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ORIGINAL ARTICLE

Soil Enzyme Activity and Stoichiometry in Relation to Different Land Uses in the Mediterranean Area

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Abstract

Mowing and grazing are among the most common grassland management practices, and the frequency and intensity with which they are applied can reduce soil nutrient availabilities. These alterations may drive microorganisms to increase the production of enzymes associated with the acquisition of limiting nutrients. This study aimed to investigate how different land uses—and their associated management practices—affect microbial strategies for soil nutrient acquisition and ecosystem responses to nutrient limitation. Thus, in the Matese mountain (Southern Italy), adjacent areas under different land uses (forest-FO, meadow-ME, pasture-PA) were selected, and the soils sampled at four times along the year (T1, T2, T3 and T4), corresponding to specific management practices in ME and PA. Total soil nutrient (seven macro- and four micronutrient) concentrations, and twelve soil enzymatic activities involved in carbon, nitrogen, and phosphorus acquisition by the microbial community were analyzed. Enzyme stoichiometric ratios (E_{CN}, E_{CP}, E_{NP}) as well as length and angle of a vector combining E_{CN} and E_{CP} were calculated to assess soil nutrient acquisition strategies. E_{CN} and E_{CP} values highlighted a greater microbial investment in nitrogen and phosphorus acquisition across all land uses, whereas the higher vector length value observed at T2 and T3 in PA and at T3 in ME suggested increased microbial allocation toward carbon acquisition. E_{NP} and the vector angle values highlighted a greater phosphorus acquisition in FO, nitrogen in PA, and balanced nitrogen and phosphorus acquisition in ME. These patterns suggest that land use and management practices influence microbial resource-allocation strategies and ecosystem responses to nutrient limitation by altering extracellular enzyme production. Our findings further show that practices such as annual mowing and low grazing pressure have helped preserve the balance of soil microbial nutrients,

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while also indicating the value of enzymatic stoichiometry as an effective useful approach for understanding microbial adaptation to nutrient limitation and ecosystem functioning under different land uses.

HIGHLIGHTS

- Land use and management shape microbial nutrient acquisition strategies.
- Microbes invest more in N, increasing C acquisition under pasture management.
- Annual mowing and light grazing preserved the balance of soil microbial nutrients.

Key words: Enzyme stoichiometric ratios; Land use; Grazing; Mowing; Grassland; Nutrient acquisition.

INTRODUCTION

Soil microbial community performs a series of ecological functions related to the matter cycle (Huang and others 2022; Mencel and others 2022). Fungi and decomposer bacteria, in particular, through the production of extracellular enzymes, promote the release of nutrients from the organic matter (Sinsabaugh and others 2009). These enzymes reflect the energy balance of the microbiota, which adapts the production of specific enzymes on the actual availability of resources (Burns and others 2013). Indeed, microorganisms require balanced levels of micro- and macronutrients (Dai and others 2023) and tend to acquire carbon, nitrogen and phosphorus in similar proportions, with a ratio of 1:1:1 (Sinsabaugh and others 2008), that can change in relation to environmental factors, available inorganic nutrients, as well as litter quantity and quality (Burns and others 2013; Zheng and others 2018). Building on the framework of ecological stoichiometry theory, Sinsabaugh and others (2008) proposed to analyze the metabolic nutrient demands of soil microorganisms by measuring the relationships among enzymatic activities linked to microbial carbon, nitrogen and phosphorus acquisition. This approach provides an effective tool for assessing microbial nutritional limitations over the long term (Moorhead and others, 2023). In recent years, the study of enzymatic stoichiometry has quickly evolved due to the introduction of vector analysis of enzymatic activities (Moorhead and others 2016), which considers two main components: the length and angle of the vector. Vector analysis can simultaneously represent the specific metabolic needs of the microbial community, being unaffected by fluctuations in overall enzymatic activity (Moorhead and others 2016). In general, enzymatic stoichiometry is affected by environmental factors (Feyissa and others 2022), among which the land use (de Medeiros and others 2023) and the associated management practices (He and others 2020; Reinhart and others 2022).

Grasslands cover 736,000 km^2 in Europe (https://ec.europa.eu/eu-grassland-watch/); they are mostly disturbance-dependent ecosystems, requiring human management, such as grazing in pasture and mowing in meadows (Mayel and others 2021), to maintain their diversity and productivity and provide a wide range of ecosystem services, including feed supply (Dibari and others 2021). Grazing and mowing, through the removal of vegetation cover and compaction caused by trampling of livestock, can reduce the supply of organic carbon and inorganic nutrients to the soil (Mayel and others 2021). These changes may force microorganisms to invest more in the production of enzymes associated with the acquisition of less available nutrients (Fu and others 2023; Zhao and others 2025), especially when the soil is subject to practices of intense management (Zhao and others 2025). Although numerous studies have separately investigated the effects of grazing (He and others 2020; Xu and others 2024) or mowing (Reinhart and others 2022; Fu and others 2023) on microbial nutritional balance, it remains unclear whether these management practices cause similar or different microbial nutrient demand. This knowledge is essential for identifying more sustainable management strategies, especially in the Mediterranean area, where environmental factors strongly affect nutrient cycles and ecosystem functions. It is therefore important to provide a general recommendation on the preferred management option, especially in relation to the future climate change scenario, which may lead to more frequent drought periods, resulting in a decrease in soil nutrients in grasslands (Liu and others 2023a; Mayel and others 2021; Jiao and others 2016).

With the aim to fill the described gaps, this study evaluated the impact of different land uses—pastures, meadows and forests—on microbial soil nutrient acquisition strategies. To achieve this purpose, extracellular enzymatic activities involved in carbon, nitrogen and phosphorus acquisition by the soil microbial community were analyzed; their enzymatic stoichiometric ratios (C:N, C:P, N:P) and vector analysis were also investigated to assess microbial nutrient demand (Moorhead and others 2013). We hypothesize that grazing in pasture and mowing in meadow, alter the metabolic strategies of soil microorganisms, leading to a nutritional imbalance, differently from forest soil, where management practices were absent.

MATERIALS AND METHODS

Study Area and Soil Sample Collections

In Mount Matese (southern Italy), three adjacent sites characterized by different land uses were selected: pasture – PA (41°24′56.3″ N 14°25′11.3″ E), meadow – ME (41°24′8.4″ N 14°26′56.5″ E) and forest – FO (41°25′00.5″N 14°25′53.2″E). All the sites are located at an elevation of approximately 1010 m a.s.l. and are developed on an Andosol Pachy-Eutrisilic substrate (FAO 1976). The area is characterized by an average annual temperature of about 17 °C and a total annual precipitation of about 1385 mm (https://agricoltura.regione.campania.it/meteo/archivio_meteo.html). The PA site has been used for mixed grazing of cattle, sheep and horses since 1982. Grazing typically lasts six months per year, from early summer to early winter, followed by a five-month spring-rest grazing. The ME site is managed as a forage meadow since 1982, fertilized with manure (applied annually at a rate of approximately 30 t ha^{-1}) and sown with a seed mixture including *Lolium perenne* L., *Lolium multiflorum* L., *Trifolium pratense* L., *Dactylis glomerata* L., *Festuca arundinacea* L., *Phleum pratense* L., *Lotus corniculatus* L. and *Trifolium repens* L. The FO site, dominated by *Fagus sylvatica* L., represents an undisturbed forest ecosystem that has remained unmanaged since the beginning of the last century.

A completely randomized experimental design was applied, consisting of three plots (5 × 5 m) chosen for each land-use type. Four sampling (T1–T4) were conducted on the same dates for all land uses. The corresponding management phases for PA and ME associated with each date are reported in Table 1. In each plot, eight soil cores (0–10 cm depth) were collected and pooled into a composite sample. This procedure was followed during every sampling time.

Soil Laboratory Analyses

Each composite soil sample was sieved (< 2 mm) and analyzed in triplicate. On fresh soil samples, twelve enzymatic activities were quantified by fluorometric assay using a Synergy HT microplate reader (BIO-TEK) after applying a heteromolecular exchange procedure, as described in Bardelli and others (2017). Enzymatic activities were expressed as nmol of product released h^{-1} g^{-1} soil dry weight (d.w.). The functions and the substrates associated with each activity are shown in detail in Table 2. On oven-dried (105 °C) and grounded soil samples, total C and N concentrations were determined according to Colombo & Teodoro (2015) using a CHNS elemental analyzer (Carlo Erba 1500). Total macro- (P, Ca, K, Mg) and micronutrient (Fe, Zn, Mn, Cu) concentrations were measured by optical emission spectrometry with inductively coupled plasma (Thermo Scientific TM iCAP PRO XP Duo) after microwave-assisted (Anton Paar GmbH Multiwave-3000) acid digestion using concentrated HNO_3 (USEPA 1996).

Data Treatment

The measured soil enzymatic activities were grouped and summed to calculate the soil enzymes involved in the acquisition of C, N and P (Liu and others 2020; Yang and others 2022). The hydrolytic activities related to carbon (Hy-C) acquisition were obtained by summing β-glucosidase (BG), cellobiohydrolase (Cell), xilosidase (Xilo) and β-galactosidase (BGAL), those related to nitrogen (Hy-N) by summing chitinase (NAG), leucine-aminopeptidase (LAP) and serin – like – protease (CBZ); while for phosphorous (Hy-P), acid phosphomonoesterase (AP), pyrophosphate—phosphodiesterase (pyroP) and phosphodiesterase (bisP) were considered. For oxidative enzymes (Ox-C), which catalyze the oxidation of complex molecules

Table 1. Sampling Dates for All Land Use (T1–T4) and the Corresponding Management Phases for PA = Pasture and ME = Meadow

Sampling time	Date	PA	ME
T1	4 June 2023	Beginning of the grazing	Sowing
T2	24 July 2023	After one month of grazing	Mowing
T3	4 December 2023	After six months of grazing	Five months after mowing
T4	6 May 2024	After the five-month resting grazing	One year after sowing

Table 2. Soil Enzyme Groups (Hy-C, Hy-N, Hy-P, Ox-C) and Their Individual Activities, with Acronyms, Functions, and Substrates

Abbreviations	Name	Acronym	Function	Substrate
Hy-C	β-glucosidase	BG	Cellulose degradation	4-methylumbelliferyl-beta-D-glucopyranoside
	β-galactosidase	BGAL	Galactose degradation	4-Methylumbelliferyl-beta-D-galactopyranoside
	Cellobiohydrolase	cell	Cellobiose degradation	4-methylumbelliferyl beta-D-cellobioside
	Xilosidase	xylo	Xylan degradation	4-methylumbelliferyl beta-D-xilopyranoside
Hy-N	Chitinase	NAG	Chitin degradation	4-methylumbelliferyl-N-acetyl-beta-D-glucosaminide
	Serin-like-protease	CBZ	Protein degradation	N-alpha-CBZ-L-Arginine 7-amido-4-methylcoumarin hydrochloride
	Leucine-aminopeptidase	LAP	Peptide hydrolysis	L-Leucine 7-amido-4methyl-coumarine hydrochloride
Hy-P	Acid phosphomonoesterase	AP	Organic phosphorous mineralization	4-methylumbelliferyl phosphate
	Phosphodiesterase	bisP	Phosphoric diester hydrolase	bis(4-methylumbelliferyl)phosphoric acid
	Pirophosphate-phosphodiesterase	piroP	Hydrolysis of pyrophosphate	bis(4-methylumbelliferyl)pyrophosphoric acid
OX-C	Peroxidase	PEROX	Degrade lignolytic, catalyzes oxidation reactions via the reduction of H_2O_2	10-Acetyl-3,7-dihydroxyphenoxazine + H2O2
	Phenol oxidase	OX	Oxidized lignin	10-Acetyl-3,7-dihydroxyphenoxazine

Abbreviations: hydrolytic activities related to carbon, nitrogen and phosphorus acquisition (Hy-C, -N and -P); oxidative enzymes (Ox-C)

such as lignin (Yang and others 2022), the activities of phenol oxidase (OX) and peroxidase (PEROX) were summed.

In order to identify microbial soil nutrient acquisition strategies, the approach based on the enzymatic stoichiometry ratios, focusing on *C*, *N* and *P*, was applied. The *C*:*N* (E_{CN}), *C*:*P* (E_{CP}), *N*:*P* (E_{NP}) ratios were determined by applying the following equations (Sinsabaugh and others 2008; Zhou and others 2020):

$$E_{CN} = \ln\mathrm{BG}/\ln(\mathrm{NAG} + \mathrm{LAP}) \tag{1}$$

$$E_{CP} = \ln\mathrm{BG}/\ln(\mathrm{AP}) \tag{2}$$

$$E_{NP} = \ln(\mathrm{NAG} + \mathrm{LAP})/\ln(\mathrm{AP}) \tag{3}$$

E_{CN}, E_{CP}, and E_{NP} values < 1 indicate a demand for *N* and *P*, while values > 1 indicate a demand for *C* and *N*.

To further assess microbial nutrient demand, vector analysis based on enzyme stoichiometry ratio was applied, as described by Moorhead and others (2013). This approach combines the E_{CN} and E_{CP} ratios into a vector characterized by the equation:

$$\text{Vector length} = \mathrm{SQRT}(X^2 + Y^2) \tag{4}$$

$$\text{Vector angle} = \mathrm{Degrees}(ATAN2(X, Y)) \tag{5}$$

where $X = \ln(\mathrm{BG})/\ln(\mathrm{AP})$ and $Y = \ln(\mathrm{BG})/\ln(\mathrm{NAG} + \mathrm{LAP})$. A longer vector length (VL) indicates a greater carbon acquisition relative to nutrient acquisition, while the vector angle (VA) $< 45°$ and $> 45°$ represent relatively higher nitrogen *versus* phosphorus acquisition, and higher phosphorus *versus* nitrogen acquisition, respectively.

Statistical Analysis

The permutation analysis of variance (PERMANOVA) was applied to test the overall response of total soil micro- and macronutrient concentrations, OX-C enzyme, Hy-C, -P, and -N enzymes against land use and sampling times (fixed factors). *P*-values were calculated using the Monte Carlo permutation test (999 permutations) together with the permutation of residuals under an unrestricted permutation model. To visualize the variation of the previously mentioned parameters among land uses and sampling times, we performed the non-metric multidimensional scaling (NMDS), with the superimposition of the confidence ellipses ($\alpha = 0.05$) for land use, as well as the temporal gradient.

All parameters of this study were evaluated by two-way repeated measurements ANOVAs (two-way RM ANOVAs) using land use and sampling time as fixed factors. Post hoc Tukey's HSD tests ($\alpha = 0.05$) were performed to compare means among land-use types and among sampling times within each land use.

Two distinct redundancy analyses (RDAs) were carried out to identify relationships among soil total micro- and macronutrient concentrations and, respectively, enzymatic activities (OX-C, Hy-C, Hy-N, and Hy-P) and stoichiometric ratios (E_{CN}, E_{CP}, and E_{NP}). First, a forward selection of soil chemical properties was applied, based on 999 permutations, to identify the most parsimonious model. Then, multicollinearity among soil chemical properties was checked, excluding parameters with a variance inflation factor (VIF) greater than 20.

The ''vegan'', ''nlme'', ''emmeans'', ''multcomp'', ''dplyr'' and ''ggplot2'' packages in the R 4.1.2 programming environment (R Core Team 2021) were used.

RESULTS

Overall, PERMANOVA performed on soil total nutrient concentrations, oxidative enzymes (OX-C) and hydrolytic activities related to *N*, *P* and *C* acquisition at FO, PA and ME along the year, highlighted significant differences among land uses ($F = 15$; $p < 0.001$) and among sampling times ($F = 22$; $p < 0.01$). The NMDS, performed on the above-mentioned parameters, showed a clear separation of PA and ME from FO (Figure 1). PA was characterized by the highest total N and C concentrations that increased at T3 and by the highest total P, Cu and Zn concentrations that decreased at T4. FO was characterized by higher OX-C, Hy-P and -C values; in particular, Hy-P and -C decreased at T4 (Figure 1; Table S1–S3). ME showed intermediate values of the analyzed parameters.

Enzyme activities, grouped into Hy-C, Hy-P, and OX-C, showed significant differences between land uses and sampling times. Among the enzymes included in Hy-C, BG and xilo were higher in FO than in ME, and both decreased at T4 in FO and increased at T3 in PA (at least $p < 0.05$), whereas Cell was higher in FO and PA than in ME and increased at T3 in each land use (at least $p < 0.01$). Regarding the enzymes included in Hy-P, AP showed differences among land uses, with higher values in FO compared to PA and ME, and a decrease at T4 in FO (at least $p < 0.05$). Finally, among the enzymes included in OX-C, OX and PEROX were higher in FO than in PA and ME, with OX increasing in T3 in FO and PEROX increasing in T3 in PA and FO (at least $p < 0.05$; Table S4).

Regarding the soil enzymatic stoichiometric ratios, both E_{CN} and E_{CP} showed values slightly lower than 1 at all sampling times in each land use (Figure 2a, b). In particular, the E_{CN} ratio did not show significant differences among land uses (Table 3), but in FO it significantly decreased at T4 respect to T1 and T3, and in ME increased at T3 in respect to T1 and T2 (Figure 2a; $p < 0.05$). The E_{CP} ratio was significantly higher in PA than in FO (Table 3; $p < 0.01$), with FO showing a decrease at T4 compared to T1 and T3. In PA, the E_{CP} ratio showed an increase at T2 and T3 in respect to T1, and in ME an increase at T3 in respect to T2 (Figure 2b; $p < 0.001$). In contrast, the E_{NP} ratio showed values slightly above 1 in PA and ME, but only PA significantly differed from FO, that showed values below 1 (Table 3; $p < 0.01$). In ME the values were equal to 1. In addition, the E_{NP} ratio did not show significant temporal variations within each land use (Figure 2c).

The vector length did not differ (for $\alpha = 0.05$) among land uses (Table 3); however, significant differences were observed among sampling times within each land use (Figure 3a). In FO, the vector length decreased at T4 compared to the other times ($p < 0.001$); in PA it showed higher values at T2 and T3 than T1 ($p < 0.001$), while in ME it increased at T3 compared to the other times ($p < 0.001$). The vector angle values showed values significantly higher in FO (VA = 47°) than in ME (VA = 44°) and PA (VA = 43°) (Table 3; $p < 0.001$). In addition, the vector angle did not show significant temporal variations within each land use (Figure 3c).

The RDA analysis explained 65% and 69% of the total variance for soil enzymatic activities and their stoichiometric ratios, respectively (Figure 4a,b). The total *P* concentration was the variable mostly contributing (36%) to the explained variance of enzymatic activities (Table S5a), whereas total Ca concentration was the most influential variable (41%) for the stoichiometric enzymatic ratios (Table S5b). Regarding enzymatic activities (Figure 4a), Hy-P exerted the main influence in FO and was correlated with total Mn concentration,

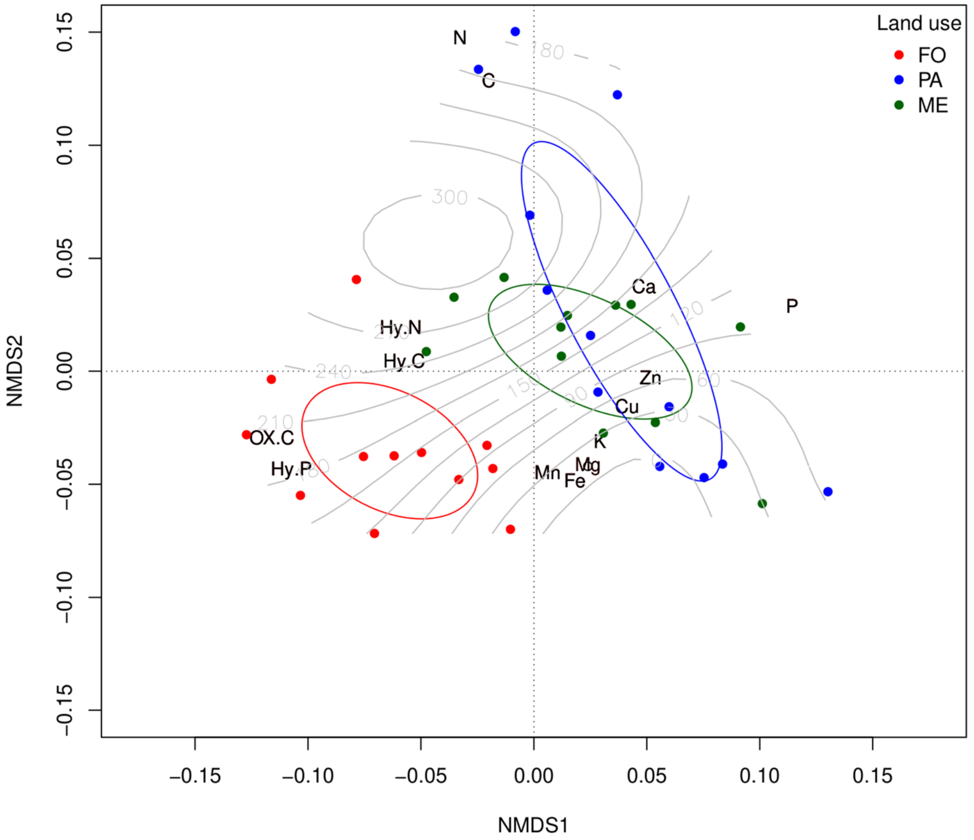

Figure 1. Non-metric multidimensional scaling (NMDS) biplot, with the superimposition of the temporal gradient (0 -T1; 30 – T2; 180 – T3; 365 – T4) and of the confidence ellipses (α = 0.05), showing the differentiation among land uses (forest, pasture and meadow) in relation to soil total nutrient (C, N, P, Ca, Fe, K, Mg, Cu, Mn, Zn) concentrations, oxidative enzymes (OX-C) and four hydrolytic activities related to N, P and C acquisition (Hy-N, P and C). FO = forest (red), PA = pasture (blue), ME = meadow (green).

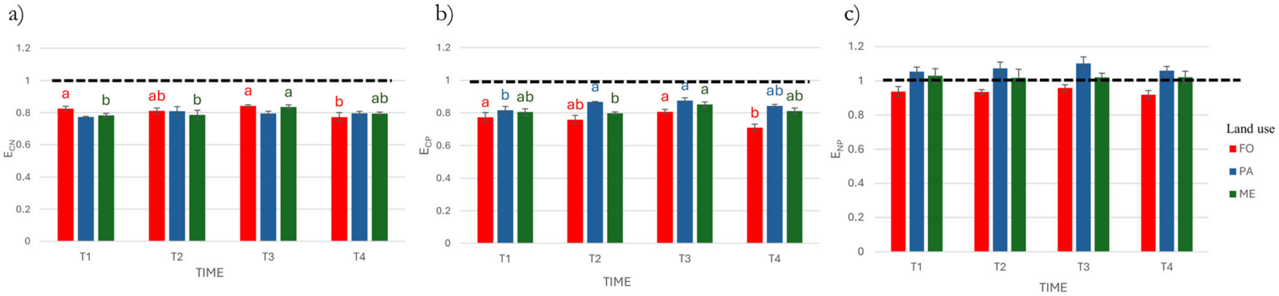

Figure 2. Bar plots (mean values ± s.e.) of the soil stoichiometric activities – E_{CN} (**a**), E_{CP} (**b**) and E_{NP} (**c**) – in FO = forest (red), PA = pasture (blue), ME = meadow (green), at each sampling time (T1, T2, T3 and T4). Different letters indicate significant differences among sampling times in each land use ($p < 0.05$).

whereas Hy-N represented the most influential variable in PA and was associated with total Ca concentration. In contrast, Hy-C showed a predominant influence in PA and was correlated with total P concentration. Regarding the stoichiometric ratios of enzymatic activities (Figure 4b), E_{CN} exerted the main influence in FO and was associated with total Mn concentration, while E_{NP} was the most influential variable in PA, correlated with total Ca concentration.

DISCUSSION

The studied forest soil showed a greater microbial demand for phosphorus relative to nitrogen compared to the pasture and meadow soils, as indicated

Table 3. E_{CN}, E_{CP}, E_{NP}, Vector Length and Angle (Mean ± S.E) in Forest (FO), Pasture (PA) and Meadow (ME) Across the Year. Significant Differences ($\alpha = 0.05$) Among Land Uses Are Indicated by Different Letters; $N = 12$

	FO	PA	ME
E_{CN}	0.81 ± 0.011	0.79 ± 0.0082	0.80 ± 0.010
E_{CP}	0.76 ± 0.015 b	0.85 ± 0.010 a	0.82 ± 0.0094 ab
E_{NP}	0.94 ± 0.011 b	1.1 ± 0.015 a	1.0 ± 0.017 ab
Vector length	1.1 ± 0.017	1.2 ± 0.010	1.1 ± 0.010
Vector angle	47 ± 0.32 a	43 ± 0.39 b	44 ± 0.46 b

Enzymes used for the calculation of E_{CN}, E_{CP}, E_{NP}, *vector length, and angle are given in Table 2 and described in the Materials and Methods section*

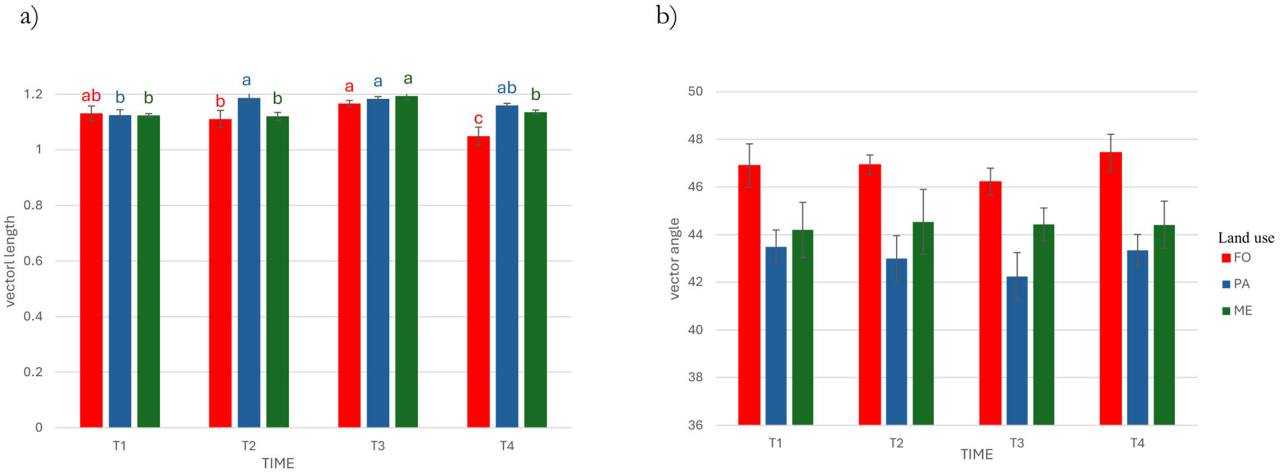

Figure 3. Bar plots (mean values ± s.e.) of the vector length (**a**) and vector angle (**b**) in FO = forest (red), PA = pasture (blue), ME = meadow (green), at each sampling time (T1, T2, T3 and T4). Different letters indicate significant differences among sampling times in each land use ($p < 0.05$).

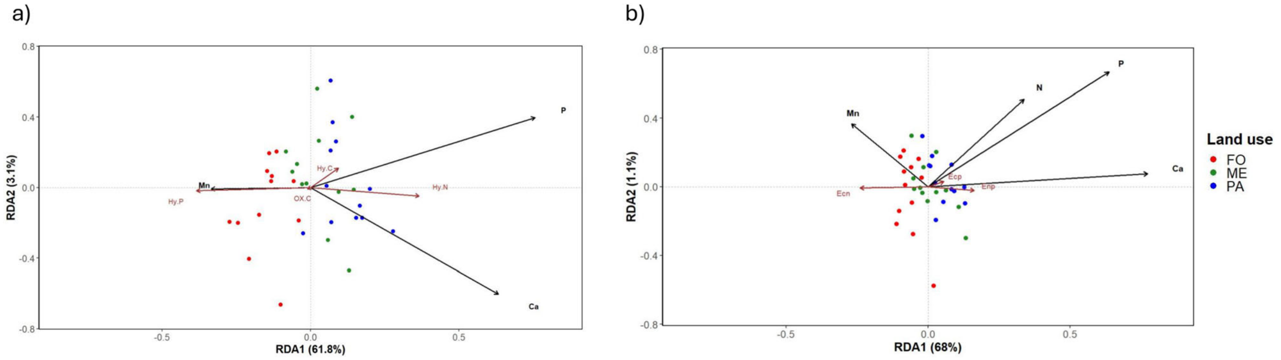

Figure 4. Redundancy analysis of the correlations between total nutrient concentrations selected from the model and (**a**) enzyme activity or (**b**) stoichiometric enzyme activity ratios in each land use. FO = forest (red), PA = pasture (blue) and ME = meadow (green).

by the vector angle. This result suggests a possible limitation of phosphorus, also confirmed by the high soil enzyme activity of acid phosphomonoesterase observed (Table S4–Table S6). This is likely attributable to the lower phosphorus concentration observed at this site. Several studies (Asensio and others 2021; Zuccarini and others 2023) have highlighted a limitation of phosphorus in Mediterranean forests, in particular in holm oak (*Quercus ilex* L.) forests. Although in our case the total phosphorus concentrations in forest soil are lower than those of pasture and meadow soils, they are still within the typical range for phosphorus-rich soils of European beech forests (1–3 g kg^{-1}; Lang and others 2017). This finding could be attributed to the addition of livestock residues that lead to an increase in phosphorus content in grassland ecosystems, as highlighted by Xu and others (2025).

The higher microbial demand for phosphorus observed could be attributed to the limited availability of immediately assimilable forms of phosphorus. This reduced availability is primarily attributed to its immobilization in microbial biomass or in complex organic forms, which are not immediately accessible (Aponte and others 2010). Indeed, we observed that forest soils have a relatively lower content of recalcitrant organic matter than pasture and meadow soils (Table S7). The average value observed in forest soil is only slightly lower than the range (4–6 mg g^{-1}) reported in studies conducted in the same forest by Picariello & De Nicola (2024). Thus, the lower recalcitrant organic matter fraction observed in forest soil could be attributed to the high enzymatic activity, particularly of phenol oxidases and peroxidases, involved in the degradation of the more recalcitrant organic matter fraction. Studies conducted by Craine and others (2007), Park and others (2022) have, in fact, highlighted that, in conditions of reduced nutrient availability, microorganisms increase the synthesis of extracellular enzymes responsible for the degradation of complex organic matrices, in order to acquire the necessary nutritional elements. In this context, the elevated enzymatic activities observed in our study may also reflect nutrient limitations integrated over a broader temporal scale (Moorhead and others 2023).

In our study, the higher manganese concentration in forest soil compared to meadows is probably related to the high concentrations of this element in beech leaves, whose decomposition can enrich the soil in available manganese, as evidenced by Michopoulos and others (2021). The high manganese concentration in forest soil and the observed correlation between manganese and the enzymes involved in phosphorus acquisition, which emerged from the redundancy analysis, may be due both to the role of manganese as a cofactor in several microbial enzymes—particularly acid phosphomonoesterase (O'Sullivan and others 2026; Burnell, 1988; Hemkemeyer and others 2021)—and to the release of carboxylic acids by beech roots. These compounds promote the mobilization of phosphorus bound to mineral particles and, at the same time, increase the availability of other elements, such as manganese, as reported by Lambers and others (2022). This process suggests that manganese can be considered an indicator of phosphorus acquisition strategies adopted by plants and soil microorganisms.

In our study, we found a greater microbial demand for nitrogen relative to phosphorus in pasture soils compared to forest soils, as indicated by the vector angle. Moreover, microorganisms appeared to invest more in carbon acquisition than in nutrient acquisition, particularly after one and six months of grazing, as highlighted by vector length. These results suggest that, although the total nitrogen concentration in pasture soils was higher than in forest and meadow soils — with values within the typical range for pastures with moderate or light grazing (Han and others 2008)—the microbial community may be affected a relatively low labile organic matter compared to meadow, especially after six months of grazing (Table S7). Such a condition may lead microorganisms to invest more resources in carbon acquisition and, consequently, to increase their demand for nitrogen more than for phosphorus under limited carbon availability, consistent with the findings of as also reported by Zhao and others (2025). Grazing may have resulted in reduced availability of labile carbon, possibly due to reduced inputs of litter to the soil and continued removal of vegetation cover, which resulted in decreased carbon flux from plants to soil through lower production of root exudates (Stark & Kytöviita, 2006). The increased microbial investment in carbon acquisition at T3 may also be partly due to climatic conditions, as a similar pattern has been observed in forest and meadow. This result is likely related to temperatures in previous months (mean temperature ~ 18 °C), which increased enzyme activity, as reported by Li and others (2022). Overall, the results suggest that grazing and temperature can alter the availability of labile carbon, thereby modifying microbial nutrient demand and affecting both extracellular enzymatic activity and soil enzyme stoichiometry. Furthermore, the high concentration of nutrients such as calcium, phosphorus, zinc and copper found in pasture compared to forest and meadow soils could be attributed to the continuous presence of livestock excreta, which are an important source of micro- and macronutrients and tend to accumulate in the soil (Garcia and others 2008; Assmann and others 2017; Indraratne and others 2021).

Among the analyzed nutrients, phosphorus and calcium were found to be the main factors affecting enzymatic activity and stoichiometry, respectively, as indicated by redundancy analysis. The availability of phosphorus in the soil depends largely on mineral decomposition, microbial activity, and on soil physical and chemical characteristics. Increased grazing pressure can slow down these processes, reducing phosphorus concentrations, while prolonged grazing can decrease vegetation cover, increasing the risk of erosion and phosphorus loss (Lemma and others 2017). According to several authors (Yang and others 2019; Liu and others, 2023b), the total phosphorus concentration in pasture soil ranges from 0.09 to 2.70 g kg^{-1} d.w. Some studies have found no significant variations among different grazing pressures (Zhang and others 2022; Usman and others 2024), while others have shown an increase in total phosphorus in the presence of light grazing (Dong and others 2012; Moghbeli and others 2021).

Calcium can affect enzymatic activity by acting as a cofactor, directly activating enzymes involved in the decomposition of organic matter (He and others 2025; Hemkemeyer and others 2021). This condition was observed in our study; in fact, in pasture, calcium was found to be correlated with enzyme involved in nitrogen acquisition, as indicated by redundancy analyses. This correlation can be explained by the dependence of some extracellular enzymes on calcium ions. These include degradative enzymes such as chitinases, which are involved in nitrogen acquisition, as reported by Hemkemeyer and others (2021). Overall, these results suggest that calcium may indirectly affect microbial nitrogen demand in grazed soils. In our study, the total calcium concentration in the pasture soil showed values between 6 and 10 g kg^{-1} d.w. at different sampling times, highlighting an increase in this nutrient after six months of grazing. These values, although higher than those reported by Assmann and others (2017) in pasture soils, still lie well above the critical level of 0.072 g kg^{-1} d.w. indicated by Khan and others (2007) and Rhue & Kidder (1983), confirming the adequate calcium concentration in the pasture soil. The critical level in fact represents the minimum threshold of the nutrient concentration, below which the quantity of the element is no longer sufficient to guarantee the correct functioning of the soil ecosystem.

Contrary to our hypothesis, pastures and meadows did not show strong nutritional imbalance, a condition particularly evident in the meadow. The low enzymatic activities found in these ecosystems compared to the forest confirm this condition and suggest that nutrients were not strongly limiting. This can be explained by a considerable energy cost associated with enzyme synthesis, making it advantageous only when nutrients represent a truly scarce resource for the microbial community (Burns and others 2013). In meadow, five months after mowing, less microbial investment in nitrogen and phosphorus acquisition than carbon was observed, as indicated by the E_{CN} and E_{CP} ratios. When the stoichiometric ratios of soil enzymes approach the theoretical equilibrium value of 1:1:1 defined by Sinsabaugh and others (2008), the enzymatic activities associated with the carbon, nitrogen and phosphorus cycles are balanced and stable, indicating the ability of soil enzymes to adapt to environmental changes and maintain a functional balance between the main biogeochemical cycles. In the meadow, the microbial community did not show a marked nutrient demand to stimulate intense microbial activity; this is probably due to mowing, which is carried out only once a year, may not have drastically reduced nutrient availability. In fact, well-calibrated mowing can even increase the carbon and nitrogen content of the soil (Mayel and others 2021). Conversely, increasing the frequency of mowing reduces plant species diversity and the number of soil microorganisms, consequently decreasing soil carbon and nitrogen turnover (Li and others 2017; Mayel and others 2021).

CONCLUSIONS

In forest soils, we observed an increased demand for nutrients (P). In meadow and pasture, no strong nutrient demand was detected, except in pasture, where a greater microbial acquisition of nitrogen compared to phosphorus was observed. These patterns suggest that land use influences microbial resource-allocation strategies and ecosystem responses to nutrient limitation through shifts in extracellular enzyme production. Our results show that practices, such as annual mowing and low grazing pressure, thanks to reduced disturbance and maintenance of organic inputs to the soil, have helped to preserve soil microbial nutrient balance and can therefore be considered sustainable management practices in the Mediterranean area. In addition, our findings highlight the potential of enzymatic stoichiometry as a useful framework for understanding microbial nutrient acquisition strategies, soil nutrient availability, and ecosystem functioning under different land uses.

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DATA AVAILABILITY

Data are available from Mendeley Data: https://doi.org/10.17632/jc68n5mr86.1

Declarations

Conflict of interest There are no conflicts of interest to declare.

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