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## SHORT COMMUNICATION

# Water availability is a stronger driver of soil microbial processing of organic nitrogen than tree species composition

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## Abstract

Soil organic nitrogen (N) cycling processes constitute a bottleneck of soil N cycling, yet little is known about how tree species composition may influence these rates, and even less under changes in soil water availability such as those that are being induced by climate change. In this study, we used a 12-year-old tree biodiversity experiment in southwestern France to assess the interactive effects of soil water availability (half of the blocks seasonally irrigated to double precipitation) and tree species composition (monocultural vs. mixed plots of coniferous *Pinus pinaster*, and of broadleaf *Betula pendula*). We measured gross protein depolymerisation rates using a novel high-throughput isotope pool dilution method, along with soil microbial biomass carbon and N to calculate microbial biomass-specific activities of soil organic N processes. Overall, high soil water availability led to a 42% increase in soil protein depolymerisation rates compared to the unirrigated plots, but we found no effect of species composition on these soil organic N cycling processes. When investigating the interactive effect of tree species mixing and soil water availability, the results suggest that mixing tree species had a negative effect on soil organic N cycling processes in the non-irrigated blocks subject to dry summers, but that this effect tended to become positive at higher soil water availability in irrigated plots. These results put forth that soil water availability could influence potential tree species mixing effects on soil organic N cycling processes in dry conditions.

## Highlights:

- Tree species (with different litter C:N ratios) had little effect on protein depolymerisation
- Increasing water availability *via* irrigation accelerated depolymerisation rates
- No interactive effect between tree species mixing and water availability, although trends emerged

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- Positive trend of mixing under high water availability and negative trend under low water

**KEYWORDS**

amino acid uptake, biodiversity, microbial biomass, precipitation, protein depolymerisation, TreeDivNet

## 1 | INTRODUCTION

Nitrogen (N) is one of the most important macronutrients which constrains the growth and metabolic activity of all living organisms (Vitousek & Howarth, 1991). Because over 50% of total soil N is represented by peptidic structures (Schulten & Schnitzer, 1998), depolymerisation of proteins to oligopeptides and free amino acids (i.e., the soil organic N processing prior to mineralisation) drives the soil N cycle and determines the amount of N becoming available to plants and soil microbes (Jan et al., 2009; Jones et al., 2002). The emergence of isotopic techniques has allowed the quantification of gross protein depolymerisation in soils (Wanek et al., 2010), recently improved with a high throughput approach (Noll, Zhang, & Wanek, 2019). The studies using these methods have evidenced that soil organic N processes respond differently to various global change factors, such as drought, warming, and elevated CO<sub>2</sub> (Andresen et al., 2015; Fuchslueger et al., 2019; Maxwell, Canarini, et al., 2022; Wild et al., 2018). However, other factors such as substrate quality may contribute to context-dependency of protein depolymerisation, and little is known about whether and how litter quality interact with environmental conditions in explaining soil organic N cycling.

High litter quality was shown to accelerate nutrient cycling by stimulating soil microbial activity and increasing the nutrient supply (Mooshammer et al., 2012), yet there is a lack of studies on how different species, such as coniferous or broadleaf species, may influence gross protein depolymerisation. Previous studies found that protein depolymerisation is a substrate-limited rather than an enzyme-limited process (Maxwell, Canarini, et al., 2022; Noll, Zhang, Zheng, et al., 2019; Wanek et al., 2010), and that it is stoichiometrically regulated, with lower depolymerisation for higher litter C:nutrient ratios (Mooshammer et al., 2012). Furthermore, mixing tree species has been shown to increase N mineralisation rates (Gartner & Cardon, 2004), as a species with nutrient-rich litter may enhance the decomposition and nutrient release from a species with lower quality litter (Chapman et al., 1988; Finzi & Canham, 1998). Therefore, investigating the effects of a broadleaf tree species

(low litter C:nutrient ratio), a coniferous tree species (higher litter C:nutrient ratio), and the mixture of the two on protein depolymerisation could give us insight on the influence of tree species identity and mixing on belowground soil nutrient cycling. However, the effect of tree mixing may depend on environmental conditions, with greater effects under low water availability due to increased complementarity in space partitioning or resource acquisition (Ratcliffe et al., 2017). This highlights that the interactive effects between different tree species are prevalent in understanding how litter quality effects may differ with changing water conditions.

Soil water availability may increase soil nutrient cycling rates due to the stimulation of microbial activity (Brockett et al., 2012). For example, an increase in water availability often increases litter decomposition (Anaya et al., 2012; Liu et al., 2021), soil extracellular enzyme activities (Maxwell et al., 2020), and N mineralisation rates (Paul et al., 2003; Santiago & Geisseler, 2022). However, other studies investigating the impact of drought on soil organic N cycling found that a decrease in soil water availability led to an increase in protein depolymerisation in montane and subalpine grasslands (Fuchslueger et al., 2019; Maxwell, Canarini, et al., 2022). While a decrease in rates with low water availability may be due to water-limiting soil conditions for microbial activity (Schimel, 2018), the increase in rates may potentially be due to a decrease in plant N uptake under drought conditions coupled to a sustained activity of soil microorganisms that remain hydrated in microsites during punctual droughts (Harris et al., 2021; Homyak et al., 2017). Yet, whether the context-dependency of water availability effects depend also on the nature of litter substrates has rarely been investigated.

In this study, we used a 12-year-old experimental forest in which both tree species identity and diversity as well as soil water availability are manipulated, to test the interactive effect of these factors on soil organic N cycling processes. First, we hypothesized (H<sub>1a</sub>) that tree identity would affect organic N process rates, with faster rates for the deciduous species. We further hypothesized (H<sub>1b</sub>) that species mixing would lead to an increase in soil organic N process rates, since mixing different litter

C:nutrient ratios may lead to synergistic effects on litter decomposition (Chapman et al., 2013; Cornwell et al., 2008) and an increase in soil microbial biomass (Chen et al., 2019; Lucas-Borja et al., 2012; Thakur et al., 2015). Second, we hypothesized ( $H_{2a}$ ) that an increase in soil water availability would increase processes rates due to the stimulation of microbial activity (Brockett et al., 2012), and especially for the high quality litter of the broadleaf species. We further hypothesized ( $H_{2b}$ ) that the positive effect of tree species mixing would be strongest in the plots subject to dry conditions, due to facilitation mechanisms between microorganisms, as this has been found to be stronger under limiting soil water conditions (Ratcliffe et al., 2017).

## 2 | METHODS

This study was conducted at the ORPHEE experimental site in southwestern France (44°44.35' N–00°47.9' W), composed of several species combinations, randomly located within six blocks (three control and three irrigated blocks, for low and high soil water availability, paired into 'superblocks'). Irrigation was initiated in 2015 and applied seasonally (3 mm per day from May to October) at the block level, to alleviate summer water shortage (Figure S1). We selected two monocultures: 'birch' (*Betula pendula*, low C:nutrient ratios), 'pine' (*Pinus pinaster*, high C:nutrient ratios) (Maxwell, Fanin, et al., 2022), and their mixture. Each plot was composed of 100 trees within a 20 × 20 m<sup>2</sup> area, and the soil type was a coarse-textured podzol (94% sand, pH = 4.3).

We collected three soil cores per plot (corer, 8 cm diameter, 15 cm depth) in July 2020, after a 4 week-period characterised by dry conditions (2 mm precipitation). Each soil sample was sieved to 2 mm, homogenized, and stored at 4°C. Aliquots of fresh soil were weighed and dried (50°C until constant weight, at least 72 h) to calculate the soil fresh weight to dry weight ratios and gravimetric soil water content. Soil microbial biomass carbon and nitrogen were measured on aliquots of fresh soil samples using the chloroform fumigation-extraction method (Vance et al., 1987), analysed using a TOC/TN analyser, and adjusted using the 0.45 conversion factor (Jenkinson et al., 2004). Total extractable N from the non-fumigated soils is referred hereafter as 'extractable N'.

To measure protein depolymerisation and amino acid uptake by microorganisms, we applied an isotope pool dilution technique (Kirkham & Bartholomew, 1954; Wanek et al., 2010), modified to a novel high-throughput method (Noll, Zhang, & Wanek, 2019). Briefly, a solution of <sup>15</sup>N-AA (98 % <sup>15</sup>N algal amino acid mix, Cambridge

Isotopes), calculated as approximately 20% of the original free amino acid concentration, was added to two aliquots of fresh soil (stopped 15 min and 45 min after tracer addition and extracted with 1 M KCl). In the extracts, ammonium was removed (Noll, Zhang, & Wanek, 2019) and amino acids were converted to N<sub>2</sub>O gas, then analysed by purge-and-trap isotope ratio mass spectrometry (Lachouani et al., 2010; Noll, Zhang, & Wanek, 2019). Using the change of <sup>15</sup>N:<sup>14</sup>N amino acids over time, soil organic N cycling rates were calculated (Kirkham & Bartholomew, 1954).

All statistical analyses were done using R software (version 4.1.0). Linear mixed effects models from the 'nlme' R package (Pinheiro & Bates, 2020) were used to assess the effect of tree species composition (birch, pine, mixed birch-pine), water availability (low, high), and their interaction on the measured variables (rows in Table 1). Superblock was included as a random effect, in which plot identity was nested. Variance homogeneity and normality of the distribution of residuals were verified visually. Biomass-specific rates were log-transformed to satisfy model assumptions. Pearson's correlations were run separately for each water availability treatment (Figure 2 and Figure S4). For further site, analytical, and statistical details please refer to the Supplementary Information S1, published code (Maxwell et al., 2023), and to Maxwell (2021).

## 3 | RESULTS AND DISCUSSION

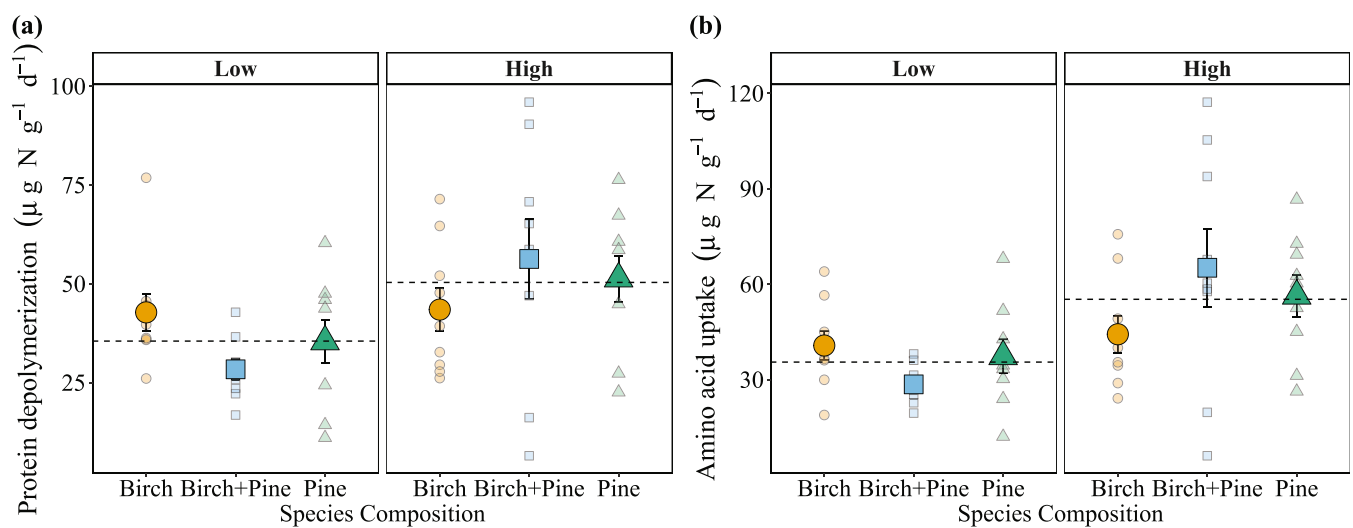
Overall, the free amino acid content (Figure S2A) averaged around 2.95 ± 0.24 μg N g<sup>-1</sup> (mean ± s.e.), gross protein depolymerisation rates ranged between 6.51 and 96.0 μg N g<sup>-1</sup> soil dry weight d<sup>-1</sup> (Figure 1a), and amino acid uptake rates ranged between 6.09 and 117 μg N g<sup>-1</sup> soil dry weight d<sup>-1</sup> (Figure 1b). These values are within the range of those found in previous studies (Fuchslueger et al., 2019; Noll, Zhang, Zheng, et al., 2019); however, at the lower range, due to the nutrient poverty (moderate soil organic C, low soil total N and P) of the sandy podzol soil at the field site (Achat et al., 2009; Augusto et al., 2010) (Table S1).

In contrast to our first hypotheses ( $H_{1a}$  and  $H_{1b}$ ), no general species identity or mixture effects on organic N cycling rates emerged (Table 1; Figure 1a,b). However, there were variations within species treatments (Figure 1a,b). Because soil enzyme activity did not differ between species treatments in the top 0–15 cm soil layer at our site (Maxwell et al., 2020), these results suggest that the variability and the trends of soil organic N cycling rates within species treatments may be caused by differences in substrate availability (i.e., soil organic N or

**TABLE 1** Results from linear mixed effects models testing the effect of tree species composition (birch vs. pine vs. birch + pine), soil water availability ( $H_2O$ ), and their interaction on soil organic N cycling processes, microbial biomass C and N, and the process rates based on microbial biomass C.

	Tree species composition (Sp)		Soil water availability ( $H_2O$ )		Sp $\times$ $H_2O$	
	F-value	p-value	F-value	p-value	F-value	p-value
Protein depolymerisation (PD)	0.015	0.985	8.483	<b>0.015</b>	2.387	0.142
Amino acid uptake (AAU)	0.201	0.821	9.424	<b>0.012</b>	2.213	0.160
Microbial biomass C	0.484	0.630	0.479	0.505	0.58	0.578
Microbial biomass N	0.053	0.949	0.285	0.605	0.656	0.540
Specific PD activity	0.249	0.785	0.719	0.416	0.041	0.960
Specific AAU activity	0.350	0.713	0.643	0.441	0.134	0.876

Note: F-values and p-values for fixed effects are presented. Significant ( $p < 0.05$ ) effects are denoted in bold font.

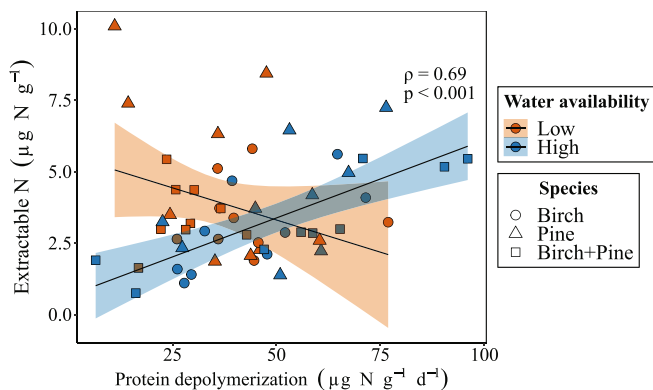


**FIGURE 1** Effect of tree species composition and soil water availability (low vs. high) on (a) protein depolymerisation (PD) and (b) amino acid uptake (AAU). Raw data are presented in partial translucence, with the mean and standard error superposed ( $n = 9$ ). Dashed lines correspond to the average protein depolymerisation of both monocultures (i.e., mean of pine and birch) calculated for each water availability treatment separately (i.e., low and high water availability).

soil protein content) and soil microbial biomass (Figure S2), rather than through changes in litter stoichiometry (Figure S3).

In partial agreement with our hypothesis  $H_{2a}$ , we found a slight positive effect of soil water availability on both protein depolymerisation and amino acid uptake (Table 1; Figure 1a,b), but no differences across species or with the mixture. At this sampling period during peak summer dry conditions in July, gravimetric soil water content differed between control ( $5.70 \pm 0.50\%$ , mean  $\pm$  s.e.) and irrigated blocks ( $10.5 \pm 0.81\%$ ; Figure S5). These results indicate that soil organic N processing by microbial communities is strongly dependent on soil moisture (Bengtson et al., 2005), probably because soil microorganisms have a greater C and N demand for biosynthesis and growth under favourable water conditions

(Cui et al., 2019; Tiemann & Billings, 2011), though this can depend on drought intensity (Canarini et al., 2020). However, the lack of effect on microbial biomass C and N (Figure S2C,D) or on microbial biomass-based specific activities (Figure S2E,F) suggests that the effect of low soil water availability on protein depolymerisation was mainly due to a decrease in substrate solubility and/or in the encounter between substrates and enzymes (Fanin, Mooshammer, et al., 2022). In line with this idea, the difference in soil moisture between low and high water treatments also significantly affected the correlation between total extractable N and protein depolymerisation ( $p < 0.001$ , Figure 2): the relationship was positive under high soil water availability ( $\rho = 0.69$ ,  $p < 0.001$ ), whereas there was a negative trend under low soil water availability ( $\rho = -0.30$ ,  $p = 0.128$ ). This reinforces the idea that



**FIGURE 2** Relationship between protein depolymerisation and total extractable nitrogen [N] for each of the soil water availability treatments (low, red, vs. high, blue). The different species compositions are identified by the symbols (pure birch, circles; pure pine, triangles; mixed birch + pine, squares).

protein depolymerisation depends directly on substrate accessibility in the soil solution, a hypothesis that was further supported by the positive relationship between protein depolymerisation and soil moisture for birch and birch + pine under low and high soil water conditions (Figure S4). Although we observed an increase in extractable N with higher protein depolymerisation (Figure 2), this was not accompanied with an increase in microbial biomass (Figure S2C,D). This suggests that soil microorganisms may be limited by other factors, since they did not increase in biomass with either more extractable soil N nor with increasing water. For instance, microbes are likely more limited by soil phosphorus availability in these P-poor podzols (Achat et al., 2009), which is supported by a previous study in the same experimental site which found that alleviating the P limitation led to a stronger energy constraint and investment into enzymes to acquire more C (Maxwell et al., 2020). Thus, the extra N was likely taken up in situ by the trees, promoting plant growth under high water availability (Maxwell, Fanin, et al., 2022).

Finally, and contrary to our hypothesis H<sub>2b</sub>, we did not find a significant interactive effect of tree species mixing and soil water availability on soil organic N cycling processes (Table 1, Figure 1). However, trends did seem to emerge: under dry soil conditions, mixing birch and pine tended to have a negative effect on both protein depolymerisation and amino acid uptake, while this tended to be negative under high soil water conditions (Figure 1a,b). Because mixing pine and birch has been found to increase the litterfall mass flux (Maxwell, Fanin, et al., 2022), and thereby increases organic substrate availability, an increase in soil moisture in irrigated plots could have led to an increase in protein depolymerisation

with tree mixing. In contrast, the low soil water availability in non-irrigated plots did not allow microbial communities to thrive and to exploit these additional litter resources in mixed plots. Instead, the greater biomass of understory vegetation in birch plots (Maxwell, Fanin, et al., 2022) increased soil moisture (Figure S5), which in turn may have triggered a positive effect on protein depolymerisation rates in these plots. Altogether, these results suggest that soil water availability plays a key role in determining potential tree species mixing effects on soil organic N cycling processes in forests. An acceleration of soil organic nitrogen (protein) breakdown with higher water availability led to higher free amino acid concentrations (Figure S2A), which may lead to higher mineralisation rates and higher concentrations of mineral N in soils (Jan et al., 2009; Schimel & Bennett, 2004). This could in turn increase nutrient uptake by the trees, which could be one explanation for the greater tree growth with higher soil moisture in our experimental site (Maxwell, Fanin, et al., 2022). Nevertheless, considering that climate change in Europe is expected to lead to drier summers but wetter winters (Russo et al., 2013), we anticipate that organic processing and subsequent mineralisation would become important for tree nutrition during the spring-autumn seasons, when both temperature, water availability, and tree demand are high enough.

## 4 | CONCLUSIONS AND PERSPECTIVES

Using a 12-year-old experimental plantation in southwestern France and a novel high-throughput isotope dilution technique, we found little tree species identity and mixture effects on soil organic N cycling rates. In general, soil water availability was the strongest driver, increasing these rates in all plots. This was likely due to higher substrate availability in the high soil water availability plots, but also by connecting the soil microorganisms and their extracellular enzymes to their respective substrates. We propose the following to gain a better understanding of these mechanisms. First, sampling of different soil layers may provide more in-depth understanding of the underlying patterns, as previous studies found synergistic tree species mixing effects at intermediate soil depth (15–30 cm), that is, on soil organic C stocks and enzyme activities (Fanin, Maxwell, et al., 2022; Maxwell et al., 2020). Second, sampling at various time points during the year may elucidate potential temporal asynchronies between species at key phenological time points. Finally, combining measurements of gross organic N process rates with those of gross inorganic N process rates (mineralisation, nitrification) may further reveal to which extent protein depolymerisation represents the bottleneck

for overall N cycling in nutrient-impooverished soils (Noll, Zhang, Zheng, et al., 2019; Schimel & Bennett, 2004).

## AUTHOR CONTRIBUTIONS

**Tania L. Maxwell:** Funding acquisition; writing – original draft; visualization; formal analysis; data curation; investigation; conceptualization; methodology. **Laurent Augusto:** Conceptualization; funding acquisition; writing – review and editing; supervision. **Ye Tian:** Methodology; writing – review and editing. **Wolfgang Wanek:** Methodology; funding acquisition; writing – review and editing; supervision. **Nicolas Fanin:** Conceptualization; funding acquisition; writing – review and editing; supervision.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## DATA AVAILABILITY STATEMENT

The data and code that support the findings of this study are openly available in Zenodo: <https://doi.org/10.5281/zenodo.7025493>.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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