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Assessing microbial activity in soil samples collected during the transition period between seasons using the Biolog EcoPlate method

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Abstract. Seasonal variations influence the composition, diversity and metabolic activity of soil microbial communities. Understanding these changes is essential for ecosystem monitoring and the objective assessment of agricultural practices. The aim of this study was to assess soil microbial activity and community structure during the transition period between two seasons using the Biolog EcoPlate method. Soil samples were collected from the experimental field of Agricultural University-Plovdiv in February and April 2023. The assessment of microbial metabolic activity was based on optical density measurements. These data were used for calculation of summarized metabolic activity (average well-color development, AWCD), the Area Under the Curve (AUC), metabolic activity towards specific substrate guilds, substrate richness (SR), various functional indexes, and heatmap plotting. The results showed an elevated summarized metabolic activity in the samples collected in April, and in particular towards carboxylic acids, carbohydrates, polymers, and phenolic compounds. In contrast, the bacterial community in the February sample was more active in utilizing amino acids and amines. The functional indexes indicated higher biodiversity in the April samples; however, this does not imply an unbalanced or impaired community in the February sample. The noted preferences in substrates utilization suggest the presence and/or activity of different species within the microbial communities or shifts towards other metabolic pathways depending on the temperature range and precipitation regime.

Key words: Biolog Ecoplate technique, soil microbial activity, substrate richness, functional indexes

Introduction

Microbial communities are the most abundant and diverse biological soil entity. Their composition, structure and properties can vary significantly depending on the soil type and climatic conditions. They are invariably and intrinsically involved in nutrient cycling, organic matter decomposition, and energy flows which empower the functioning of, either natural or artificial ecosystems (Fierer, 2017; Fan et al., 2023). Bacterial communities occupy specific habitats based on their properties, such as bulk soil and the rhizosphere. They colonize soil layers both horizontally and vertically soil layers, creating a distinct pattern of distribution. In these habitats the functioning and structure of bacterial communities are dynamically shaped by nutrient availability, biotic interactions, and various edaphic factors. Bacterial communities are also affected by global change, carbon dioxide level, and factors related to anthropogenic activities. The changes in community structure, however, are closely related to the specificities of each studied ecosystem, including agroecosystems, and incorporation of community responses to abiotic and biotic factors into predictive models has not been achieved yet (Lladó et al., 2017). The precise assessment of bacterial community contribution to ecosystem

functioning is still under exploration and examination, but since the shift in the structure of microbial communities will affect the ecosystems globally it is of utmost importance to understand communities' response to ongoing climate changes and its possible effects on ecosystem services (Abirami et al., 2021; Saez-Sandino et al., 2023).

The most important factors influencing the activity of soil microbial communities include soil type and composition, climate and environmental conditions, land use and management practices, plant roots and rhizosphere interactions, nutrient availability, pH levels and oxygen availability, soil disturbances, and successions driven by natural processes. Temperature and moisture are critical factors that significantly influence soil microbial communities. Either independently or interactively, they impact the composition of these communities (Zhou et al., 2017; Zhao et al., 2024; Zeng et al., 2024). The effect of temperature on soil microbial communities is related to soil moisture, as liquid water is essential for most metabolic reactions of living organisms (Klimek, 2013). The changes in temperature and moisture have been found to alter the taxonomic and functional profiles of microbial communities in soil ecosystems (Barboza et al., 2018). Variations in air temperature, rainfall intensity, and frequency due to climate change have direct consequences on soil moisture content and temperature, thereby affecting soil microbial communities (Asano et al., 2023). Alteration in soil moisture affect soil microbial community diversity, structure, and phenotypic characteristics, with implications for ecosystem functions and nutrient cycling (Wei et al., 2021). Additionally, variations in soil moisture due to irrigation or precipitation can impact soil microbial community composition and soil organic carbon storage, leading to imbalances in the terrestrial ecosystem carbon cycle (Qi et al., 2021).

Soil bacterial communities are notably influenced not only by soil temperature, soil moisture, cropping systems, infection status, and weed communities but also by the sampling date within the growing season (Ishaq et al., 2020). Seasons and transition between seasons significantly influence soil microbial community structure and functioning. Yang et al. (2018) demonstrated that forest age and season impact soil microbial community structure and enzyme activities and similarly Guo et al. (2022) highlighted the impact of seasons, showing that warming altered community structures and increased network complexity. Broadbent et al. (2021) further emphasized how climate change-induced alterations, such as earlier snowmelt, can lead to shifts in soil microbial community composition, functioning, and biogeochemical cycling. Moreover, Wang et al. (2018) illustrated changes in microbial communities' structure in relation to seasonal variations in soil pH and organic matter content. Pajares et al. (2018) also noted the influence of seasonal soil heterogeneity and rainfall on soil bacterial community structure and function in a tropical forest. Yan et al. (2017) explored the effects of nitrogen additions on soil microbial communities across seasons in a boreal forest, demonstrating that soil nitrogen concentrations and seasonal fluctuations shaped microbial community composition. Cao (2023) delved into the interplay between elevation, season, and soil microbial communities, revealing the dynamic nature of interactions between factors. Additionally, the observed seasonal variations in the tropical zone, emphasized the role of soil moisture in stimulating microbial biomass and activity during specific seasons (Araújo et al., 2013). Shigyo et al. (2019) investigated the seasonal dynamics of soil fungal and bacterial communities in cool-temperate montane forests, showcasing the relationship between soil fungal communities and seasonal variations in soil organic matter composition. Understanding the intricate effects of seasons and the specifics of the transition period between seasons on the activity and structure of soil microbial communities is important for predicting how these communities will respond to ongoing environmental changes, such as climate warming and land use alterations. This highlights the importance of considering seasonal dynamics in soil microbial research.

The aim of the current study was to assess soil microbial activity and community structure at soil sampling, conducted at the transition period between two seasons, by utilizing the Biolog EcoPlate method.

Materials and methods

Soil samples

The analyzed soil samples were collected on the 23rd of February and the 20th of April from a depth of 0 to 10 cm at the Experimental Field of

the Agricultural University in Plovdiv, Bulgaria. The field is located at 42.14°N latitude and 24.80°E longitude, with an altitude of 156 meters above sea level. The soil type was classified as silty clay loam (Mollic fluvisols) - 19.9% sand, 46.9% silt, 33.2% clay. Analysis of soil mineral content provided data about the main soil parameters, such as nitrogen – 7.94 ppm, phosphorus (P_2O_5) – 214.6 ppm, potassium (K_2O) – 423.5 ppm, $CaCO_3$ – 8.75 g/kg, exchangeable cations (Ca^{2+} and Mg^{2+}) – 16.2 meq/100g, conductivity 108.1 µS/cm, soil organic matter – 2.66%, pH – 8.8. The plots from which samples were taken were kept free of plants and devoid of agronomic interventions.

Meteorological data

Meteorological data (temperature and precipitation) for February and April 2023 was retrieved from Meteoblue and are presented in Fig. 1 and Fig. 2, respectively.

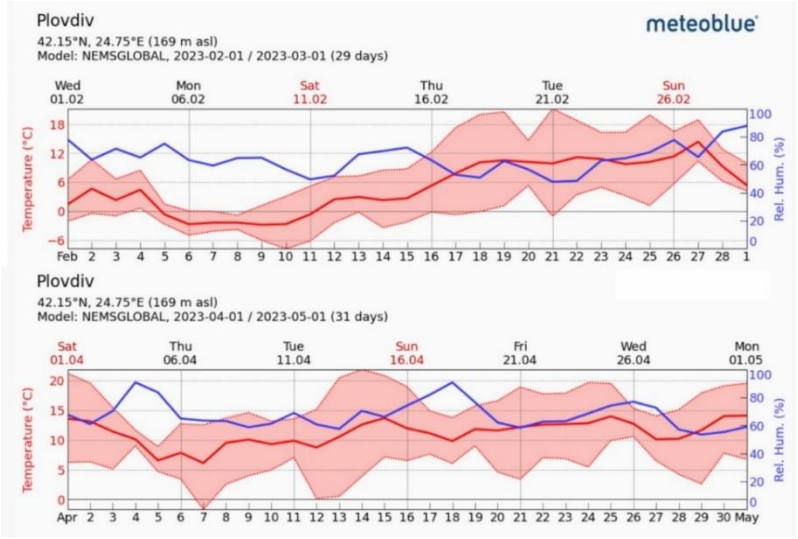

Fig. 1. Diurnal temperature variation in February and April 2023

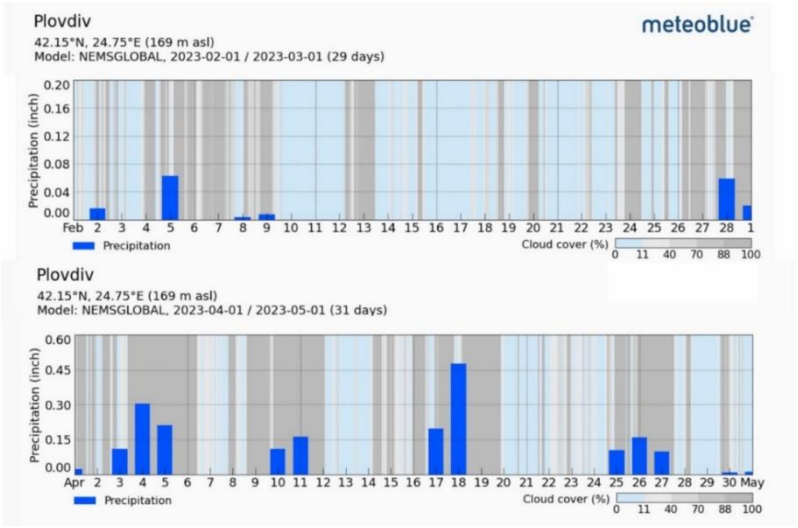

Fig. 2. Diurnal precipitation variation in February and April 2023

Biolog Ecoplate

Metabolic activity of microbial communities was assessed using the 96-well Eco MicroPlates™ of BIOLOG® (Biolog Inc., USA). Each EcoPlate is comprised of 31 different substrates organized in the following guilds - carbohydrates, carboxylic acids, polymers, amino acids, amines and phenolic compounds (Table 1).

The EcoPlate contains three replicates of each guild.

Table 1. Substrates in the Biolog EcoPlate

Guild	Substrates	Guild	Substrates
Amino acids	L-Arginine	*Carbohydrates*	D-Cellobiose
	L-Asparagine		α-D-Lactose
	L-Phenylalanine		β methyl D Glucoside
	L-Serine		D -Xylose
	L-Threonine		i-Erythritol
	Glycyl-L-glutamic acid		D-Mannitol
Amines	Phenylethylamine		N-acetyl-D-glucosamine
	Putrescine		Glucose-1-phosphate
Carboxylic acids	Pyruvic acid methyl ester		D, L-α-Glycerol phosphate
	D-Glucosaminic acid		D-Galactonic acid γ-lactone
	D-Galacturonic acid	*Polymers*	Tween 40
	γ-Amino butyric acid		Tween 80
	Itaconic acid		α cyclodextrin
	α-keto Butyric acid		Glycogen
	D-Malic acid		
Phenolic compounds	2-Hydroxybenzoic acid 4-Hydroxybenzoic acid		

The soil samples collected in February and April was processed uniformly. One gram of soil, cleaned of stones and debris, was sieved with 2 mm sieve and suspended in 9 ml sterile distilled water. It was thoroughly mixed for 10 min and left to settle for 5 min. The inoculation of Biolog® EcoPlates was done with 150 µl of a 10^{-3} dilution. The plates were incubated at 24±1°C and read spectrophotometrically at 24-hour intervals for 7 days (168h) with the MicroStation™ Reader provided by the BIOLOG® System. The calculations of average well-color development (AWCD) and separately for each substrate guild were based on the optical density (OD) measured at 590 nm and 750 nm according to the procedure described by Sofo & Ricciuti (2019), except for the formula for AWCD which was according to Huang et al. (2012) as follows:

$$\mathbf{AWCD} = \sum(\boldsymbol{C_i} - \boldsymbol{R})/\mathbf{31},$$

where R is the control well (water) and C_i is the value of any substrate-containing well.

The measurement at 24-hour was used for data normalization by subtracting each consecutive measurement with the corresponding values at 24-hour in order to avoid so called background noise according to Urakawa et al. (2013). Negative values obtained during calculation and normalization of the data were set to zero (Garland, 1996).

The Area Under the Curve (AUC) was calculated using the trapezoidal rule (Klimek, 2013) and expressed in Equation 1:

$$\boldsymbol{AUC} = \sum_{\boldsymbol{i}}^{\boldsymbol{n}} \left(\frac{\boldsymbol{OD_i} + \boldsymbol{OD_{i+1}}}{\mathbf{2}}\right) \times (\boldsymbol{t_{i-1}} - \boldsymbol{t})$$

where t_n is the value of each consecutive time intervals (in the current experiment, i=24, n=168) and OD_i is the optical density value at each point of the measured time interval.

Functional indexes

Sofo & Ricciuti (2019) recommended calculation of functional indexes based on wells with an OD ≥0.250 and in the current study was obtained the aforementioned approach and presented indexes were calculated using the measurements done on the 120th hour of EcoPlate incubation. The chosen time point (120th hour) corresponds to the inflexion point of AWCD graph of OD changes during incubation.

The formulas and corresponding references for index calculations are listed in Table 2.

Table 2. Formulas for functional indexes calculation

Functional index	Formula	References
Shannon-Wiener index, H′	$H' = -\sum p_i \times (ln\ p_i)$ where p_i is C_i, divided by the sum of C_i, wells with value ≥ 0.250	Jurkšienė et al. (2020)
Pielou index, E	$E = \frac{H'}{ln\ S}$ where H′ is Shannon-Wiener index S – number of wells with value ≥ 0.250	Pielou (1966) Jurkšienė et al. (2020)
Simpson index, D	$D = 1 - \sum P_i^2$ where P_i is C_i, divided by the sum of C_i, wells with value ≥ 0.250	Chen et al. (2020)
Margalef index, d	$d = \frac{(S-1)}{lnN}$ where, S – number of wells with value ≥ 0.250, N – number of substates, i.e. 31	Türkmen & Kazanci (2010)
McIntosh index, U	$U = \sqrt{\sum P_i^2}$ where P_i is C_i, divided by the sum of C_i wells with value ≥ 0.250	McIntosh (1967) Huang et al. (2012)
McIntosh evenness, McI	$McI = N - U/N - (N/\sqrt{S})$ where U – McIntosh diversity index, N – sum of wells with value ≥ 0.250, S – number of substrates, i.e. 31	Xu et al. (2015)
Gini coefficient, G	$G = \frac{\sum_{i=1}^{N}\sum_{j=1}^{N}\lvert x_i - x_j\rvert}{2N^2\bar{x}}$ where x_i and x_j represent each pair of OD readings, $\bar{x}$ - AWCD, N - number of substrates. The final value was further multiplied with n/(n-1)	Weiner & Solbrig (1984) Harch et al. (1997)

Data analysis

The AWCD were expressed as total activity and as per guilds, and graphs were done with Microsoft Excel. The three sets of substrates in each EcoPlate were considered replicates (n=3). The error bars represent the standard deviation and different letters indicate statistically significant difference. Functional indexes of community structure of samples collected in February and April and the substrate richness were computed at the 120th hour of EcoPlate incubation and compared by independent-samples t-test with SPSS program (IBM, ver. 26) The statistical difference in total substrate richness (SR) and per guilds of the samples is indicated with an asterisk.

Results

The activity of microbial communities was presented both as average well-color development (AWCD) (Fig. 3) and as values for the Area Under the Curve (Table 3), providing different aspects of activity. The AWCD graph showed a typical lag phase during the first 24 hours of EcoPlate incubition. This lag phase is associated with the natural adaptation of microorganisms to available substrates. After the lag phase till the end of the incubation a steady increase of OD was observed at each reading time point except for the measurements done between 120 and 168 hours when the AWCD curve became relatively flat. The initial lag phase at the beginning of EcoPlate incubation is highly dependent on the primary source of collected samples. After 48th hour the metabolic activity of the microbial community in the sample collected in April was higher than in the February sample. At the end of the incubation period, the OD reached 1.014±0.062 and 0.831±0.11, respectively. The Area Under the Curve (Table 3) indicated the same trend, with a higher value for sample collected in April compared to the sample taken in February, with corresponding values of 94.81 and 71.16, respectively.

Apart from summarizing the metabolic activity of microbial communities expressed as AWCD, the Biolog EcoPlate provides an option for estimating specific substrate metabolic patterns. The thirty-one substrates in the EcoPlate belong to six chemically different guilds: amino acids, carbohydrates, carboxylic acids, amines, polymers, and phenolic compounds (Table 2). Each set is repeated three times on the plate, facilitating calculation and permitting statistical analysis.

The trend of amino acids utilization did not differ between the two samples up to 96 hours (Fig. 4). However, measurements - between 120 and 168 hours revealed that the microbial community in the sample collected in February was 15% more active in amino acid utilization compared to the sample collected in April and the corresponding values were 1.182 ±0.185 and 1.013 ±0.087. The observed difference was mainly due to higher utilization of L-arginine, L-asparagine, L-serine, and glycyl-L-glutamic acid of the microbial community in sample collected in February. The calculated value for the Area Under the Curve was higher for February sample (104.09) and lower for the April sample (93.37).

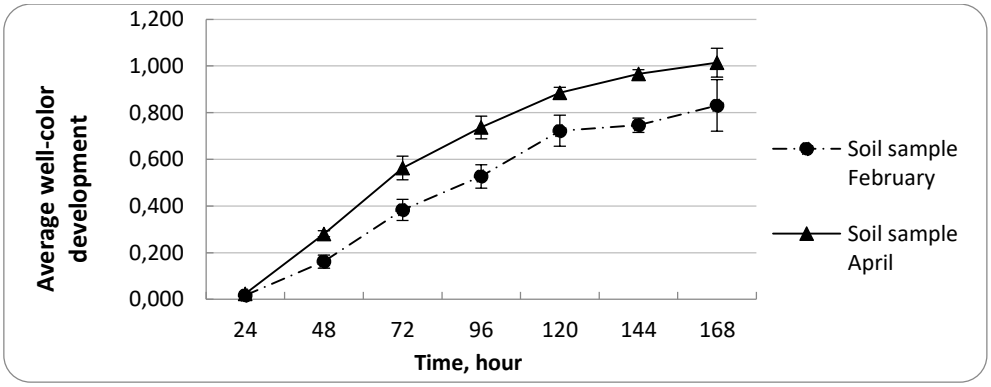

Fig. 3. Microbial metabolic activity in soil samples taken in February and April

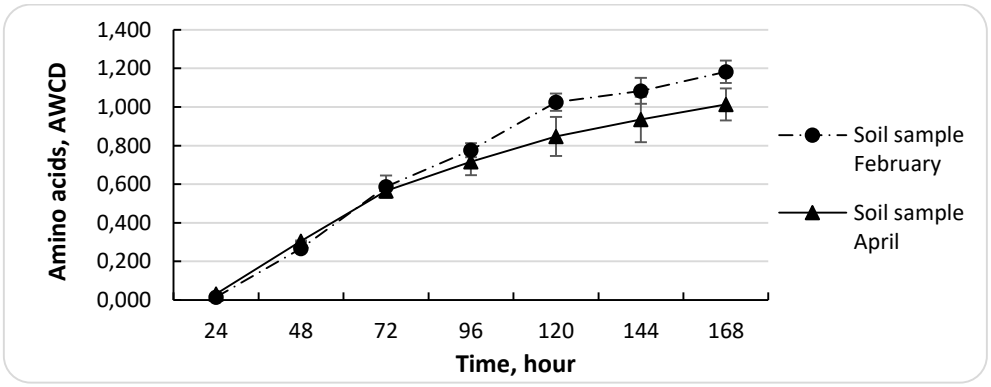

Fig. 4. Utilization of amino acids by microbial communities in soil samples collected in February and April using the Biolog EcoPlate method

Sample	Total microbial activity	Substrates					
		Amino acids	Amines	Carboxylic acids	Carbohydrates	Polymers	Phenolic compounds
February	71.16	104.09	76.75	82.63	51.32	71.70	23.33
April	94.81	93.37	68.41	120.99	85.46	88.12	94.11

After the 72nd hour until the end of the incubation period, the utilization of amines was more effective in the soil sample collected in February (Fig. 5). To some extent, the difference could be attributed to the relatively larger standard deviation of the mean values calculated for the April sample. It should be noted that the community in the April sample showed a more balanced utilization of both amine compounds (phenylethylamine and putrescine) compared to the February sample. On the other hand, between the 96th-168th hours, putrescine utilization in the February sample was almost two-fold higher than in the April sample, surpassing its counterpart as the mean value. However, at the end of the incubition period, the numerical difference between the samples collected in February and April was insignificant – 0.816 ±0.196 and 0.755 ±0.161, respectively. This was also reflected in the small difference in the Area Under the Curve values, which were very similar. For the February sample, it reached 76.75 and for April, it was 68.41 (Table 3).

The bacterial community in the sample collected in April significantly outperformed its February counterpart in terms of metabolic activity for carboxylic acid utilization, with the Area Under the Curve being 120.99 and 82.63, respectively (Table 3). The difference became evident very early in the EcoPlate incubation (48 hours) and remained unchanged until the end of the incubation period (Fig. 6).

Similar to the trend observed for carboxylic acid utilization, microbial activity in terms of carbohydrate utilization showed significantly higher metabolic activity in the sample collected in April compared to the sample collected in February, as indicated by the values of the Area under the Curve. For the sample taken in April, the AUC was 85.46, and for the February sample - 51.32 (Table 3). At the end of the incubation period, the OD reached 0.625 ±0.150 and 0.998 ±0.069 for samples collected in February and April, respectively (Fig. 7). Among all other guilds, except phenolic compounds utilization and, to some extent amines, the measured OD for carbohydrates utilization showed the lowest values.

At the 168th hour, the mean value of microbial activity reached 0.998 ± 0.069 and 0.625 ±0.150 in sample collected in April and February, respectively.

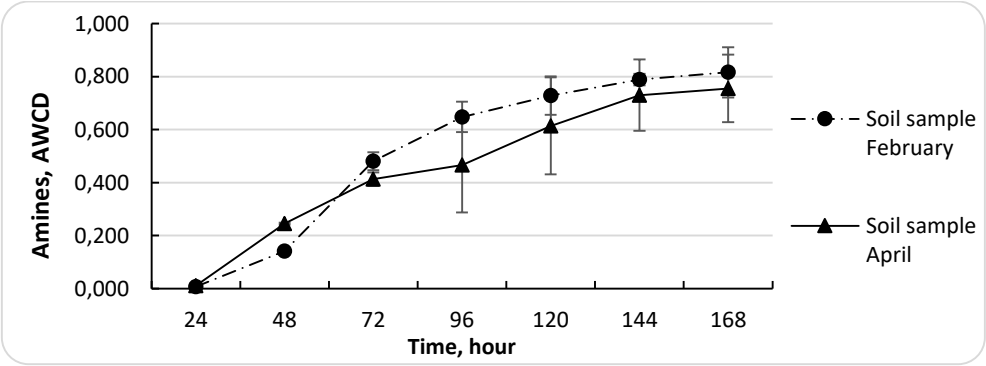

Fig. 5. Utilization of amines by microbial communities in soil samples collected in February and April using the Biolog EcoPlate method

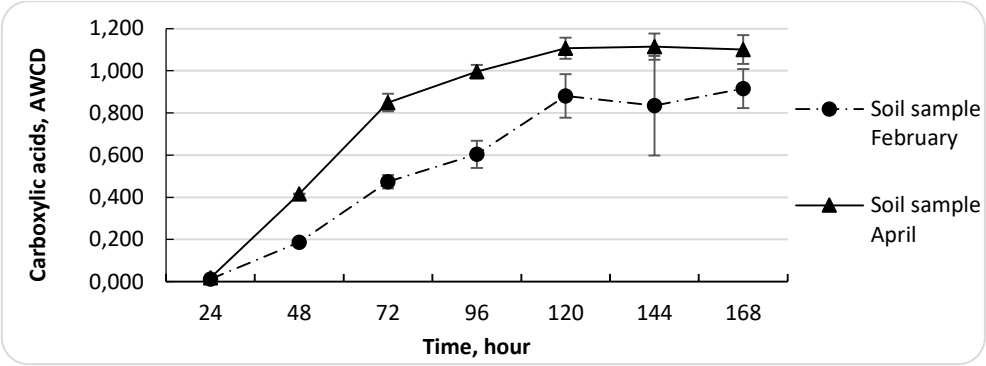

Fig. 6. Utilization of carboxylic acids by microbial communities in soil samples collected in February and April using the Biolog EcoPlate method

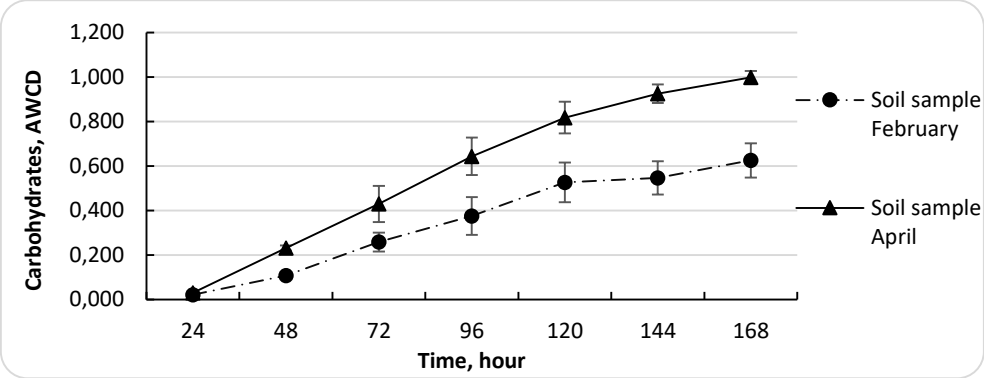

Fig. 7. Utilization of carbohydrates by microbial communities in soil samples collected in February and April using the Biolog EcoPlate method

The bacterial community in the sample collected in April utilized the polymers present in the EcoPlate more actively (the AUC = 88.12) compared to the sample collected in February (the AUC = 71.70). Nonetheless, the increase of the measured OD was proportional and consistent for both samples through the incubation period (Fig. 8). At the end of the incubation period, the average OD for polymer utilization reached 0.894 ± 0.133 and 1.099 ±0.064 for samples collected in February and April, respectively. The average OD calculation for the interval between 96 and 168 hours indicated that microbial communities in the sample collected in February utilized the substrates Tween 40 and Tween 80 more effectively by 26% and 42%, respectively. In contrast, the utilization of glycogen by microbial communities in the sample collected in April was three-fold higher than in the February sample. The most significant difference between the two samples was the utilization of α-cyclodextrin, which was virtually non-metabolized by the microbial community collected in February.

The most profound difference between the metabolic abilities of samples collected in February and April appeared in the metabolization of phenolic compounds. This difference was clearly indicated by the values of the Area under the Curve. For the sample taken in April, the AUC was 94.11, while for the February sample, it was only 23.33 (Table 3). The sample collected in February revealed a prolonged lag phase and low activity up to the 120th hour of incubation. After that, the curve of metabolic activity was smooth and showed a moderate increase, with a final value of 0.395 ± 0.128 (Fig. 9). The curve of metabolic activity for the community in the sample collected in April was spike-like up to the 72nd hour, followed by a gradual increase up to the 120th hour, and then plateaued at the last two measurements, with a final mean value of 0.885 ± 0.005. The difference between the samples was attributed entirely to the significantly higher utilization of 4-hydroxybenzoic acid in the sample collected in April

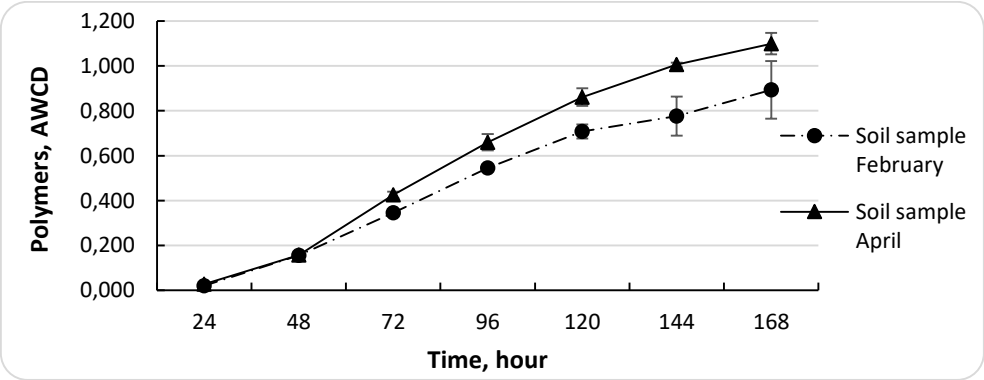

Fig. 8. Utilization of polymers by microbial communities in soil samples collected in February and April using the Biolog EcoPlate method

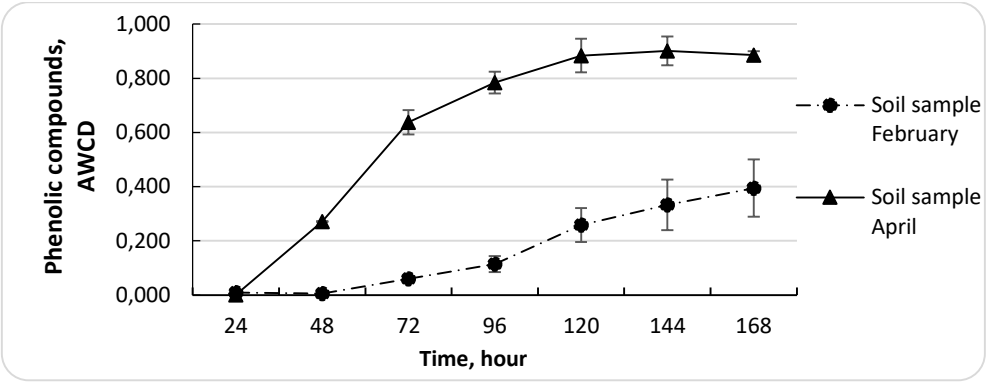

Fig. 9. Utilization of phenolic compounds by microbial communities in soil samples collected in February and April using the Biolog EcoPlate method

The calculation of functional indexes, which represent the structure of microbial communities, was based on the estimated OD values in the wells of the EcoPlate, as provided by the Biolog MicroStation™ Reader and proposed by Zak et al. (1994). The estimated functional indexes (Table 4), except for the McIntosh index, clearly indicate higher diversity and evenness for microbial communities in the sample collected in April. Furthermore, the difference in the functional indexes, except for the Pielou index, between samples collected in February and April was statistically proven.

Table 4. Functional indexes of the soil microbial communities structure based on average values of OD at the 120th hour of EcoPlate incubation

Samples	**Functional indexes**						
	Shannon-Wiener index, H'	*Pielou index, E*	*Simpson index, D*	*Margalef index, d*	*McIntosh index, U*	*McIntosh evenness, McI*	*Gini coefficient, G*
Soil sample February	2.769 ± 0.075	0.965 ±0.014	0.932 ± 0.007	4.854 ± 0.445	0.260 ± 0.012	1.204 ± 0.001	0.446 ± 0.029
Soil sample April	3.218 ± 0.009	0.973 ± 0.007	0.957 ± 0.001	7.668 ± 0.169	0.206 ± 0.001	1.210 ± 0.001	0.227 ± 0.016
p value	0.000	0.449	0.003	0.001	0.002	0.002	0.001

Legend: the data are presented as mean ± standard deviation

The generated heatmap displayed a detailed metabolic profile based on the averaged OD values measured at the 120th hour of EcoPlate incubation. However, the applied color scheme, with its precision up to three decimal places, highlighted even the tiniest differences between samples. While this approach leverages the Biolog EcoPlate data effectively, it can exaggerate minor numerical differences and obscure some important similarities between samples. The samples collected in February and April shared 58% similarity in substrate utilization, despite the range of measured values not being particularly wide. Both samples showed high and moderate metabolic activity towards utilization of L-arginine, L-asparagine, L-serine, pyruvic acid methyl ester, D-mannitol, glucose-1-phosphate, D-galactonic acid γ-lactone, D-glucosaminic acid, N-acetyl-D-glucosamine, Tween 40, and glycogen. Similarly, both samples showed low (β methyl-D-glucoside, D-malic acid), very low (D, L-α-glycerol phosphate, L-phenylalanine), and virtually no utilization of D-xylose and 2-hydroxy benzoic acid. The major difference between samples lay in the utilization of several substrates. In the sample collected in February, substrates such as glycyl-L-glutamic acid, putrescine, γ-amino butyric acid, and Tween 80 were utilized almost two-fold more effectively than in the sample collected in April. Conversely, the sample collected in April showed activity towards substrates such as itaconic acid, D-galacturonic acid, D-cellobiose, phenylethylamine, L-threonine, 4-hydroxybenzoic acid, α-keto-butyric acid, α-D-lactose, and α-cyclodextrin, which had low utilization in the sample collected in February, and in the case of the last three substrates, even non-existent.

The estimated substrate richness (SR) showed a higher number of positive wells (OD>0.15) for sample taken in April compared to sample taken in February. The t-test indicated statistical difference between samples in terms of total SR and utilization of carboxylic acids, carbohydrates and polymers.

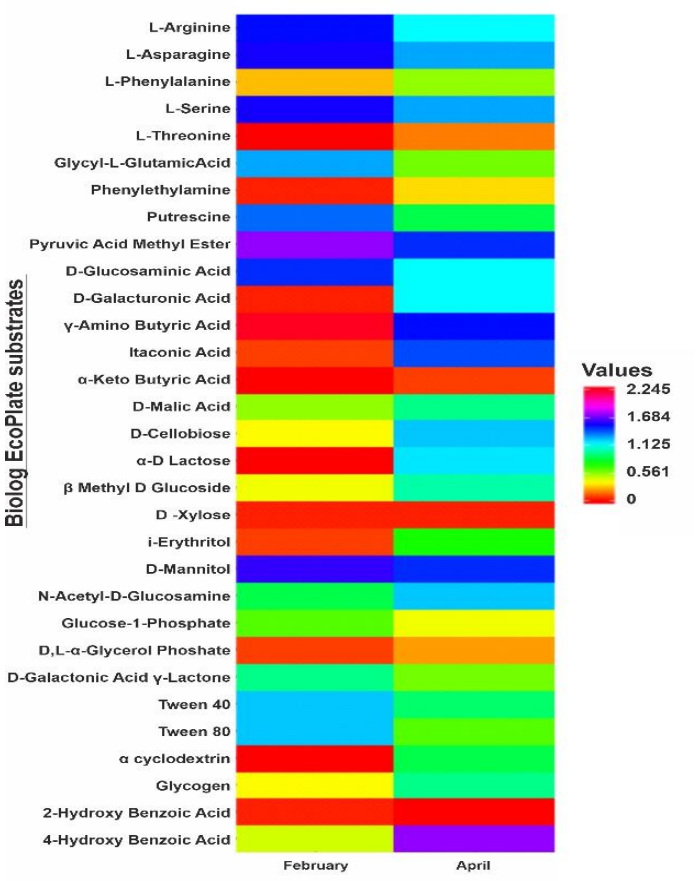

Fig. 10. Heatmap of substrates utilization of soil bacterial communities in samples collected in February and April and based on the average values of OD at the 120th hour of EcoPlate incubation

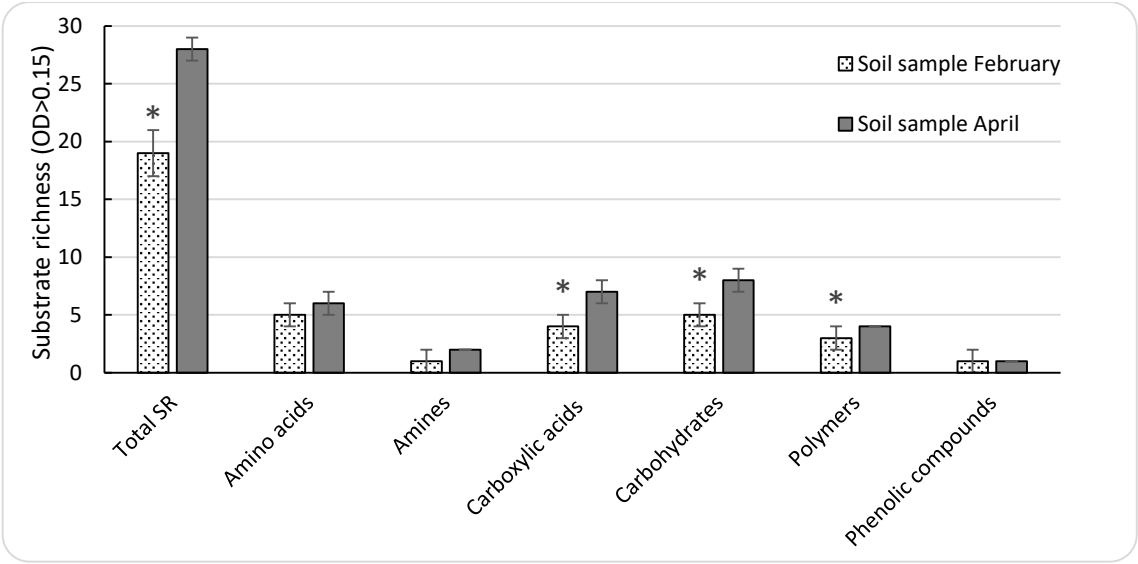

Fig. 11. Substrate richness indicated by the number of carbon sources (OD > 0.15) utilized by microbial communities in the soil samples collected in February and April (statistical difference marked with an asterisk).

Discussion

The Biolog EcoPlate method offers several advantages that enhance the understanding of microbial dynamics in various ecosystems. The method allows researchers to evaluate the physiological profiles of microbial communities, revealing how they respond to changing environmental conditions and facilitates the identification of specific metabolic pathways that are active during different seasons. This reveals how microbial communities adapt to seasonal changes, resource availability, and environmental stressors.

Rodrigues-Oliveira et al. (2014) demonstrated that the seasonal changes in microbial communities inhabiting lake sediment were associated with variations in nutrient content and such changes were effectively monitored using the Biolog EcoPlate. Palladino et al. (2021) noted that during summer, the microbial community associated with the jewel anemone shifted towards a more heterotrophic fermentative pathway, indicating changes in the substrate utilization patterns. Wang et al. (2020) found that microbial community composition changed more significantly between seasons than within them, underscoring the importance of using methods like Biolog and the options it provides to capture these dynamics. The AWCD curve is used for the general characterization of the metabolic activity of the studied microbial communities. Ge et al. (2018) who worked with microbial communities of stored rice and Sun et al. (2012) who used the sludge-amended soil observed a lag phase of 24 and 60 hours, respectively. Usually, AWCD curve has a typical sigmoid shape characterized by a gradual increase in OD and flat graph segments in the late hours of incubation (Stefanowicz, 2006; Lima et al., 2015). In the current study, the AWCD of samples collected in February and April showed a significant difference in the trend of OD increase with the obvious microbial activity in the sample collected in the warmer and rainy period of the year (Fig. 1 and Fig. 2). Estimating the effect of temperature and water-holding capacity, Klimek (2013) reported values for the Area Under the Curve in the range of 21.43 to 120.33. In contrast to the current study, none of the samples reached an activity higher than 104.09, with the lowest value being 23.33. Zogg et al. (1997) discussed that soil warming leads to an increase in the metabolic activity of microbes due to higher growth efficiency and facilitated diffusional processes. The authors also considered that temperature-induced changes in diffusional processes would only increase the rate at which substrates are utilized. However, this does not necessarily affect the quantity of substrates utilized, as their exact necessity is controlled by microbial metabolism. However, estimating the number of positive wells in the EcoPlate is a good indicator of utilized substrates. The results from the current study are similar to those of Garau et al. (2007), who reported numbers below 5 and over 20 for utilized substrates in microbial communities extracted from two different soils. Feigl et al. (2017) reported a broader range of SR, from 0 to 15 positive wells. Assessing the effect of environmental factors on growth efficiencies or substrate utilization individually for all participants in the microbial community is a difficult, if not impossible task. Therefore, utilizing as many approaches as possible seems more reasonable.

The results from the current study, in terms of substrates utilization and particularly to amino acid utilization do not comply with the findings of Gomez et al. (2000), who analyzed four samples collected from locations differing in several traits such as vegetation and management system, but did not find any difference.

Gomez et al. (2000) considered that utilization of amines in the Biolog EcoPlate is relatively low. However, the mean values for amines utilization in the current study (168th hour) reached – 0.816 and 0.755 for samples collected in February and April, respectively, which comply with the study of Ge et al. (2018) who reported an average OD of 0.600 – 0.800 for amines utilization at the end of the incubation period. Biogenic amines such as phenylethylamine and putrescine are important compounds for bacterial physiology and ecology. Putrescine has been associated with the formation of biofilms (Patel et al., 2006) and the ability of bacteria to respond to putrescine produced by other species was related to intercellular signaling and microbial interactions (Armbruster & Mobley, 2012). Phenylethylamine was reported to be utilized by some strains *Escherichia coli* as a sole source of carbon and energy (Parrot et al., 1987).

Carboxylic acids serve as key substrates for energy generation and cellular building blocks in bacterial metabolism (Obernosterer et al., 1999). Haiming et al. (2020) considered carboxylic acids as the most important types of exogenous carbon

source, followed by amino acid and carbohydrates, and the complex compounds with the least contribution to the metabolism of microbial communities. According to Resmer & White (2011), the ability of bacteria to metabolize carboxylic acids contributes to the cycling of organic matter in soil ecosystems, influencing nutrient availability and microbial community dynamics. This observation complies with the higher metabolic activity estimated for the April sample in the current study. In this aspect, Sierra et al. (2015) pointed out that the temperatures not only affect the kinetics of soil microorganisms but also impact water availability which is necessary for sufficient substrate dissolution. Salazar et al. (2016) conducted a study on soil respiration under different temperatures and moisture levels, finding that microbial specific growth rates were influenced. This indicates that the availability of substrates for microorganisms was linked to the variations in temperature and moisture. Similarly, Zhang et al. (2022) demonstrated that changes in moisture and temperature conditions affected organic substrates available to soil microorganisms, and these changes were further related to changes in the growth of aboveground plants.

The relatively low temperatures recorded at the sampling date in February and the higher utilization of nonionic surfactants such as Tween 40 are in good compliance with the results of Pessi et al. (2012). The researchers studied soil microbial communities collected from a glacier in the South Pole and hypothesized that Tween 40 and Tween 80 provide adaptation to low temperatures. Furthermore, they considered that the accumulation of these nonionic surfactants could play a particular role in cell protection in recurrent freeze-and-thaw events.

Among the polymers present in the EcoPlate, α-cyclodextrin is often considered the least utilized polymeric substrate. However, some hyperthermophilic archaea, such as *Thermococcus* sp., *Pyrococcus furiosus*, and *Archaeoglobus fulgidus*, as well as mesophilic bacteria like *Klebsiella oxytoca* and *Bacillus subtilis*, have been reported to utilize α-cyclodextrin (Centeno-Leija et al., 2022; Kamionka & Dahl, 2001). This relatively rare biochemical pathway implies that α-cyclodextrin could be utilized by only a few species in the soil samples. In contrast, Oros et al. (1990) found that a high percentage of cyclodextrins (α-cyclodextrin and β-cyclodextrin) were utilized by the studied bacterial strains. Similarly, the high utilization of α-cyclodextrin observed in the samples collected in April in the current study aligns with the results reported by Sala et al. (2005).

Campbell et al. (1997), among other researchers, significantly contributed to the development of the contemporary Biolog EcoPlate design. They observed that long-chain aliphatic acids and phenolic acids were the most slowly utilized substrates, and even prolonged incubation did not result in higher AWCD values. However, these substrates have important and versatile functions in bacterial metabolism and ecology. In terms of utilization and biological role, the phenolic compounds included in the EcoPlate are likely the most multifaceted. Aerobic and anaerobic degradation of these substrates were associated with denitrifying bacteria (Altenschmidt et al., 1993; Bonting & Fuchs, 1996). On the other hand, 4-hydroxybenzoic acid, can act as a signaling molecule in bacteria and affects biofilm formation and strains virulence potential (Wang et al., 2023). Microbial communities inhabiting Arctic seawater utilized these phenolic substrates extensively (Sala et al., 2005). Similarly, Pessi et al. (2012) found that 2-hydroxybenzoic acid and D-xylose, compounds expelled by the plant roots into the soil, were extensively metabolized. However, the current study revealed an opposite trend. The utilization of 2-hydroxybenzoic acid was insignificant and its contribution to the AWCD was negligible in both samples. The average metabolization of phenolic compounds was primarily based on data for 4-hydroxybenzoic acid utilization. In the samples collected in April, the OD values, measured in the interval between 48 and 168 hours of EcoPlate incubation, exceeded from twofold to manifold those of the samples collected in February.

Functional indexes are used to characterize the distribution, diversity, and evenness of participating species within a particular community. In the case of the Biolog EcoPlate method, substrate utilization indicates specific metabolic pathways that can be assigned to different species and are employed to assess community structure. The t-test between samples collected in February and April showed that the difference was statistically significant for most of the calculated functional indexes. The values of 2.97–3.24 and 0.94–0.96 for

Shannon-Wiener and Simpson's indexes, respectively, were considered indicative of high species diversity (Janniche et al., 2012). According to Jurkšienė et al. (2020), the Shannon-Wiener index usually ranges between 0 and 5 but rarely exceeds 4.5. In most cases, values above 3.0 define the community as well-structured and functioning, while values below 1.0 indicate perturbation and impairment of the community. However, in the current study, despite the statistically significant lower value of the sample collected in February compared to April, the Shannon-Wiener index of 2.769 ± 0.075 did not indicate a disbalance in the microbial community. It is more likely that different species participate or are active in the community. Overall, the microbial community is vigorous, functioning, and adapted to the environmental conditions typical for the season.

In their study, Huang et al. (2012) reported Shannon-Wiener, McIntosh, and Simpson indexes similar to those calculated in the current study. In contrast, Zhang et al. (2013) outlined a wider range for both Simpson and Shannon-Wiener indexes: 0.650-0.873 and 0.076-2.343, respectively. In the current study, the Margalef index was used to compare collected samples, following the approach of Türkmen & Kazanci (2010). The index calculation was based on the number of positive wells in the EcoPlate, with higher values indicating greater biodiversity. The sample collected in April exhibited higher biodiversity, with a Margalef index of 7.668 ± 0.169, nearly double that of the sample collected in February (4.854 ± 0.445).

Unlike other indexes where higher values indicate greater community diversity and evenness, the interpretation of the Gini coefficient is the opposite. Lower values of the Gini coefficient indicate higher microbial diversity, as explained by Harch et al. (1997). The Gini coefficient is used in biology and anthropology (Ceriani & Verme, 2011) and is a useful indicator for estimating the detrimental and beneficial effects on microorganisms' growth and activity (Wittebolle et al., 2009). Some researchers have highlighted the sensitivity and accuracy of the Gini coefficient compared to other indexes (Beaugrand & Edwards, 2001). In this study, the almost two-fold lower value of the Gini coefficient for sample collected in April (0.227 ± 0.016) indicated higher diversity compared to the community characteristics of sample collected in February (0.446 ± 0.029).

The utilization of substrates by microbial communities creates their specific metabolic profiles and allows for comparison between them. This characteristic is known as community-level physiological profiling (Classen et al., 2003). The approach of characterizing samples by substrate utilization in the Biolog EcoPlate, as presented through the heatmap in the current study, was also employed by Sala et al. (2005) and Janniche et al. (2012). In contrast to the current study, Sala et al. (2005) observed utilization of 2-hydroxy benzoic acid but not of D-galactonic acid γ-lactone, i-erythritol, L-arginine, L-asparagine, and D-malic acid, which showed low or moderate utilization in the samples collected in February and April. Pessi et al. (2012) and Sala et al. (2006) also reported specific metabolic patterns for substrate utilization in the analyzed samples. Chen et al. (2020) found that gram-negative bacteria grew well with sufficient soil moisture, while gram-positive bacteria and fungi thrived at lower moisture levels. However, Classen et al. (2003) pointed out that substrate utilization in the EcoPlate may not fully align with community metabolic activity in their natural environment. Zogg et al. (1997) suggested that soil warming could increase microbial metabolic activity, but establishing a clear connection between microorganism growth efficiency and the metabolic fingerprint of individual soil microorganisms within a specific community is quite challenging. Despite the Biolog EcoPlate method not accounting for substrate preference among individual members of a microbial community, it provides important insights into the community's metabolic activity towards specific substrates.

Conclusions

The seasonal changes in the metabolic activity of soil microbial communities affect organic matter decomposition, nutrient availability, and biochemical cycles in all ecosystems. These shifts in microbial activity are sensitive to temperature variations and irregular precipitation. Research in this area aims to increase knowledge of microbial soil processes and their potential impacts on ecosystem functions and services. This study examines soil microbial activity and community structure at the transition period between two seasons, utilizing the Biolog EcoPlate method. The results revealed specific metabolic activities of

microbial communities in both samples, with elevated activity in the April sample, characterizing the period with higher average temperatures and more regular precipitation. In general, the functional indexes which characterized the microbial communities' structure indicated higher diversity and evenness for samples collected in more favorable environmental conditions. However, despite the lower values in the sample collected in February, the functional indexes did not imply impairment or disturbance of the community. The most likely explanation is that temperature and precipitation affect the development of different microbial species, but the relative balance between species is still preserved. This corresponds to the difference in substrates utilized by communities in the samples; increased temperature and substrates solubilization would favor the growth of different bacterial species or the shifts towards other metabolic pathways. These changes would affect the range and pace of organic matter utilization in the soil and, as a result, its properties, fertility and health.

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