



Microbial activity and habitat heterogeneity in surface soil in a high-mountain grassland landscape (Central Apennines, Italy)

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ABSTRACT

A spatially intensive sampling of surface soil layers (81 stratified-randomized sampling points across a 14 km² study area) was performed in a high-mountain grassland landscape (Central Apennines, Italy) in July 2017, in order to describe the heterogeneity of microbial habitats and measure their microbial enzymatic activity. Three different microbial habitat types were identified via Hierarchical Cluster Analysis on the basis of 14 (measured or remote-sensed) environmental variables (including chemical and physical soil properties, topographic and geomorphological features and vegetation cover): (i) sub-acidic, at high elevation, with high vegetation cover (of mostly acidophytic/mesophytic species) and high silt content, and the lowest clay content; (ii) sub-acidic, at low elevation, with a high vegetation cover and a very low stone cover, with the highest sand and the lowest silt content, and the highest available phosphorus; (iii) very shallow soil, mainly stone-covered, at high elevation, with the highest pH values, on steep slopes, with the highest content of organic matter and the highest water holding capacity. The third habitat showed the highest enzymatic activity (b-glucosidase, β-cellobiohydrolase and leucine-arylamidase) involved in C and N cycling, while the more acidic and deeper soils, typical of sinkhole or slope areas, favoured the acid phosphomonoesterase activity involved in P cycle thanks to the establishment of the rhizospheric activity of the herbaceous plant species. Moreover, we analysed the relative importance of environmental variables on the total enzyme activities via Boosted Regression Trees (BRT): the results highlighted - for the first time as far as we know - the importance of topographic factors such as slope inclination in predicting the microbial functional capacity in a mountain grassland ecosystem. We conclude that the different enzymatic activity patterns found in the three habitats suggest diverse microbial functions with respect to nutrient cycling, within a small landscape and a relatively homogeneous land-cover.

1. Introduction

Mountain environments are spatially and temporally heterogeneous systems, where soil properties and soil microbial habitat may vary widely even within very small areas. Marozzi et al. (2022) reported that the environmental factors allowing the survival and growth of microbial communities (e.g., oxygen, water, or nutrient availability) can vary substantially from the millimeter to centimeter scale. Other studies (King et al., 2010; Ranjard and Richaume, 2013; Nunan et al., 2017) evidenced that soil microorganisms are not homogeneously distributed across landscapes but rather occur in patches which are related to the distribution of biogeochemical properties (e.g. substrate and water bioavailability, pH, soil structure and plant cover). Indeed, many biotic

factors such as microbial biomass, enzymatic activities, vegetation cover and vegetation composition are expected to be highly influenced by physical and chemical soil properties and by geomorphological and (micro)climatic condition at a very fine spatial scale (Sebastià, 2004; Bach et al., 2018; Filibeck et al., 2019; Filibeck et al., 2020).

The microbial biomass and enzymatic activities play a crucial role in the regulation of some fundamental processes that determine the turnover of elements such as the mineralization of organic matter, ammonification, nitrification and atmospheric nitrogen fixation (Rapport et al., 1997). The most widely tested enzymes in mountain environment are cellobiohydrolase, α- and β-glucosidase, and phenol oxidase which are involved in the degradation of cellulose and lignin, the most abundant components of plant litter (Allison et al., 2007). Other commonly

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measured enzymes are those involved in the hydrolysis of proteins, chitin and peptidoglycan, which are the principal reservoirs of organic N (Caldwell, 2005). Furthermore, extracellular phosphatases are important for their role in mineralizing P from nucleic acids, phospholipids and other ester phosphates (Turner et al., 2002; Toor et al., 2003).

In Southern Europe, grasslands are an important ecosystem for biodiversity and landscape conservation, and for sustaining traditional farming (Habel et al., 2013; Apostolova et al., 2014). In particular, some types of secondary grasslands are of outstanding value since they are among the most diverse ecosystems in the world concerning plant species richness at small spatial scale (Wilson et al., 2012; Chytrý et al., 2015); therefore, they are protected as “priority habitat types” by the EU Habitats Directive (European Union, 1992). Secondary grasslands are semi-natural habitats originated and maintained by disturbance such as livestock grazing, in areas that would be potentially covered by forest vegetation or other vegetation types such as subalpine scrubs (e.g. Dengler et al., 2014). However, they are not totally man-made ecosystems, as livestock disturbance replaces the grazing and browsing by wild prehistoric megaherbivores and by extant ungulates (e.g. Pärtel et al., 2005; Sandom et al., 2014). In the Central Apennine mountains (Italy), there still are large areas of grasslands used for extensive grazing (e.g. Primi et al., 2016). These are extremely interesting for basic and applied studies on environmental diversity and management, as sheep grazing and transhumance were shaping Apennine landscapes already during the Bronze Age, i.e. since 6th century BCE or earlier (Brown et al., 2013); these habitats play a key role in the conservation of endangered fauna and flora (Primi et al., 2016; Cancellieri et al., 2020).

In the present paper, a comprehensive approach has been adopted to characterize soil microbial habitat heterogeneity according to soil chemical, physical and geomorphological properties of the grassland ecosystems in a sector of the Central Apennine mountains contained within Abruzzo Lazio & Molise National Park. The study area has a high heterogeneity of bedrock and landform (Bigi et al., 1986), and this work attempts to explore the interactions between abiotic and biotic components, taking into account a wide array of environmental parameters, especially concerning the topographic and geomorphological features – an approach that, as far as we know, has never been used in Italy. The specific objectives of this paper are: i) to identify the different habitats for the microbial soil community within a mountain grassland landscape; ii) to establish to which extent soil properties can affect soil functions, such as nutrient cycling by microbial biomass and activity in the different habitats; iii) to identify what are the environmental factors that can better predict soil microbial functional capacity in mountain grasslands.

2. Material and methods

2.1. Study area

The study was carried out in a sector of “Abruzzo Lazio and Molise National Park”, namely in the municipality of Picinisco and, in particular, in the area surrounding Monte Meta (Lazio, Central Italy). The study area extended over c. 1400 ha and included the grassland habitats between the 1400 m a.s.l. contour line and the mountain ridges, peaking at 2241 m a.s.l. with Monte Meta. Potential vegetation is considered to be *Fagus sylvatica* forest below c. 1900 m a.s.l., and subalpine scrubland (dominated by *Juniperus communis* subsp. *nana*) above this threshold (Blasi et al., 2010). Mean annual precipitation is estimated to be c. 1300–1500 mm/yr, with mean annual temperature between c. 8 and 4 °C, depending on elevation; there are 6–8 months with mean temperature <10 °C (Blasi, 1994; Crespi et al., 2018; Cancellieri et al., 2020). Precipitation regime is sub-Mediterranean, i.e. with marked decrease in summer (Crespi et al., 2018), leading in July–August to a relative drought stress and a fall in grassland productivity (Primi et al., 2016). Bedrock shows a very complex pattern at relatively fine spatial scale, with Mesozoic dolomite and limestone, Palaeogene jasper and

clay, Eocene limestone, and Quaternary moraine sediments (Bigi et al., 1986). As a consequence of the different geological substrata and of the wide elevation range, geomorphology shows a mixture of karst land-forms (with dolines, polje, karren, etc.), glacial land-forms (such as moraines) and other land-form types connected with high-elevation environments (such as screes and cliffs) (Cinque et al., 1990; Giraudi, 2001). Following the high lithological and geomorphological heterogeneity, and according to the different elevation belts, the grassland communities of the study area show a high variability in dominant species, floristic composition, productivity and cover. Driest habitats on limestones are dominated by the xerophytic grass *Festuca circum-mediterranea*; very shallow or rocky soils are dominated by a mixture of xerophytic grasses and chamaephytes such as *Globularia meridionalis*, with irregular cover; jasper areas are dominated by *Nardus stricta*, giving rise to dense-cover, relatively productive grasslands; clayey doline bottoms are often characterized by closed turf of *Festuca microphylla* or by dwarf - but dense - carpets of *Trifolium thalii*; steep slopes with loose debris can be dominated by the tall grass *Brachypodium genuense*; high-elevation grasslands are characterized by *Festuca laevigata*, *F. violacea* subsp. *italica*, *Poa alpina* and/or *Avenula praetutiana* (Ciaschetti et al., 2016; Primi et al., 2016; Cancellieri et al., 2020; Filibeck et al., 2022).

2.2. Sampling design

The soil samples analysed in the present study were collected during a botanical survey of grassland composition, in the frame of a wider research project on rangeland management. The grassland survey followed a stratified random approach, in order to account for the complexity of the physical environment of the study area. A 1:10,000 map of the areas that could be defined as “grasslands” (i.e. excluding screes, rocks, shrub stands, etc.) within the study area was obtained via visual photointerpretation on aerial photographs. We then overlapped the grid of the (10 m × 10 m) pixels of the Sentinel 2 satellite images on the grassland map. The c. 131,000 pixels selected in this way were then classified into 12 sampling strata, applying to each pixel the following attributes that were derived in GIS environment (ESRI ArcGIS). 1) *Elevation belt*: two belts, i.e. <1800 m and >1800 m (as this is the approximate elevation of tree line in the Central Apennines and the border between the montane and subalpine bioclimatic belts: Cancellieri et al., 2020). 2) *Bedrock category*: siliceous vs. calcareous (based on Bigi et al., 1986). 3) *NDVI*, i.e. normalized vegetation index, a remote-sensed proxy of standing biomass and productivity, obtained from the Sentinel imagery of July 12, 2016; we divided it into three levels (the most appropriate thresholds were identified applying the ArcGIS function “Jenks' Natural Break” to the frequency distribution within study area): high, i.e. >163; intermediate, i.e. 153–163; low, i.e. <153. Four of the 12 strata had a negligible area, so sampling was performed randomizing 8 points within each of the remaining 8 strata, yielding 64 points. Nineteen additional sampling points were located subjectively to include some specific grassland types that were not addressed by the randomized sampling, yielding a total of 83 sampling points. Field sampling took place in July 2017. At each sampling point (retrieved with a GPS), a square plot of 3.16 × 3.16 m (=10 m²) was delimited for the botanical survey and measurements used in the wider project. Two of the randomly extracted points had a very rocky substrate that yielded an insufficient amount of soil, so the present analyses are based on n = 81 (Fig. 1). Within each plot, the following environmental data used in the present paper were measured in the field: *elevation* (measured with a GPS); *slope inclination* (with a clinometer); *slope aspect* (with a compass); *rock and stone cover* (visually estimated, in %); *soil depth*, i.e. the depth from surface to bedrock (we followed Dengler et al., 2016: a 70 cm long iron rod was driven into the soil until the parent material was reached, at 5 fixed points within each 10 m² plot; the median of the 5 measures was used in the analyses); *total herb layer cover* (visually estimated, in %). A *heat-load index* for each sampling unit was then calculated from latitude, slope inclination and aspect (following McCune and Keon, 2002). The

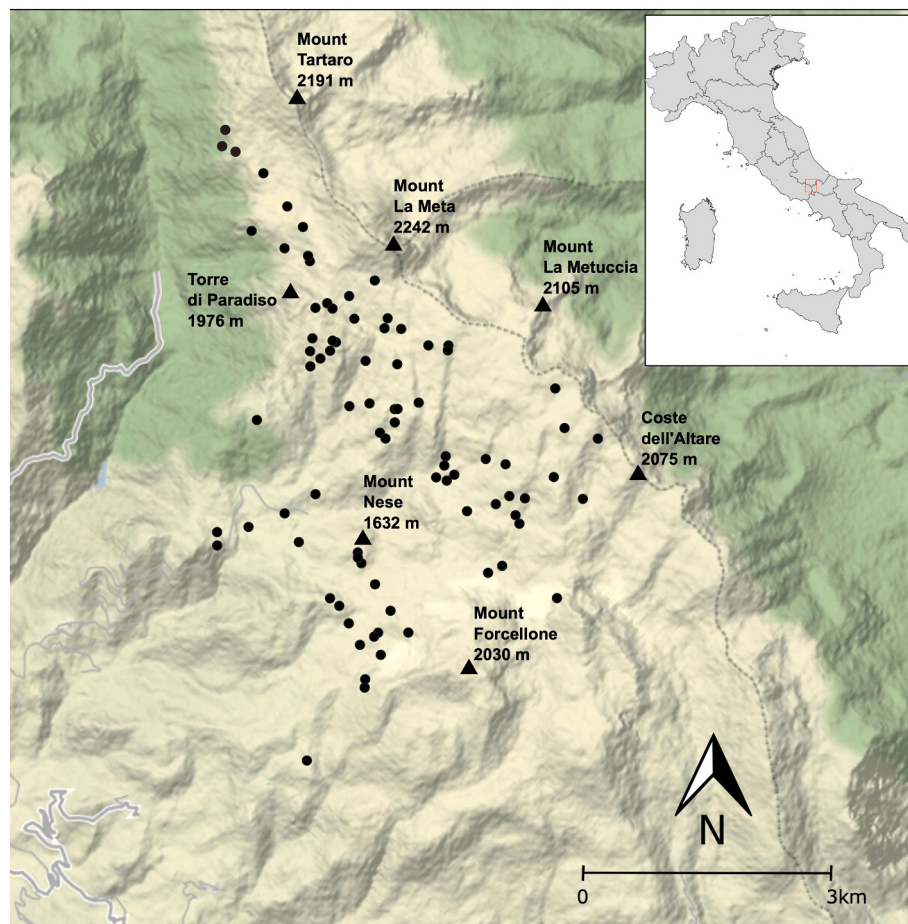


Fig. 1. Main map: outline of study area with position of sampling plots (dots). Inset: position of study area within Italy.

soil samples analysed in this paper were collected at three pre-determined points within each 10 m² plot, i.e. in the centre and at two opposite corners. At each collecting point, the surface soils were collected at maximum 5 cm depth. The soil from the 3 pits of the same plot was then joined in a mixed sample. The 81 soil samples were taken to the laboratory, air-dried, and sieved through a 2-mm mesh before proceeding to the physical, chemical and biochemical analyses.

2.3. Soil physical and chemical analyses

The particle-size distribution was determined by the pipette method (Gee and Bauder, 1986) after treatment with NaClO solution at 6 % of active chlorine to remove organic cements (Lavkulich and Wiens, 1970) and with dithionite-citrate-bicarbonate solution to remove Fe-Al oxides cements (Mehra and Jackson, 1960). The water holding capacity (WHC) was determined following the method of ISO 11465 (1993). The pH was determined potentiometrically in 1 M KCl solution after one night of solid:liquid (1:2.5 w:v ratio) contact, using a combined glass-calomel electrode immersed into the suspension. Total organic carbon (TOC) and total nitrogen (TN) were measured using the dry combustion method with Thermo Soil NC—Flash EA1112 elemental analyser. Potentially plant-available P was determined by the Olsen method (Olsen et al., 1954).

2.4. Soil biochemical analysis

Soil microbial biomass C (MBC) was determined using the fumigation-extraction method (Brookes et al., 1985; Vance et al., 1987). MBC was obtained by $EC \cdot k_{EC}$, where eC was the difference between

organic C extracted using 0.5 M K₂SO₄ solution (1:4 w/v) from fumigated and not-fumigated samples, and $k_{EC} = 2.64$ is the extraction efficiency coefficient (Joergensen, 1996). The amount of C extracted by K₂SO₄ solution from non-fumigated samples (C_{ext}) was considered the easily extractable and most labile soil organic C pool. The extracted C was determined with the TOC-V CSN and TNM-1 analysers (Shimadzu, Japan). The microbial quotient (MBC:TOC) has also been calculated.

The soil enzyme activities were measured using 4-methylumbelliferine (MUF) and 7-amino-4-methylcoumarin (AMC) fluorogenic substrates (Marx et al., 2001; Vepsäläinen et al., 2001). The selected 9 enzyme activities are involved in the main biogeochemical cycle of C (β -cellobiohydrolase = CHB, β -xylosidase, β -glucosidase = BG, α -glucosidase = AG, and butyrate esterase), N (leucine-arylamidase = LAP, and N-acetyl- β -glucosaminidase = NAG activities), P (acid phosphomonoesterase = AP activity), and S (arylsulphatase activity). Even if the pH values of the studied soil samples ranged from 3.6 to 6.9, enzymes involved in a wide range of substance degradation with optimal pH in acid and alkaline intervals were selected. Therefore, specific substrates were prepared using different buffer adjusted to the optimum for each selected enzyme (0.5 M sodium acetate pH 5.5; 0.5 M Tris acetate pH 7.5). Fluorescence (excitation 360 nm, emission 450 nm) was measured with an automatic fluorometric plate reader (Fluoroskan Ascent), and readings were performed after 0, 30, 60, 120, and 180 min at 30 °C. The MUF and the AMC standard curves were prepared and measured for each sample and buffer. The results were expressed as nmol of product (MUF or AMC) of each enzymatic reaction released per g of soil sample per unit of time in relation to a standard curve prepared with increasing MUF or AMC concentrations and incubated at the same experimental conditions. The synthetic enzymatic index (SEI), which

expresses the sum of all enzyme activities, was calculated for all samples as a synthetic measure of microbial functional capacity (Moscatelli et al., 2018). Based on the obtained data, the specific enzyme activities per unit of TOC (SEI/TOC) and per unit of MBC (SEI/MBC) were calculated. Furthermore ratios of $\ln(\text{BG}):\ln(\text{AP})$, $\ln(\text{BG}):\ln(\text{LAP}+\text{NAG})$ and $\ln(\text{LAP}+\text{NAG}):\ln(\text{AP})$ activities were calculated, which were a measures of the enzymatic resources directed towards acquisition of organic P and organic N relative to C and organic P relative to N (Sinsabaugh et al., 2008).

2.5. Statistical analyses

A hierarchical clustering on principal component (HCPC) was performed to divide the soil samples into homogeneous groups for chemical and geomorphological environmental characteristics, by using the following variables: elevation, slope inclination, heat load index, soil depth, rock and stone % cover, herb layer % cover, TN%, TOC%, pH (KCl), avail. P, WHC, sand%, silt% and clay%. The descriptive statistics of the variables are shown in Table 1.

HCPC is a useful approach when dealing with multidimensional data sets containing multiple continuous variables, as the principal component analysis (PCA) allows reducing the dimension of data into few continuous variables containing the most important information, leading to a more stable clustering. Data were first standardized and subjected to a PCA. Then, the first two dimensions (explaining a cumulative variance of ca. 50 %) were used to search for the optimal clustering by means of the function HCPC in the package FactoMineR (Le et al., 2008) of R (R Core Team, 2020).

To assess the differences in the biochemical soil properties among clusters, a one-way analysis of variance (ANOVA) was applied. The graphical analysis of residuals was used to verify the normality and homoscedasticity of the data, which were transformed when necessary. The transformation was selected by the maximum likelihood procedure suggested by Box and Cox (1964), as implemented in the boxcox function of the package MASS (Venables and Ripley, 2002). The multiple comparison tests were performed with Tukey's HSD with a significance level of 0.05.

We then assessed the relative importance of chemical soil characteristics and of topographic-geomorphologic factors on the total enzyme activities (expressed per unit of microbial biomass carbon: SEI/MBC) by means of Boosted Regression Trees (BRT: Elith et al., 2008). BRT are a class of machine learning techniques based on regression trees, which model the relationship between a given response variable with its predictors by recursive binary splits, improving model accuracy by fitting trees iteratively to training data. BRT have many desirable properties, as they have no need for prior data transformation and can handle complex nonlinear relationships and interaction effects. Moreover, BRT do not

Table 1
Summary statistics of the predictor variables.

Variables	Mean	Std. dev.	Min	Max
Elevation (m)	1794.64	175.68	1377	2134
Slope inclination (°)	17.75	8.73	2	45
Heat-load index	0.94	0.08	0.66	1.03
Soil depth (cm)	11.70	8.54	0	31
Stone and rock cover (%)	22.60	23.16	0	90
Herb layer cover (%)	76.09	19.40	23	100
pH (in KCl)	5.33	0.96	3.55	6.92
Avail. P (mg kg ⁻¹)	5.43	4.98	0.13	34.45
WHC (%)	51.9	5.65	39.02	68.32
Sand (%)	11.12	8.64	0.18	38.03
Silt (%)	85.78	10.38	61.01	99.04
Clay (%)	3.08	4.69	0.10	21.94
TN (%)	0.82	0.29	0.25	1.80
TOC (%)	8.98	2.76	2.88	16.92

Avail. P = available phosphorous; WHC = water holding capacity; TN = total nitrogen; TOC = total organic carbon.

necessarily need a prior process of variable selection, as they ignore non-informative predictors when fitting trees (Elith et al., 2008). Here, we used the function 'gbm.step' in the package 'dismo' (Hijmans et al., 2020) of R (R Core Team, 2020).

The coefficient of determination (R^2), root mean square error (RMSE), mean absolute prediction error (MAE) and correlation coefficient between predicted and observed values (r) were used to test for model accuracy. The contribution of each predictor in determining the observed SEI/MBC values has been measured by estimating the relative influence based on the average number of times a variable was selected for splitting (weighted by the squared improvement of the model). We used partial dependence plots to visualize fitted functions in BRT models, where the effect of each variable is modelled after accounting for the average effects all other variables in the model (Elith et al., 2008).

3. Results

3.1. Cluster and principal components analyses

The HCPC analysis grouped the soils into three clusters (34 plots belong to the first cluster, 19 to the second cluster and 28 to the third cluster). The results of clustering are visually reported in the PCA plot in Fig. 2a. The PCA scoring plot (Fig. 2a) showed a complete separation between the cluster 2 and 3, whereas a partial overlapping occurred between cluster 1 and cluster 2 and 3. The axes 1 and 2 of PCA explained about 37 % and 13 % of the variation contained in the original dataset, respectively. The distances between cluster 2 and 3 occurred along the PCA1 axes and appeared to be mainly driven by rock and boulder cover, nutrient content (TOC, TN) and pH (cluster 3), and by sand content, soil depth and herb cover (cluster 2). Conversely, the soils belonging to cluster 1 differed from the others mainly for elevation and Heat Load topographic index (Fig. 2b). In Fig. 3 we report the boxplots for each environmental variable across the three clusters. The sites belonging to cluster 3 had the lowest soil depth, herb cover percentage and Pav, and the highest slope inclination, rock and boulder cover, TOC and TN percentage, pH and WHC. On the contrary, the sites belonging to the cluster 2 had the highest Pav values and sand percentage and the lowest silt content, Heat Load topographic index and elevation. The sites of cluster 1 revealed for almost all parameters (slope inclination, soil depth, rock cover, herb cover, TOC, TN, pH and WHC) the same value of the sites belonging to cluster 2 and for a few other (sand, silt, elevation) were not significantly different from cluster 3. If the dominant vegetation type, resulting from the wider botanical survey performed in the area (G. Filibeck and L. Cancellieri, unpubl.), is assigned to each sampling unit, then it results that 77 % of plots in cluster 1 belonged to acidophytic and/or mesophytic grasslands (dominated by *Nardus stricta* and/or *Festuca microphylla*); in cluster 2 there was a mixture between xerophytic grasslands (dominated by *Globularia meridionalis*), with 41 % of the plots, and acidophytic/mesophytic swards (43 %); the same situation applied to cluster 3 (41 % *Nardus-Festuca* and 41 % *Globularia*-dominated communities).

3.2. Soil biochemical properties

Tables 2 and 3 show soil enzyme activities and biochemical indices in the homogeneous areas of secondary grassland, identified by cluster analysis. Among the nine selected enzyme activities, only CHB, BG, AP and LAP varied significantly among three clusters. In particular, the BG and LAP activity were higher in the clusters 3 than cluster 1 and 2, whereas the CHB activity was higher in the cluster 3 than cluster 2. Finally, AP activity was significantly higher in cluster 1 than cluster 3. For the other enzymatic activities and SEI, there were no significant differences among clusters. Conversely, SEI/TOC and SEI/MBC ratios had a similar trend, the values of both ratios were higher in the cluster 1 and 2 than cluster 3; MBC content was higher in cluster 3 than in cluster

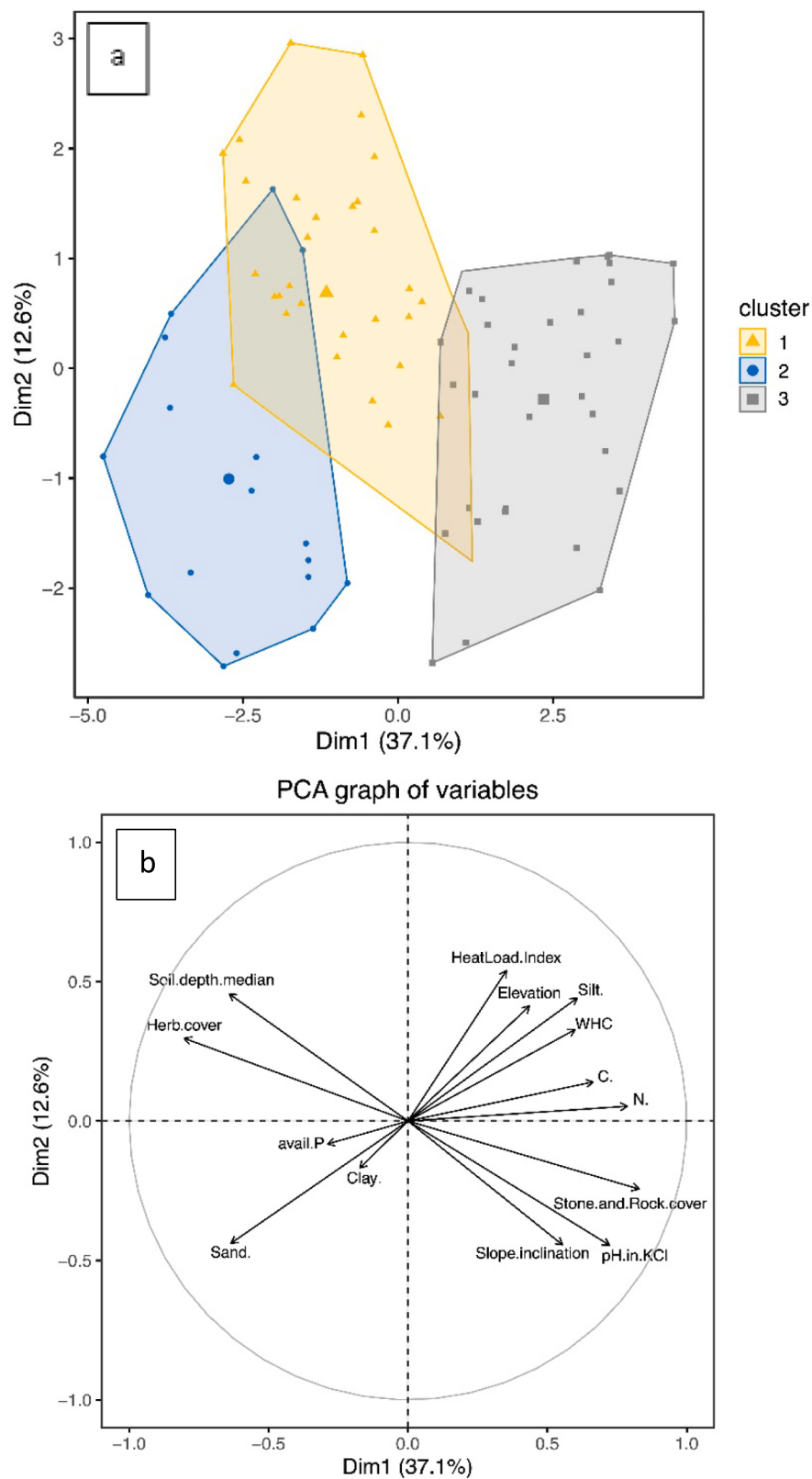


Fig. 2. a) Principal component analysis (PCA) plot of the results of Hierarchical Cluster Analyses (HCA) of sampling plots, b) PCA variables.

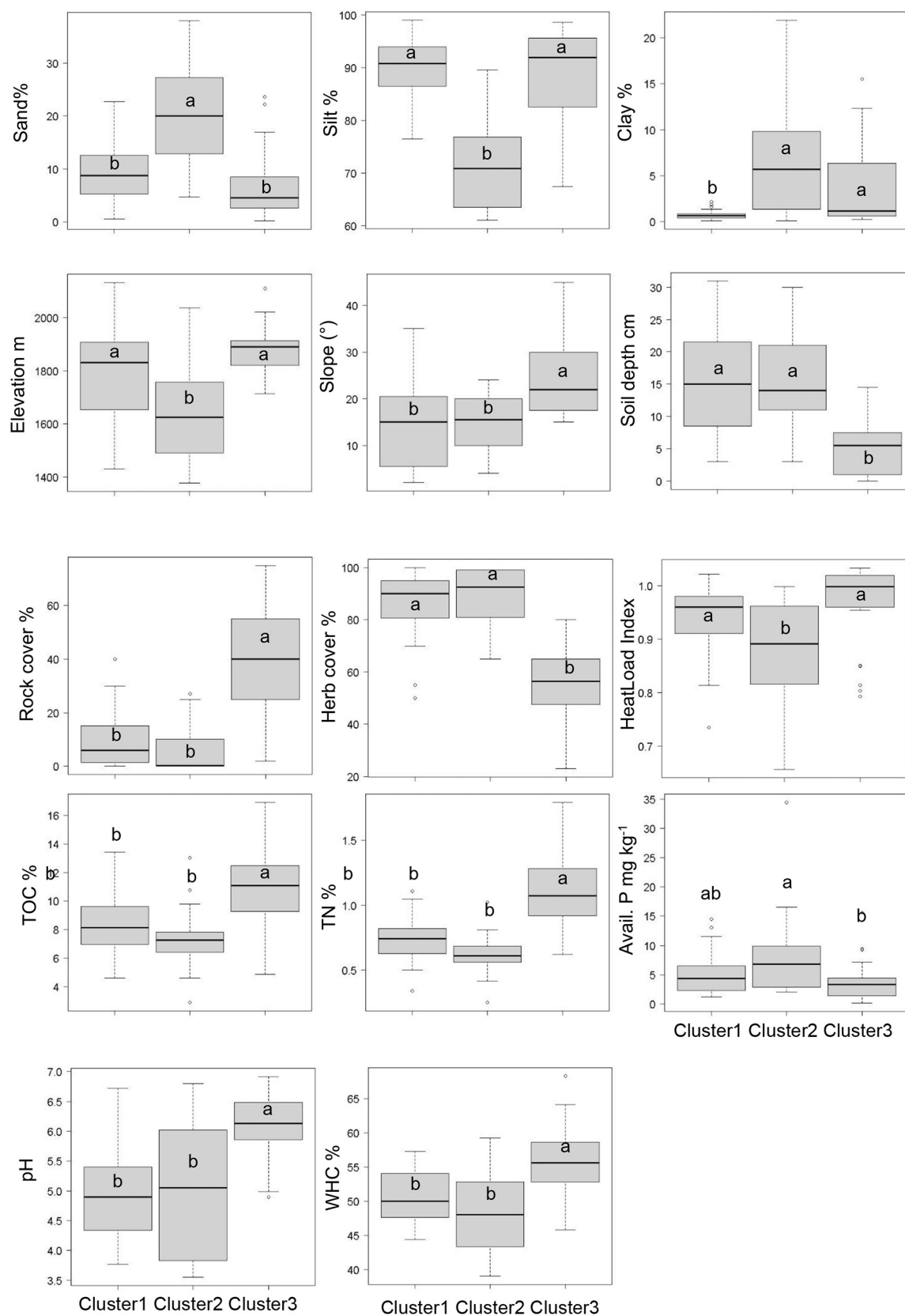


Fig. 3. Boxplots of the following variables (from left to right), across the three clusters obtained from HCPC: sand%, silt%, clay%, elevation, slope inclination, soil depth (median), rock and boulder % cover, herb layer % cover, Heat Load topographic index, TOC%, TN%, Pav, pH(KCl) and WHC. Different letters mean significant difference at p-level < 0.01.

Table 2

Synthetic enzymatic index (SEI), soil microbial biomass C (MBC) and the ratios of soil microbial biomass and organic carbon (MBC/TOC), synthetic enzymatic index per unit of organic carbon (SEI/TOC), and synthetic enzymatic index per unit of microbial biomass carbon (SEI/MBC) for the three clusters identified. Values within brackets represent the standard errors. Different letter indicates significant difference among soil clusters ($p < 0.05$).

	Cluster 1	Cluster 2	Cluster 3
SEI nmol MUF/AMC g ⁻¹ h ⁻¹	15,515.8 (983.2)a	13,492.1 (842.0)a	15,343.2 (1047.6)a
MBC mg kg ⁻¹	981.8 (60.6)b	820.7 (87.8)b	1264.9 (103.5)a
MBC/TOC	1.20 (0.1)a	1.17 (0.1)a	1.23 (0.1)a
SEI/TOC nmol MUF mg ⁻¹ TOC h ⁻¹	195.2 (13.7)a	198.7 (16.3)a	150.2 (11.7)b
SEI/MBC nmol MUF mg ⁻¹ MBC h ⁻¹	18.0 (1.5)ab	18.6 (2.0)a	14.6 (1.9)b

Table 3

Enzyme activities in the three cluster identified. Values within brackets represent the standard errors. Different letter indicates significant difference among soil clusters ($p < 0.05$).

Enzymes		Cluster 1	Cluster 2	Cluster 3
CHB	nmol MUF g ⁻¹ h ⁻¹	539.2 (58.7)a	371.5 (63.6)b	694.9 (103.0)a
NAG	nmol MUF g ⁻¹ h ⁻¹	1242.7 (157.9)a	963.5 (144.6)a	1032.2 (134.6)a
BG	nmol MUF g ⁻¹ h ⁻¹	2404.4 (171.4)b	1773.5 (169.2)c	3703.9 (276.3)a
AG	nmol MUF g ⁻¹ h ⁻¹	247.2 (16.4)a	220.5 (19.8)a	255.3 (19.2)a
AP	nmol MUF g ⁻¹ h ⁻¹	5285.4 (394.9)a	4906.1 (474.7)ab	4058.0 (378.3)b
AS	nmol MUF g ⁻¹ h ⁻¹	1884.7 (187.3)a	1493.2 (201.3)a	1680.7 (122.6)a
XO	nmol MUF g ⁻¹ h ⁻¹	605.2 (53.2)a	534.2 (58.7)a	510.1 (43.2)a
BE	nmol MUF g ⁻¹ h ⁻¹	3306.9 (153.1)a	3229.6 (166.0)a	3408.1 (142.5)a
LAP	nmol AMC g ⁻¹ h ⁻¹	88.6 (7.3)b	78.5 (10.0)b	131.1 (8.1)a

CHB = β -cellobiohydrolase; NAG = *N*-acetyl- β -glucosaminidase; BG = β -glucosidase; AG = α -glucosidase; AP = acid phosphomonoesterase; AS = arylsulphatase; XO = β -xylosidase; BE = butyrate esterase; LAP = leucine-arylaminidase.

1 and 2 (Table 2).

The $\ln(\text{BG}):\ln(\text{AP})$ and $\ln(\text{BG}):\ln(\text{NAG} + \text{LAP})$ ratios were used as an indicators of potential C:N and C:P acquisition activity respectively, both indices significantly increased from cluster 1 and 2 to cluster 3, whereas the ratio $\ln(\text{LAP} + \text{NAG}):\ln(\text{AP})$ did not significantly change among clusters (Fig. 4).

3.3. BRT analysis

The best model convergence was obtained for $lr = 0.001$, $bf = 0.75$ and $tc = 2$, with $nt = 2665$ and quite optimal accuracy values between observed and predicted SEI/MBC values ($r = 0.764$, $\text{RMSE} = 44.182$, $\text{MAE} = 4.317$, $R^2 = 0.477$; Fig. 5a). Six main predictors were able to explain >80 % of variation in SEI/MBC index, with pH alone explaining ca. 23 % and slope inclination and soil depth accounting for >35 % (Fig. 5b). Partial dependent plots showed a negative relationship between SEI/MBC and pH, with a slight decrease for increasing pH values in the range 3.5–4.5, followed by a drastic decline for pH values within 4.5–5 and stable values for pH > 5 (Fig. 4c). The same negative pattern can be observed when relating SEI/MBC with slope inclinations, observing a rapid decrease of SEI/MBC up to values of slope of c.25°, followed by a plateau indicating no significant changes in observed SEI/MBC values (Fig. 5c). Soil depth showed a completely different pattern characterized by no significant effects up to a depth of 15 cm

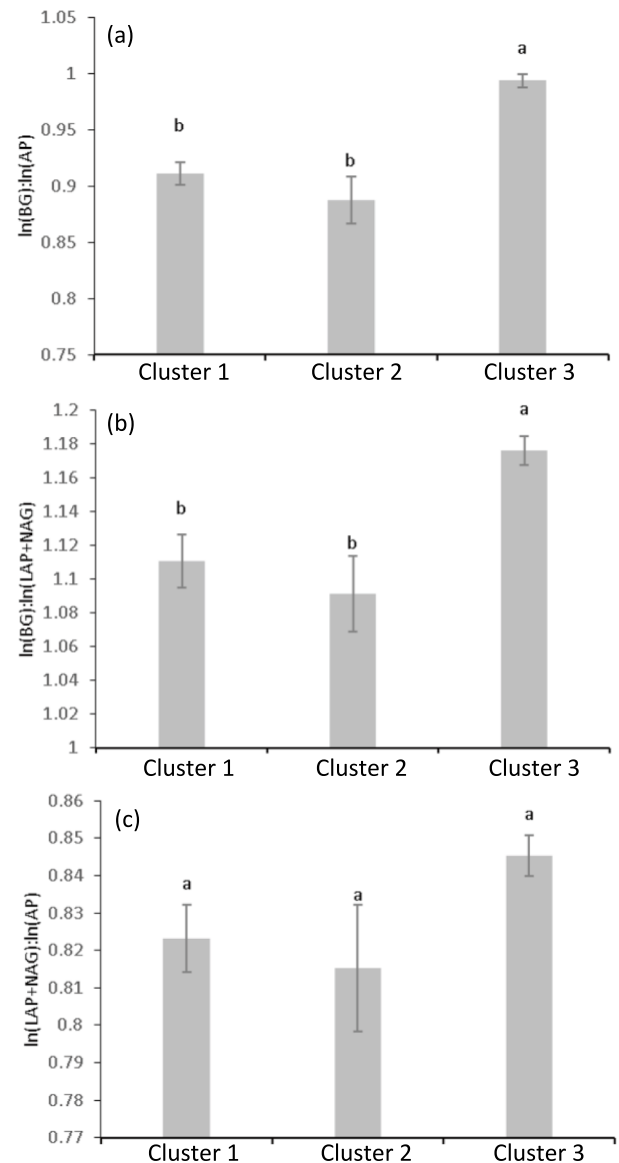


Fig. 4. Ratios of $\ln(\text{BG}):\ln(\text{AP})$ (a); $\ln(\text{BG}):\ln(\text{LAP} + \text{NAG})$ (b) and $\ln(\text{LAP} + \text{NAG}):\ln(\text{AP})$ (c) activities on the three cluster identified. Different letters mean significant difference at p-level < 0.01.

followed by a rapid increase of SEI/MBC for depth above 15 cm (Fig. 5).

4. Discussion

4.1. Fine-scale patterns of soil properties

In this study, soil microbial habitat heterogeneity was characterized in a mountain grassland landscape in central Italy, to investigate the effect of chemical-physical soil properties, vegetation cover and geomorphological features on microbial habitat.

The cluster analyses identified, in the study area, three different types of soils with homogeneous properties, that can be summarized as follows. Type 1: sub-acidic, at high elevation, with dense vegetation cover of acidophytic/mesophytic grasses and high silt content, and the lowest clay content. Type 2: sub-acidic, at low elevation, with a high vegetation cover of acidophytic/mesophytic grasses mixed with patches of xerophytic plants and a very low stone cover, with the highest sand and lowest silt content, and highest avail. p values. Type 3: very shallow soil, mainly stone-covered, with a mixed vegetation mosaic, at high

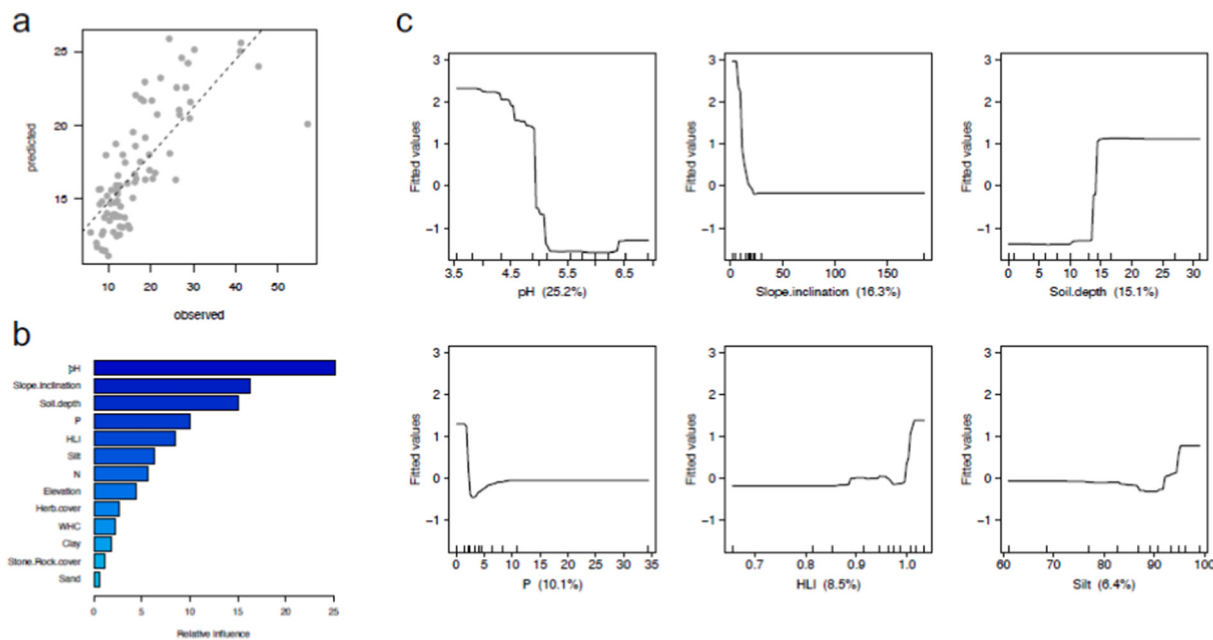


Fig. 5. Results of the Boosted Regression Trees (BRTs) relating the total enzyme activities (expressed as per unit of microbial biomass carbon, SEI/MBC) and the environmental predictors (chemical soil characteristics and topographic-geomorphologic variables) of sampling plots. a) scatterplot of the observed vs. fitted values of BRT with best regression line (dashed line); b) histograms of the relative influence (in percentage) of environmental predictors on SEI/MBC c) partial dependent plots showing the shape of the relationship between the top six predictors and SEI/MBC.

elevation, with the highest pH values (ranging from sub-acidic to neutral), on steep slopes, with the highest values of TOC, TN and WHC. The chemical and physical properties of the soils are partly due to the bedrock types, since the soils of type 1 and 2 were for the 63 % and 83 %, respectively, developed in areas classified as siliceous lithology, whereas type 3 was collected, for about 61 % of cases, in sites recorded in the geological map as calcareous substrates.

Several studies have examined the differences in various soil properties under specific parent materials over particular regions (Graham and Franco-Vizcaino, 1992; Jaiyeoba, 1995; Van de Wauw et al., 2008; Gruba and Socha, 2016) but results are generally not synthesised to draw out clear universal trends. The higher the silica content of a parent material, the generally lower the clay and base cation content of derivative soils (Grey et al., 2016). This assumption reflects partially our data regarding the soils of type 1 mostly developed on siliceous lithology and showing lower clay content and slightly lower pH. In the soil type 2, the siliceous lithology could have determined the coarser texture with respect to the soils of type 3. However, the different texture between soil type 1 and 2 could be also due to accumulation of silty deposit of volcanic origin in the soil type 2, caused by wind transportation and/or colluvial rearrangement (Giraudi, 2001). The sub-acid pH combined with the fair depth of the soils (and hence a smaller drought stress) and a lower elevation, have led to a favourable condition for herbaceous vegetation growth in the soil of type 1 and 2 (cf. Filibeck et al., 2020; Cancellieri et al., 2020). This is confirmed by the value of the herb cover percentage, which recorded the highest values in these soils. Conversely, the greater coverage of stone and boulders and the highest soil organic matter content of the soils of type 3 could be partially explained by the higher elevation. According to several authors (e.g. Dai and Huang, 2006; Follett et al., 2012), the content of TOC and TN is related to climatic conditions and especially to the temperature. The higher elevation and resulting lower temperature could have determined a reduction of the soil microbial activity favouring the organic matter accumulation (De Feudis et al., 2016; Massaccesi et al., 2020). However, other causes must be taken into consideration, such as the reduced herb-cover and the botanical composition of vegetation. Indeed, the soils of type 1 were at the same altitude as type 3 but recorded significantly lower TOC and

TN content; since type 1 features higher herb-cover values and deeper soil profiles, we can hypothesize a nutrient mobilization from native organic matter stocks operated by herbaceous rhizospheric activity, resulting in a net soil C and N loss (Cardelli et al., 2019; Massaccesi et al., 2015). Finally, the sub-acidic and neutral values of pH in soil type 3 could be explained by the shallow soil in close contact with calcareous parent material, and by the higher organic matter content (Filibeck et al., 2019, 2020).

4.2. Effects of chemical, physical and geomorphological soil properties on microbial biomass and enzymatic activity

Regarding the biochemical properties, the soils belonging to type 2 showed a lower value of CBH, BC and LAP enzymatic activity unlike soil type 3 which recorded the highest values. The above-mentioned reduced enzymatic activity and the higher SEI/TOC and SEI/MBC ratios values could be mainly attributed to the reduced content of total organic C and N, and microbial biomass in soils type 2. Conversely, the highest β -cellobiohydrolase, β -glucosidase and leucine-arylamidase activities of the soils type 3 were explained by the highest content of TOC and TN of those soils, as usually found in literature (Sinsabaugh et al., 2008; Wallenius et al., 2011; Cenini et al., 2016) the β -cellobiohydrolase, β -glucosidase and leucine-arylamidase, regulating the C and N cycling, largely depend on the amount and quality of soil organic matter. In particular, in the grassland soils, the cellulose represents a significant portion of the plant litter reaching the soil, and the production of β -glucosidase is indispensable because it catalyses the hydrolysis of cellobiose to glucose (Sinsabaugh and Follstad Shah, 2011).

The opposite trend was reordered for acid phosphomonoesterase which led a higher activity in soils type 1 than soil type 3, and it could be primarily linked to the pH values. Indeed, the average higher pH values of the soils type 3 than soils type 1 could be able to inhibit the acidic phosphomonoesterase activity. Further, the higher enzymatic activities (β -cellobiohydrolase, β -glucosidase, and leucine aminopeptidase) found in soil type 3 might be attributed to the high microbial biomass content, than to plant roots and the consequent exudates release, almost absent in these soils.

The increase of $\ln(\text{BG}):\ln(\text{AP})$ and $\ln(\text{BG}):\ln(\text{NAG}+\text{LAP})$ ratios from soils type 1 and 2 to soils type 3, according to the approach developed by Sinsabaugh et al. (2008), would reflect an increase of microbial C-shortage relative to P and a microbial C-shortage relative to N from soils type 1 and 2 with respect to soils type 3. This was confirmed by the amount of TOC and MBC, which was lower in the soil type 1 and 2 compared to type 3, and by the lower Pav value of soil type 3. Mori (2020) suggested a new conceptual model to distinguish when $\ln(\text{BG}):\ln(\text{NAG}+\text{LAP})$ reflects microbial C vs. N limitation, that happens when cellulose is the predominant C source (relative to chitin, peptidoglycan, and protein).

4.3. Predictors of the biochemical soil activity evaluated by SEI indices

BRT analysis was performed to identify the relative importance of each variable in regulating SEI/MBC ratio in the studied mountain grasslands. The results showed that pH, slope inclination and soil depth together explain 57 % of variation in the SEI/MBC ratio. A global scale meta-analysis reported by Sinsabaugh et al. (2008) has demonstrated that pH is the primary control of soil enzyme activity.

However, a novel finding in our results is the fact that slope inclination could represent a predictor of enzymatic activity in mountain environments, as suggested by the rapid decrease of SEI/MBC up to a slope inclination of c. 25°. We can hypothesize an indirect effect of slope inclination on soil properties through the regulation of the amount of solar radiation received (Sariyildiz et al., 2005; Sidari et al., 2008). The amount of insolation regulates soil temperature and water availability which in turn affects soil properties. Moreover, slope inclination, influencing run-off and erosion, also influences soil depth and texture (Moeslund et al., 2013). Soil depth could also represent a good predictor because the shallower soil profiles resulted to be those richer in TOC and TN, which are the main driver of microbial community composition and activity (Grayston et al., 2004; Franklin and Mills, 2009; Katsalirou et al., 2010) and consequently of the enzymatic activity.

5. Conclusion

In this study, a spatially intensive sampling and a multidisciplinary approach that included botanical and topographical data allowed to describe the heterogeneity of microbial habitats within a mountain grassland landscape. Three different microbial habitats in soil surface layers were identified on the basis of chemical and physical soil properties, topographic and geomorphological features and vegetation cover. While the third habitat (high pH, shallow soil on steep slopes) is the one with the highest microbial activity involved in C and N cycling, the more acidic and deeper soils (soils of type 1 and 2), typical of sinkhole or slope areas, favoured the enzyme activity involved in P cycle thanks to the establishment of the rhizospheric activity of the herbaceous plant species.

The different enzymatic activity patterns found in the three habitats suggest diverse microbial functions, with respect to nutrient cycling, within a small landscape and a relatively homogeneous land-cover. Finally, the results of the BRT analysis showed - for the first time as far as we know - the importance of topographic factors such as slope inclination in predicting the microbial functional capacity in mountain grassland ecosystems.

The multidisciplinary approach we adopted can provide the basis for diachronic monitoring and assessment of ecosystem functions in mountain landscapes. For instance, the mountain ranges of Central Italy host a major network of National Parks. The ecosystems of this region were shaped by millennia of pastoral activities, and developing methodological tools to assess the role and impacts of traditional land use on all grassland components can prove essential to guide the policies of protected areas.

CRedit authorship contribution statement

GF designed and supervised the wider research project during which soils were sampled. SM conceived the paper aims. LC coordinated field sampling and measurements. BB and LM performed statistical analyses. RM performed soil analyses. The manuscript was drafted by LM, with major contributions by GF and SM. All Authors commented and approved the manuscript.

Declaration of competing interest

The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript.

Data availability

Data will be made available on request.

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