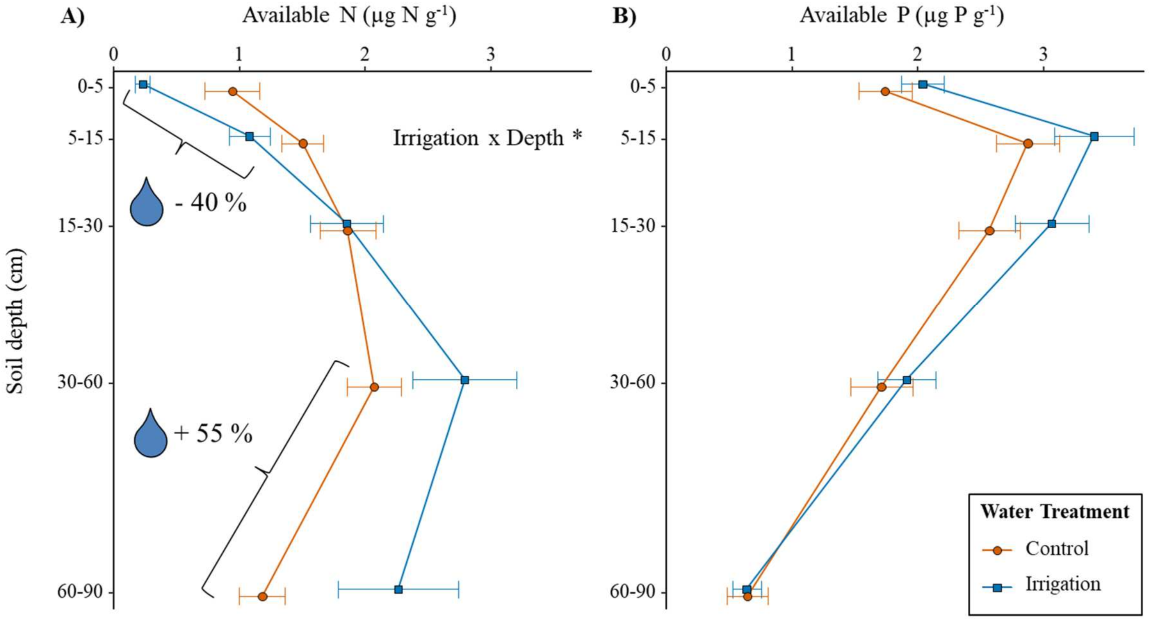

Figure 4

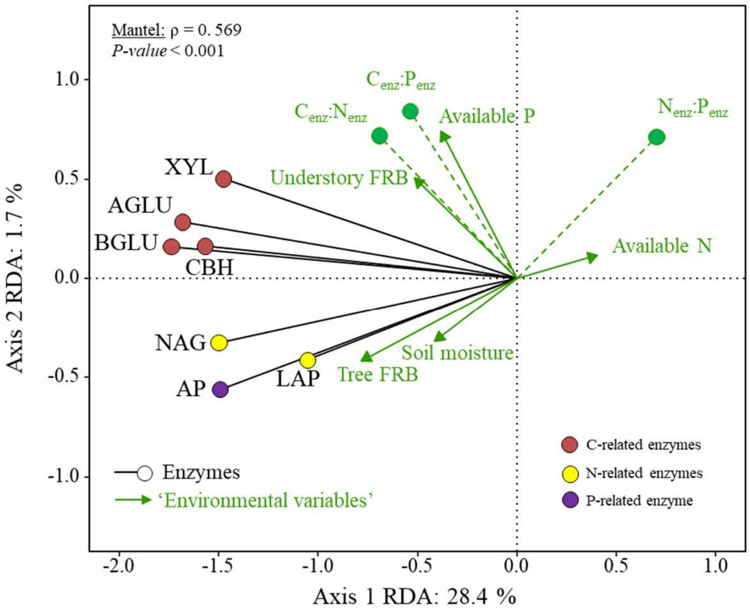

Figure 5

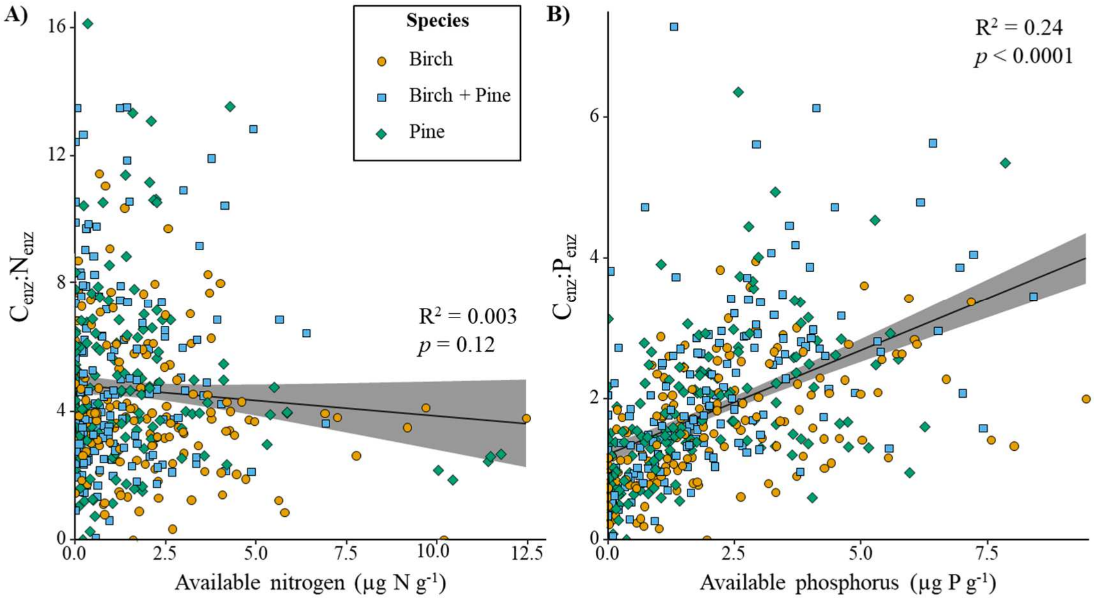

Figure 6

Tables

Table 1

Selected properties of the study site in the three different tree species compositions, each in both dry control and irrigated blocks. Data are mean ± standard error of the mean (n = the total number of samples per treatment).

		Birch		**Birch + Pine**		**Pine**	
	n	Control	Irrigation	Control	Irrigation	Control	Irrigation
Litterfall C:N	3	39.88 ± 3.69	50.96 ± 3.47	99.50 ± 21.78	110.19 ± 27.54	149.39 ± 9.22	160.43 ± 4.22
Forest floor C:N	4	35.85 ± 0.88	37.42 ± 2.78	42.15 ± 4.61	61.00 ± 4.79	51.58 ± 2.95	69.61 ± 8.05
Soil moisture (%)	16	13.21 ± 0.45	12.79 ± 0.45	12.46 ± 0.61	12.86 ± 0.76	12.54 ± 0.81	13.91 ± 0.63
Soil pH [H_2O]	4	4.50 ± 0.04	4.62 ± 0.06	4.45 ± 0.05	4.46 ± 0.04	4.49 ± 0.05	4.67 ± 0.17

Note: Litterfall was collected in the fall, while forest floor, soil moisture and soil pH were collected in early spring. Three blocks of each water availability treatment were sampled for litterfall while four blocks were sampled for the other variables. We analyzed one composite sample per plot for litterfall, forest floor and pH or four replicates per plot for soil moisture. Weighted means were calculated for soil pH and soil moisture along the soil profile and detailed data per soil depth layer can be found in Table S2.

Table 2

Results of the linear mixed effects models to test for the effects of tree species composition (pure birch, pure pine, mixed birch +pine), water availability (irrigation, dry control), soil depth and their interactions on C-related EEAs (sum of XYL, AGLU, BGLU, and CBH), N-related EEAs (sum of NAG and LAP), P-related EEA (AP), available N and available P. Soil moisture was used as a covariable in the model and not shown here. Significant results are in bold font.

Variables	**C-related EEAs**				**N-related EEAs**				**P-related EEA**				**Available N**				**Available P**			
	num DF	*den DF*	*F*	*p*	*num DF*	*den DF*	*F*	*p*	*num DF*	*den DF*	*F*	*p*	*num DF*	*den DF*	*F*	*p*	*num DF*	*den DF*	*F*	*p*
Species (S)	2	12	2.87	0.096	2	12	0.95	0.414	2	12	0.26	0.774	2	12	3.30	0.072	2	12	0.48	0.631
Irrigation (I)	1	6	4.89	0.069	1	6	**6.91**	**0.039**	1	6	1.38	0.285	1	6	0.05	0.835	1	6	0.36	0.570
Depth (D)	4	350	**159.74**	**<.0001**	4	349	**103.86**	**<.0001**	4	351	**155.38**	**<.0001**	4	351	**20.65**	**<.0001**	4	350	**49.21**	**<.0001**
S × I	2	12	0.82	0.464	2	12	2.20	0.153	2	12	0.10	0.908	2	12	3.08	0.083	2	12	0.14	0.869
S × D	8	350	**7.20**	**<.0001**	8	349	**2.45**	**0.014**	8	351	**2.31**	**0.020**	8	351	1.66	0.107	8	350	1.60	0.123
I × D	4	350	1.88	0.113	4	349	1.77	0.134	4	351	**2.51**	**0.042**	4	351	**6.16**	**<.0001**	4	350	0.45	0.775
S × I × D	8	350	0.58	0.794	8	349	0.43	0.901	8	351	0.20	0.990	8	351	1.33	0.228	8	350	1.35	0.216