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Selection of soil biochemical indicators according to seasonal variation and vegetation cover for long-term soil monitoring in a mountain valley of the Alps

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Abstract

The complexity of soil organic matter and the multifunctional role of its components on soil processes make the characterization of soil ecological status challenging. Due to its ready responsiveness to environmental changes, the soil microbial community has gained increasing attention for its relationship to the dynamics of C pools and soil chemical and physical processes. Its activity can be monitored by the enzymatic profile, which enables the detection of early changes in soil status, supported by direct or indirect measurement – e.g., by double-stranded DNA (dsDNA) – of microbial biomass and parameters, such as dissolved fractions of C and N, which are linked to soil activity as rapidly available energy sources. This study analyzed the seasonal response of these indicators in a subalpine ecosystem, using sampling date and vegetation cover as predictors capable of capturing long-term and short-term changes in the ecosystem, respectively. Most of the bioindicators showed higher values in the warmest and least rainy summer season. In the cold season, two distinct trends were evident: the values of dsDNA and enzyme activities decreased to their minimum in early winter and rose to their maximum in late winter, while those of soil organic matter (SOM), dissolved C, and N continued to decline until the end of winter. The study also found that the dynamics of SOM in the woodland and meadow ecosystems differed, with the former achieving the highest SOM content during the summer period of greatest plant and faunal activity. Overall, this study suggests that the use of bioindicators and high-throughput techniques can contribute to improving soil quality assessment and monitoring. Additionally, they can be used to characterize humus forms and motivate the preservation of Alpine meadows and surrounding wooded habitats for their non-wood products.

1 Introduction

Soil monitoring in time has become an important issue since the interest of soil science has focused on the assessment of the ecological status of soils. Due to the dynamic nature of the processes involved, soil organic matter (SOM) is one of the main objects of monitoring. At the same time, the multifunctional role of organic components that contribute to shape soil dynamics requires the determination of numerous, sometimes collinear variables, making the selection of a limited number of them non-

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trivial to address the costs of monitoring (Bünemann et al. 2018). A further aspect to consider is the scale of spatial and temporal variation of soil processes and the parameters capable of capturing their dynamics. Spatial variation is the result of the site specificity of often interlinked soil processes, which in turn are a cause and/or consequence of the high diversity of habitats in the ecosystem (Zornoza et al. 2015; Bünemann et al. 2018; Schloter et al. 2018). Although the spatial variability of monitoring indicators is well known in soil science, soil monitoring has mainly been planned on a national and regional scale (see Bünemann et al. 2018 for references), ignoring the issue of variability in soil processes within and between habitats that even regional-scale monitoring may fail to capture.

When monitoring is aimed at providing early indications of changes taking place in the soil status, a parameter with adequate sensitivity is soil microbial biomass, which, although accounting for less than 5% of the weight of the SOM (Brookes 2001, Bhaduri et al. 2022), significantly influences the dynamics of soil C pools (Blagodatskaya and Kuzyakov 2013; Liang et al. 2017; Bhaduri et al. 2022) by releasing extracellular enzymes and behaving as a labile reservoir of N, P and S for plant uptake (Brookes 2001). Hydrolases acting on biopolymers like cellulases (β-glucosidase) and chitinases (N-acetylglucosaminidase) are produced by fungi to release simple sugars, thus being respectively involved in C and organic N cycling (Baldrian and Štursová 2010; Baldrian 2014). Peptidases like leucine aminopeptidase are released to break down small peptides into their constituent amino acids, therefore influencing the rate of organic N mineralization (Sobucki et al. 2021). Phosphorus and sulfur cycles are driven by phosphatases (phosphomonoesterase, phosphodiesterase, pyrophosphodiesterase) and sulphatases (arylsulfatase), the former produced by a broad spectrum of soil organisms, the latter specifically linked to bacterial communities (Baldrian and Štursová 2010).

Given the need for many sampling points, the use of biochemical indicators in soil monitoring requires that their measurement should not be expensive and analytically burdensome. Cost reductions have been achieved with assays that characterize soil enzymes in terms of abundance, catalysed reaction and activity rate (Baldrian 2009; Nannipieri et al. 2018). In contrast, the time-consuming measurements of microbial biomass – estimated in 1.32 hours per sampling point for sample collection and analysis by Bragato et al. (2016) – sometimes conflict with the sample size required by soil monitoring activities. Over the last decade several high-throughput methods were developed for the characterization of the ecological status of the soil system employing indicators like enzymatic assays (Fornasier and Margon 2007) and double-stranded DNA (dsDNA) (Fornasier et al. 2014). They were tested to investigate the spatial variability of extracellular enzyme activities (EEAs) (Bardelli et al. 2017, Nadimi-Goki et al. 2018, Cardelli et al. 2019) and microbial biomass (Bragato et al. 2016; Semenov et al. 2018) in systematic surveys of different soils and ecosystems. Regarding sampling and analysis times, Bragato et al. (2016) calculated that those for dsDNA are about a quarter of those for microbial biomass C (0.29-0.33 hours per sample depending on whether the initial sample was air-dry or field-moist, respectively). Based on our experience, similar times are obtained for the measurement of enzymatic activities, which have similar extraction procedures and differ in terms of longer measurement times in fluorimetry, which are largely compensated for by the possibility of measuring a significant number of enzyme activities on the same extract.

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Our study was intended to be preparatory to the planning of space-time designs for soil health monitoring according to the hybrid design-based and model-based sampling approach proposed by Brus and de Gruijter (2011). We were specifically interested in the selection of soil biochemical indicators useful for monitoring mountain ecosystems managed by man, namely hay meadows and hazel-dominated groves areas, whose ecological equilibria are changing because of depopulation and the abandonment of mixed crops-livestock farming in the mountain (Mazoyer and Roudart, 2002), with the risk of losing an ecosystem typical of the Alps. From a soil perspective, hay meadows and forest habitats have provided a continuous supply of organic material, making the processes linked to the SOM cycle predominant. However, the transformation dynamics of SOM may change between groves and meadows because, even with comparable physical and chemical conditions, substantial differences in the composition of litter, soil fauna and microbial communities occur (Šťursová and Baldrian 2011; Šťursová et al. 2020).

Our aim was to assess how variable were some widely used parameters – namely SOM, total dissolved N (TDN) and dissolved organic C (DOC) – combined with biochemical indicators of mass and activity of soil microbial population indicators – i.e. dsDNA and EEAs – little used in soil monitoring over time but capable of providing information on the state of the most active component of the soil and thus able to respond relatively readily to changes in the soil system over time. Our investigation considered the vegetation cover and four seasons of a year to define a restricted set of noncollinear parameters with sufficient readiness for long-term soil monitoring of subalpine ecosystems of meadows and hazel-dominated groves, whose ecological balance is changing following the abandonment of mountain mixed crops-livestock farming.

2 Materials and methods

2.1 Site description

The investigation was done in an area of about 32 ha near the village of Cimolais (north-eastern Italy; 46.3°N, 12.5°E) at an elevation of 650 m above sea level. The vegetation of the area is linked to the practice of haymaking which is typical of the subalpine valleys of north-eastern Italy. Approximately 85% of the area consists of hay meadows dominated by tall oatgrass (*Arrhenatherum elatius* (L.) P. Beauv. ex J. & C. Press). Meadows are separated by linear, 10-20m-wide groves dominated by European hazelnut (Corylus avellana L.) that were widely propagated in the past to obtain branches, nuts and ties.

In the period 1961-2010, the average annual temperature was 9.4°C, with average minimum and maximum values of -1.9°C and 19.6°C in January and July respectively (ISPRA, 2020). Average annual precipitation is 1613 mm, with peaks in April-May and October-November. The area was also characterized by a continuous snow cover of 62 days between December and February in the period 1973-2009 (ARPA FVG, 2020).

The investigated area is located on a Pleistocene-Holocene fluvioglacial cone surrounded by Triassic reliefs belonging to the Dolomia Principale formation. Soil is a Dolomitic Rendzic Leptosol (Arenic, Hyperhumic) (IUSS Working Group WRB 2014) with a 18cm-thick A horizon characterized by a gravelly sandy loam texture, pH of 7.3 and 29% organic matter content

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(Bragato et al. 2019). In agreement with Ponge et al. (2014), it is characterized by an Amphi humus system, which occurs on base-rich soils in cold climates with hot summers and cold winters resulting in prolonged periods of biological inactivity.

2.2 Soil sampling and laboratory analyses

We planned the investigation using *Vegetation cover* and Sampling date – i.e., seasons – as treatments. The vegetation cover was either meadow or hazel groves, and *Sampling dates* were 23 May, 1 August and 6 December 2012, and 27 March 2013. Sampling locations for each combination of vegetation cover and sampling date were randomly selected with a simple random sampling design. Sampling sites were located with a Montana 650 pocket GPS detector (Garmin, USA). Soil samples were collected in the A horizon at a depth of 0-10 cm using a hand-auger for stony soils. Fresh samples were stored in a cooler and transported in the laboratory, where they sieved at 3 mm and analysed for total dissolved N and organic C. Briefly, aliquots were shaken for 30 min in a 0.5M K2SO4 solution (1, 4 w/v), centrifuged and analysed with an automated elemental TOC-VCPN analyser (Shimadzu, Japan) and standard solutions for calibration. The samples were then air-dried and sieved to 2 mm for all other analyses, which were carried out within a couple of weeks after sample collection. A TGA-601 thermogravimeter (LECO Corporation, USA) was used to determine SOM as the mass loss at 550 °C (Hoogsteen et al. 2015). The microbial biomass was quantified by dsDNA content according to the microplate fluorometric method of Fornasier et al. (2014). An amount of 0.3 g of sample was transferred to a 2-mL Eppendorf tube containing 1.4 mL 0.12 M Na3PO4 buffer at pH 8 and a mixture of glass beads and ceramic beads. Tubes were bead-beated in a MM 400 beating mill (Retsch, Germany) and centrifuged at maximum speed. Supernatants were then diluted ninety times and the dsDNA quantified fluorometrically by using PicoGreen reagent (Thermo Fischer Scientific, USA) following the manufacturer's instructions.

Similarly, soil enzymatic profiles were determined following Fornasier and Margon (2007). Aliquots of 0.3 g were diluted in 2-mL Eppendorf tube with 1.25 mL solution of Tris-HCl 50mM pH 7.5, 4% lysozyme and a mixture of glass beads and ceramic beads. Tubes were then bead-beated and centrifuged. The supernatants were directly transferred into microplates and analysed with fluorogenic 4-methylumbelliferyl-based (MUF) substrates for the determination of acid (ACP) and alkaline phosphatase (ALP), arylsulfatase (ARYS); β-glucosidase (BG); bis-phosphatase (BISP); chitinase (CHIT); esterase (EST); leucine aminopeptidase (LAP) and pyro-phosphatase (PYROP).

2.2 Data analysis

Data analysis started with the computation of the statistics of variables per sampling date and vegetation cover. Principal Component Analysis (PCA) was then performed to assess the degree of correlation between variables and their possible clustering. The effect of *Sampling date* and *Vegetation cover* was in the end assessed with the linear model approach (Venables and Ripley 2002). As the residuals of the models were homoscedastic, linear models were fitted using ordinary least squares and the best ones chosen according to Akaike's information criterion. Computations were performed with the version 4.0.3 of the R software (R Core Team 2020) using the packages Stats for descriptive statistics and PCA; Mass (Venables and Ripley 2002) for linear modelling, and ggplot2 (Wickham 2016) for graphic sessions.

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3 Results

The descriptive statistics of SOM, TDN and DOC are reported in Table 1 and in the error bar graphs of Figure 1.

Table 1 – Mean and standard errors (in parenthesis) of the data partitioned by *Sampling date* and *Vegetation cover* (n = 75 for enzyme activities; n = 79 for the other variables).

Sampling date	Vegetation cover	SOM	DOC	TDN	dsDNA	ARYS	ACP	ALP	BISP	PYROP	LAP	CHIT	BG	EST
			g kg^{-1}		mg kg^{-1}					nM MUF h^{-1} g^{-1}				
May 2012	Meadows	220	41	6.7	60	17.1	192	364	58	34	91	5.4	11.7	3,341
		(8)	(2)	(0.4)	(4)	(1.1)	(32)	(67)	(4)	(2)	(8)	(0.6)	(1.1)	(234)
	Hazel	236	58	9.5	90	18.0	169	330	51	34	87	3.9	8.4	2,940
	groves	(16)	(5)	(1.0)	(9)	(1.7)	(36)	(70)	(4)	(5)	(8)	(0.6)	(0.7)	(232)
Aug 2012	Meadows	220	44	7.4	101	17.2	189	330	51	33	95	8.5	10.8	3,113
		(7)	(3)	(0.5)	(9)	(0.7)	(22)	(40)	(2)	(3)	(7)	(0.9)	(0.7)	(139)
	Hazel	322	81	11.7	131	23.0	241	362	49	29	121	11.0	9.6	3,244
	groves	(31)	(8)	(0.8)	(13)	(1.4)	(35)	(45)	(3)	(3)	(4)	(2.4)	(1.3)	(135)
Dec 2012	Meadows	203	51	8.5	72	8.2	103	292	34	33	75	7.9	7.6	1,921
		(14)	(3)	(0.5)	(5)	(1.6)	(50)	(142)	(4)	(7)	(13)	(1.3)	(1.4)	(206)
	Hazel	258	64	9.4	84	13.6	129	347	40	24	92	6.6	6.3	1,967
	groves	(24)	(7)	(1.0)	(10)	(2.3)	(62)	(201)	(5)	(3)	(21)	(2.6)	(1.2)	(237)
Mar 2013	Meadows	170	24	4.3	106	16.5	174	347	50	30	92	7.7	9.9	2,764
		(12)	(2)	(0.4)	(9)	(1.0)	(34)	(59)	(3)	(3)	(6)	(0.9)	(0.7)	(134)
	Hazel	206	36	6.0	104	21.0	195	314	50	37	90	7.8	9.4	2,853
	groves	(19)	(4)	(0.8)	(12)	(2.4)	(62)	(77)	(4)	(6)	(13)	(1.8)	(1.5)	(237)

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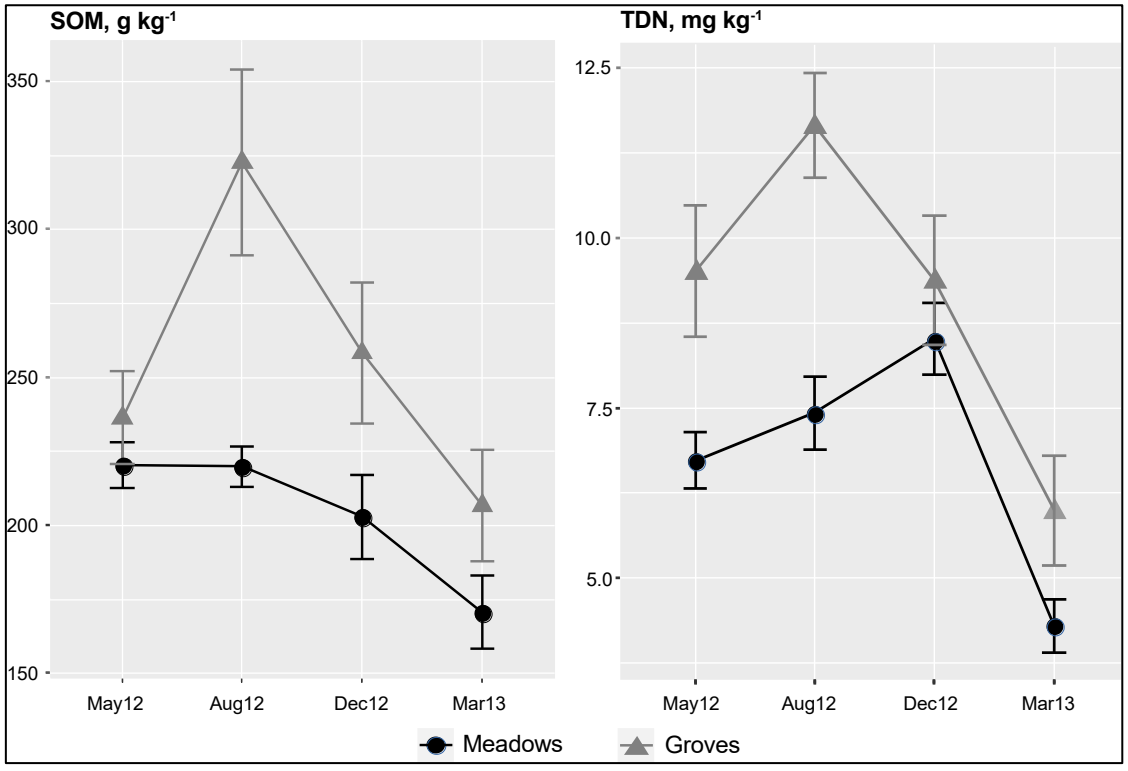

Figure 1 – Mean and standard error of SOM and TDN recorded in the two vegetation covers and the four sampling dates.

Since TDN and DOC showed the same seasonal trend, we chose former to represent their trend in Fig. 1. The SOM content was high in August 2012 and low in March 2013, but its values were notably affected by vegetation cover, with hazel groves having 52 g kg^{-1} more SOM than meadows. Hazel groves also displayed a greater decrease of SOM from summer to winter, in meadows the mean value of March 2013 – 170 g kg^{-1} – was about 23% lower than that of August 2012, while in hazel groves the decrease – from 322 g kg^{-1} – was in the order of 36%. TDN and DOC showed a trend like SOM, with minimum values in March 2012 equal to 6.0 and 4.3 mg kg^{-1} for hazel groves and meadows, respectively. The highest values, on the other hand, show a temporal deviation between hazel groves and meadows, with the former persisting in August 2012 and the latter moving to December 2012.

Table 1 also shows the dsDNA and EEAs data. In addition, the seasonal trends of dsDNA and ACP are shown as error bar graphs in Figure 2, where ACP was chosen as a reference for the pattern of seasonal variation of ARYS, BG, BISP, EST and LAP.

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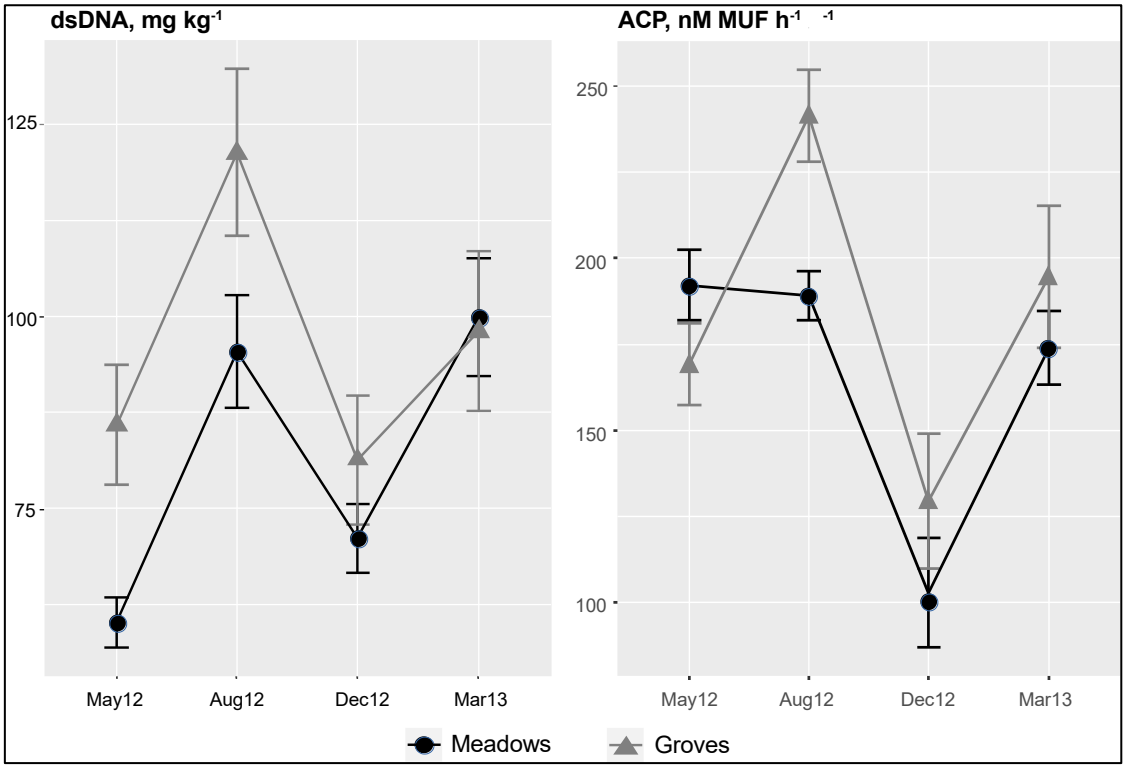

Figure 2 – Mean and standard error of dsDNA and ARYS recorded in the two vegetation covers and the four sampling dates.

The dsDNA showed an alternating seasonal trend, with values around 75 mg kg^{-1} in May and December 2012 and higher than 100 mg kg^{-1} in August 2012 and March 2013 (Fig. 2, left side). The ACP also reached a minimum point in December 2012, but its values remained relatively constant in the other three sampling dates (Fig. 2, right side).

The dsDNA, ACP, ARYS and LAP also showed variations in relation to *Vegetation cover*, with higher values in hazel groves on separate dates: dsDNA in May and August 2012; ACP, ARYS and LAP in August 2012. The effect of vegetation cover reversed in the case of BG, with higher values in meadows and maximum difference in May 2012. Finally, the enzymes ALP, CHIT and PYROP were highly variable, therefore showing no variation in relation to either *Sampling date* or *Vegetation cover*. The relations between variables observed in Tab. 1 are also supported by the results of PCA in Table 2.

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Table 2 – Variance and factor loadings of the Principal Component Analysis. Variables are ordered according to the factor loadings of the three components.

	PC1	PC2	PC3
Variance	5.79	2.50	1.34
Relative variance, %	44.6	19.3	10.3
Factor loadings			
ACP	0.90	-0.13	-0.27
EST	0.89	-0.22	-0.09
ARYS	0.88	-0.03	-0.25
LAP	0.86	-0.03	-0.06
BG	0.80	-0.27	-0.10
BISP	0.75	-0.40	0.34
dsDNA	0.66	0.27	-0.30
DOC	0.22	0.89	0.18
TDN	0.29	0.87	0.26
SOM	0.55	0.71	-0.02
ALP	0.44	-0.21	0.73
PYROP	0.49	-0.15	0.55
CHIT	0.42	0.07	-0.18

The highest loadings in PC1 component concerned six of the nine EEAs and dsDNA, while PC2 was linked to SOM and the soluble fractions of C and N. Finally, PC3 was mainly connected to ALP and PYROP. The first two components also suggest the influence of distinct environmental factors, attributable to microbial community dynamics for PC1 and biochemical processes involving soil organic matter for PC2.

Data analysis ended with linear modelling. Since EEAs are correlated with dsDNA (Tab. 2) as they are at least partly a product of the soil microbial pool (Moorhead et al., 2013), we have standardised them on dsDNA before linear model analysis. The resulting statistics are reported in Table 3.

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Table 3 – Mean and standard errors (in parenthesis) of the enzyme activities standardised to dsDNA partitioned by *Sampling date* and *Vegetation cover* (n = 75).

Sampling date	Vegetation cover	ARYS	ACP	ALP	BISP	PYROP	LAP	CHIT	BG	EST
		nM MUF h^{-1} mg^{-1} dsDNA								
May 2012	Meadows	0.30	3.32	6.3	1.01	0.61	1.56	0.10	0.21	58
		(0.03)	(0.24)	(0.5)	(0.08)	(0.07)	(0.13)	(0.02)	(0.03)	(5)
	Hazel groves	0.21	1.99	3.8	0.59	0.37	1.01	0.04	0.10	34
		(0.02)	0.17)	(0.3)	(0.05)	(0.02)	(0.08)	(0.01)	(0.01)	(2)
Aug 2012	Meadows	0.18	2.00	3.5	0.53	0.32	0.99	0.09	0.11	32
		(0.01)	(0.17)	(0.3)	(0.04)	(0.01)	(0.09)	(0.01)	(0.01)	(2)
	Hazel groves	0.18	1.86	2.8	0.38	0.22	0.94	0.08	0.08	25
		(0.02)	(0.20)	(0.3)	(0.04)	(0.02)	(0.10)	(0.02)	(0.01)	(3)
Dec 2012	Meadows	0.11	1.41	4.5	0.49	0.58	1.22	0.13	0.12	28
		(0.02)	(0.17)	(0.9)	(0.05)	(0.23)	(0.37)	(0.04)	(0.03)	(3)
	Hazel groves	0.15	1.46	5.3	0.61	0.36	0.91	0.05	0.07	24
		(0.02)	(0.12)	(1.9)	(0.20)	(0.12)	(0.08)	(0.02)	(0.01)	(2)
Mar 2013	Meadows	0.17	1.73	3.4	0.50	0.30	0.90	0.08	0.10	27
		(0.02)	(0.18)	(0.3)	(0.05)	(0.04)	(0.07)	(0.01)	(0.01)	(3)
	Hazel groves	0.22	2.02	3.4	0.53	0.39	0.90	0.08	0.09	30
		(0.01)	(0.07)	(0.3)	(0.03)	(0.04)	(0.05)	(0.01)	(0.01)	(2)

The parameters of the best-fitted models are shown in Table 4, where the states of the factors *Sampling date* and *Vegetation cover* are compared with the first one of the series – meadows on May 2012 – which, according to the conventions of the MASS package, are reported as model intercept. Estimates with a p-value <0.01 are marked in bold in the Table.

Table 4 – Linear models relating the variables with *Sampling date* and *Vegetation cover* (n = 75 for enzyme activities; n = 79 for the other variables). In bold the estimates of regression parameters significant at $p < 0.01$. Enzyme activities are standardised on dsDNA.

Factor levels	SOM	DOC	TDN	dsDNA	ARYS	ACP	ALP	BISP	PYROP	LAP	CHIT	BG	EST
		g kg^{-1}		mg kg^{-1}	nM MUF h^{-1} mg^{-1} dsDNA								
Intercept	203	**40**	**6.8**	**66**	**0.25**	**2.7**	**5.1**	**0.86**	**0.40**	**1.4**	**0.10**	**0.18**	**50**
	(14)	(4)	(0.6)	(7)	(0.01)	(0.1)	(0.6)	(0.07)	(0.04)	(0.7)	(0.01)	(0.01)	(3)
Aug 2012	41	**13**	**1.8**	**41**	**-0.08**	**-0.8**	**-1.9**	**-0.35**		**-0.4**		**-0.06**	**-18**
	(18)	(5)	(0.8)	(9)	(0.02)	(0.2)	(0.9)	(0.10)		(0.2)		(0.02)	(3)
Dec 2012	1	8	0.8	4	**-0.12**	**-1.2**	-0.2	**-0.26**		-0.2		**-0.06**	**-21**
	(18)	(5)	(0.8)	(9)	(0.02)	(0.2)	(0.8)	(0.09)		(0.2)		(0.02)	(3)
Mar 2013	-41	**-20**	**-3.0**	**31**	**-0.07**	**-0.8**	-1.7	**-0.29**		**-0.4**		**-0.06**	**- 18**
	(18)	(5)	(0.8)	(9)	(0.02)	(0.1)	(0.8)	(0.09)		(0.2)		(0.02)	(3)
Hazel groves	**52**	**19**	**2.6**	**17**							**-0.04**	**-0.05**	**-8**
	(13)	(4)	(0.5)	(7)							(0.01)	(0.01)	(2)
R^2_{adj}	0.29	0.50	0.45	0.29	0.36	0.32	0.06	0.16	-	0.09	0.07	0.30	0.46

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As already suggested by PCA, SOM, DOC, and TDN showed comparable trends, although noticeably different from that of enzyme activities and dsDNA. They displayed significantly higher values in hazel groves compared to meadows, with the greatest difference being found in August 2012 (Fig. 1). Linear modelling confirms the link of Sampling date with DOC and TDN whose values, compared to those of May 2012 and December 2012, are significantly higher in August 2012 and lower in March 2013. In the case of SOM, the differences between the estimates were less pronounced, with p-value < 0.03.

Also, in the case of DNA, significantly higher values were recorded in hazel groves compared to meadows, with a maximum difference of +30% in May 2012. Regarding the sampling date, the values for May 2012 (intercept in Tab. 4) and December 2012 were similar and significantly lower than the estimates, also similar between them, of August 2012 and March 2013.

Except for CHIT and PYROP – which were independent from Sampling date – all the other standardised EEAs showed substantially equivalent seasonal variations, with a significant decrease from May 2012 to August 2012 – from just over -40% in meadows to less than -20% in hazel groves – followed by relatively constant values, with minima generally recorded in December 2012 (Tab. 3). This behaviour can be also seen in the error bar graphs in Figure 3, where ACP and EST have been chosen as the most representative of the EEAs groups influenced or not influenced by vegetation cover throughout the year.

Finally, it is worth noting that according to both Fig. 3 and Tab. 4 all enzymes show in spring an average 40% higher activity in meadows than in hazel groves.

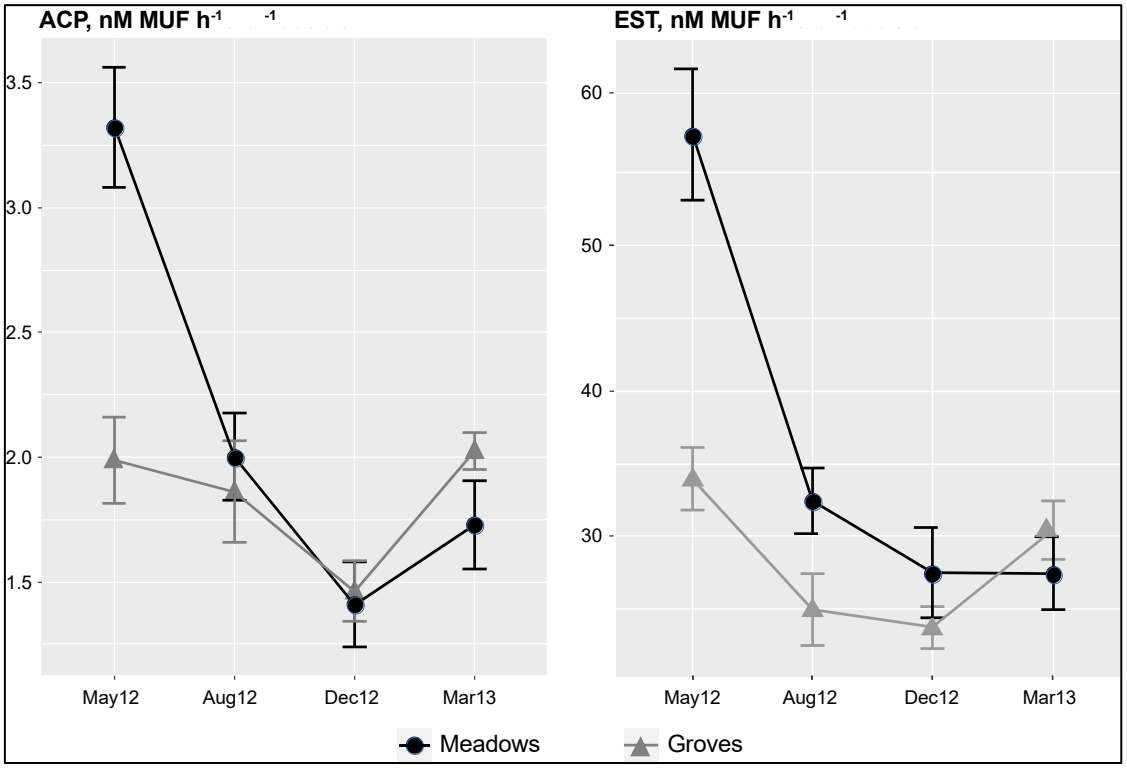

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Figure 3 – Mean and standard error of ARYS and EST recorded in the two vegetation covers and the four sampling dates. Data are standardized by dsDNA content.

4 Discussion

The factors *Vegetation cover* and *Sampling date* were used to investigate the seasonal response of soil biochemical variables as indicators for the temporal monitoring of meadow and hazel groves habitats in subalpine ecosystems, *Vegetation cover* being chosen as indicative of long-term man management of the area, *Sampling date* as relating to short-term variability driven by seasonal climate variation.

The selection of a set of indicators for temporal variations in soil processes in the habitats of interest must be based on the type of information these indicators provide and, all other costs being equal, on their analytical costs. Before selecting the soil indicators to be monitored, it is therefore crucial to assess the relationships between the potential indicators and the soil processes that influence SOM and the microbial pool. In our investigation, most variables showed high values in the warmest and least rainy summer season. In the cold season, instead, two distinct trends appeared. SOM, DOC and TDN continue to decline until the end of winter, while dsDNA and standardized EEAs decrease to their minimum in early winter and rise to their maximum in late winter. These trends suggest a partial separation between the overall transformation cycle of organic matter and the biodegradation of litter by soil microorganisms.

4.1 Soil organic matter, dissolved organic C and total dissolved N

As already observed by Horvath (2007), the soil A horizon in the wooded areas showed a significantly higher content of SOM than meadows but, despite the homogeneity of climate, humus system and soil type, the SOM dynamics appeared different in the two habitats. In the hazel groves, the highest SOM content was detected in summer, which corresponds to the period of greatest faunal activity, particularly that of burrowing earthworm species that fragment the leaf litter and transport organic materials along the soil profile (Vesterdal et al. 2013). The sharp reduction of SOM in late autumn also suggests that the incorporation of organic materials into soil is an intense but time-limited process that ends with the arrival of the first snowfall. In the meadows, where grass litter is more readily decomposable and less variable than leaf litter (Chapman et al. 2006), incorporation of organic materials is limited because the litter released by roots is already underground, resulting in less marked variation of SOM. Fluctuations in DOC and TDN are consistent with SOM content across the seasons and their close relationship – indicated by both Tab. 2 and Tab. 3 – can be explained by the prevalence of organic N in the soluble component of SOM. From a monitoring point of view, the three variables provide the same type of information, with a higher percentage of variance explained by DOC and TDN.

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4.2 Double stranded DNA

Changes in microbial biomass measured through microbial dsDNA are likely explainable by seasonal variations in temperature and moisture, and by nutrient cycles in soil. In their annual life cycle, plants regulate the availability of belowground C and N through exudation and uptake, inducing shifts in microbial physiology (Koranda et al. 2013). In spring the root systems absorb nutrients to support the photosynthetic activity (Warren and Adams 2002; Seufert et al. 2019) and the dsDNA minima recorded in May 2012 suggest a limitation in the growth of microbial biomass caused by the increased competitiveness of plants for N. On the other hand, the maximum dsDNA abundance is observed while heading to the summer season in concomitance with high amounts of TOC and TDN.

The second peak of dsDNA at the end of winter and, according to the data of May 2012, its decrease in early spring appears to be influenced by processes unrelated to plant growth. Comparable peaks in microbial biomass N were recorded in alpine meadow habitats under late winter snowpack (Lipson et al. 1999; Schmidt and Lipson 2004) and in a beech forest occasionally covered by snow (Kaiser et al. 2011). All these authors recorded a peak of soluble N in summer and its gradual decrease towards the winter season and hypothesized that part of the available N would be stored in the soil microbial biomass during winter and released in spring, when a microbial biomass turnover occurs after snow melt (Schmidt and Lipson 2004). Similar processes seem to occur in our study, where the peak of dsDNA in late winter coexists with the minimum values of TDN and, similarly to what we observed in May 2012, we could expect a fast decline in microbial biomass in spring accompanied by an increase in dissolved N. Based on these considerations, it seems possible to apply to the investigated subalpine meadows and hazel groves the conceptual model of Schmidt et al. (2007) for seasonal snowed ecosystems, which postulates an autumn/winter cycle of microbial build-up and immobilization of N into microbial cells fed by plant litter, and a summer cycle mostly fuelled by plant rhizodeposition.

4.3 Extracellular enzyme activities

In contrast with the variables considered above, the mean values of most EEAs did not change with *Vegetation cover* and, according to Tab. 4, only BG, CHIT and EST showed greater activity in meadows than in hazel groves. Differences with the temporal trend of SOM and soluble C and N also occurred for *Sampling date*, with relatively constant values in three of them and a significant reduction in early winter (Figs. 1 and 2).

There are more similarities with the seasonal variation of dsDNA, further supporting indications of PCA that EEAs were linked to the dynamics of the soil microbial pool, which differed from those governing the SOM cycle. Once the effect of microbial biomass has been removed through standardisation on dsDNA, EEAs showed a different trend of seasonal change, with maximum values in spring, a marked decrease in summer and a slight increase in late winter (Fig. 3). This variation support the hypothesis of Puissant et al. (2015) on a partial decoupling between microbial biomass and enzyme pool attributable to seasonal changes in soil microbial community structure.

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A second option, which does not exclude the previous one, is that the high values recorded in May 2012 are partly due to an increased release EEAs by the root systems at a time when plant's metabolism is at its peak. The sampling strategy we used did not aim to disaggregate enzyme production of microbial origin from that of the roots, but future research could provide more detailed answers on this hypothesis.

4.4 Selection of indicators for soil monitoring

The results of the PCA (Tab. 2) provide an initial selection criterion for groups of collinear variables by suggesting a minimum number of three variables representative of the biochemical and microbiological processes prevalent in subalpine valley floor soils on coarse alluvium, where meadows are interspersed by hazel-dominated groves.

A further selection within these groups can be based on the results of the linear models shown in Tab. 3, using the effect of sampling dates and vegetation cover as selection criteria, along with the percentage of explained variance as measured by R^2 adjusted (R^2_{adj}).

In the case of biochemical variables, DOC and TDN are preferable to SOM because, in addition to responding to the change in vegetation cover, they are more sensitive to seasonal variations, allowing greater detail on the transformation processes of organic matter over the course of the year. A further advantage is that they are usually determined sequentially in the same extract, allowing soluble C and N to be considered together. Regarding costs, instrumentation depreciation and energy consumption are lower than those for measuring SOM or organic C (if using an elemental C analyser).

As the PCA already suggested, the variables measuring the mass and activity of the soil microbial component show seasonal trends different between them and distinct from those of the SOM and its soluble fractions. For the soil microbial biomass, we opted for dsDNA as a proxy for microbial biomass C measured by the fumigation-extraction method of Vance et al. (1987) because it ensures significantly lower costs by reducing sampling and analysis time by more than 75% (Bragato et al., 2016). The problem of collinearity with EEAs can then be solved by standardising EEAs on dsDNA – as we have done in the present investigation – which allows the environmental response of the microbial mass to be separated from the activity of the extracellular enzymes it produces.

Turning to EEAs, costs are limited because enzyme activities are measured sequentially in the same microplate when using fluorometric techniques. Costs can be further reduced in the data processing phase by analysing a smaller number of noncollinear EEAs that can be selected based on the soil nutrient cycles in which they are involved as a further criterion. In the subalpine ecosystem investigated, the EEAs that best meet this criterion are represented by EST for the C-cycle (higher R^2_{adj} than BG); LAP for the N-cycle (higher R^2_{adj} than CHIT, which moreover does not vary with the sampling date); ARYS for the S-cycle; ACP for the P-cycle (higher R^2_{adj} than BISP, combined with the lack of variation of ALP and PYROP with the two variability factors considered).

As regards the most suitable period for sampling, excluding the winter season when snowfall limits access to sites and lengthens sampling times, the choice depends on the objective pursued by the monitoring: i) analysis of long-term trends with measures taken annually/every few years; or, ii) shorter-term surveys that also focus on intra-annual variability in soil

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biological and biochemical cycles. Our survey shows that late spring and late summer/early autumn are useful periods, which also coincide with the two periods of greatest plant activity in continental and humid temperate climates: if the focus is on intra-annual variations in microbial mass and activity, sampling should be planned for both periods; if, on the other hand, the main focus is on longer-term trends, it is advisable to choose one of the two periods, taking care to keep the sampling season constant.

Conclusions

The combination of traditional and most recent biochemical indicators would implement the soil quality assessment in view of preserving soil from degradation. Although chemical or physical soil indicators remain popular and reliable monitoring tools of soil threatened by both climatic and anthropogenic activities, an effective and accurate early warning set of indicators is lacking. The data we have presented would like to illustrate how biochemical indicators could be adopted to investigate the ecosystem parameters by employing high throughput techniques. They might represent a cost-effective alternative for long-term longitudinal studies and the space-time monitoring of biological and biochemical processes in pre-alpine and alpine ecosystems in the future. Such an approach would also be a stimulus to further explore mass-specific enzyme activities to better understand the dynamics of the microbial pool across the seasons. Moreover, the dsDNA and enzyme activities could be used as quantitative parameters for the characterization of humus forms, focusing on their most stable organo-mineral Ah horizon, therefore partially emancipating the characterization and classification of humus forms from the high inter-annual variability that characterizes organic horizons.

Data availability

All raw data can be provided by the corresponding author upon rerquest.

Author contributions

NG: investigation, validation, writing (original draft – equal, review and editing – equal). FF: investigation. GB: conceptualization, methodology, supervision, formal analysis, writing (original draft – equal, review and editing – equal). All authors read, commented and approved the final manuscript.

Competing interests

The authors declare that they have no conflict of interest.

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