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Soil Properties and Forest Decline in the North-Western Part of Romania

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Abstract: The paper presents the study of the soil quality and health expressed by the chemical and biological properties in a research field placed at Varciorog, Bihor County, Romania. The soil samples were collected from 3 soil variants in March 2023. In each soil variant, some soil chemical parameters and the abundance of bacteria were determined. The frequency and intensity of colonization, along with arbuscules and vesicles, were scored to determine the mycorrhizal potential of each soil. The community-level physiological profile was used to determine the functional microbiome and its ability to decompose a specific set of substrates. In the control variant (CTRL), which is a functional forest cultivated with beech in a proportion of 90%, the soil properties were compared with those determined from Site 1 (a declined mixed forest) and from Site 2 (chestnut forest in a stage of complete drying). The data were statistically processed with a one-way ANOVA test, followed by the Duncan post-hoc test, which revealed significant variation in the potential of microbial functional communities across the analyzed sites. Also, the soil parameters that significantly varied in the 3 soil variants were bacterial number, pH, humus, exchangeable aluminum, coarse sand, dust, and fine sand. The Pearson correlation was computed to study the links between bacterial numbers and chemical parameters. The results showed strong correlations between most of the studied soil properties. The Ecoplates approach to soil functional microbiome highlighted various differences between the microbial communities of the three tested sites. Mycorrhizal colonization shows different potentials for symbiosis formation. The peak of mycorrhizal colonization was in declined forest, with 43.36% colonization frequency and 24.56% intensity. Arbuscules reached 11.36% in declined forest, while in control and decayed sites, the indicator was under 4%. Vesicles are more associated with control and decayed forests, with values of presence over 1.30%. As an indicator of microbial general activity, the sum of recorded activities was higher in declined and decayed forests. At these sites, the activity of the functional microbiome was amplified. The decline process activates a higher diversity of functional groups and is associated with a larger area of substrate decomposition capacity, which indicates a more extensive range of microbial functions related to breaking down organic matter.

Keywords: microbial abundance; arbuscular mycorrhizal potential; chemical soil properties; pH; nutrient content; humus; texture; soil quality; forest decline; community level physiological profile

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1. Introduction

Mountain ecosystems provide unique opportunities for studying microbial communities due to the wide range of climate and soil variables that change over short distances. This environmental heterogeneity allows for the examination of how different factors influence the diversity and structure of microbial communities. Soil pH is recognized as one of the key variables that directly impacts soil microorganisms. This pH variation

influences the diversity and distribution of microbial communities, as certain taxa may be favored or inhibited by specific pH conditions. In addition to soil pH, other factors such as soil characteristics (e.g., texture, organic matter content), soil quality, seasonality, climate, and elevation also contribute to the variation in soil microbial communities in mountain ecosystems [1]. These factors interact in complex ways, creating a dynamic environment that influences the recruitment and composition of root endophytes, the microorganisms that live within plant roots [2]. Studying microbial communities in mountain ecosystems provides valuable insights into the relationships between environmental variables and microbial diversity. Here are some key reasons why these studies are beneficial. This knowledge is crucial for understanding ecosystem functioning, nutrient cycling, and plant–microbe interactions in these unique and environmentally sensitive habitats [3–7]. On a larger geographical scale, several factors, including soil characteristics, soil quality, season, climate, and elevation, can collectively impact the soil microbial community composition. For instance, soil composition and nutrient availability vary with location, and these factors can shape the types of microorganisms present in the soil.

Another factor that impacts soil quality and health is soil erosion, which can have detrimental effects on forest productivity due to the loss of soil water and nutrients [8,9].

Erosion can lead to the removal of the topsoil, which is the most fertile and moisture-retaining layer of the soil. This topsoil is rich in organic matter and has a high water-holding capacity. When erosion occurs, the loss of topsoil reduces the soil's ability to retain water. As a result, the available soil water for forest growth decreases, which can negatively impact the health and productivity of trees and other vegetation. Insufficient soil moisture can lead to water stress, reduced growth rates, and even mortality in severe cases [10,11]. Eroded sediment often carries away valuable nutrients that are essential for plant growth. The topsoil contains a significant portion of the nutrients necessary for supporting the forest ecosystem. When erosion removes the topsoil, these nutrients are lost from the site. Nutrients such as nitrogen, phosphorus, potassium, and various micronutrients are crucial for plant growth and productivity. The loss of these nutrients can result in nutrient deficiencies and hinder the forest's ability to thrive and regenerate. Over time, if nutrient losses are not replenished, soil fertility decreases, further impacting forest productivity [12,13]. Harvesting activities reduce surface cover and compact the soil, leading to increased runoff and erosion. The effects of soil erosion go beyond the loss of fertile land. Erosion generally decreases the productivity of forests by decreasing the available soil water for forest growth and through the loss of nutrients in eroded sediment. These processes lead further to forest degradation, which can impair the ability of the entire landscape to adapt to environmental change. Forests play a crucial role in maintaining ecosystem functions and services, and when they become degraded, it can have far-reaching consequences for nutrient cycles and ecosystem dynamics beyond the forest ecosystem itself [14].

Forest degradation disrupts the natural nutrient cycles that operate within forest ecosystems. This disruption can lead to imbalances in nutrient availability, impacting the growth and health of plants, including those in neighboring ecosystems. Nutrients that would typically be retained within the forest through cycling processes may be lost due to degradation, leading to reduced nutrient availability in downstream or adjacent ecosystems [15]. When forest soils are degraded, the ability to restore the original plant community and recover ecosystem functions is compromised. Disturbed nutrient cycles and decreased soil fertility can hinder the establishment and growth of desired plant species during restoration efforts. This can lead to difficulties in restoring the ecological integrity of the degraded forest and the associated landscape.

Elevation gradients in mountain ecosystems provide a unique opportunity to study how plants recruit microbial symbionts in response to changing environmental conditions. As elevation increases, various biotic and abiotic factors change rapidly, including temperature, precipitation, oxygen levels, and vegetation types. These changes affect the soil properties and consequently influence the microbial community. Plants rely on beneficial

microbial symbionts, such as root endophytes, to enhance their nutrient uptake, disease resistance, and overall fitness. As environmental conditions shift along elevation gradients, plant hosts may need to adapt and recruit different microbial symbionts to maintain their symbiotic relationships. The availability and composition of microbial symbionts may vary with elevation due to the altered soil characteristics and associated microclimatic conditions [8].

The main goal of the study was to evaluate the stress factors that cause the premature and progressive loss of trees and to study the chemical and biological parameters of the soil in order to characterize the soil and their influence on the state of health of the forest. This study emphasizes the intricate connections between microbial functional communities, substrate utilization, and vegetation in functional and declined forest sites. Changes in soil conditions due to forest decline can impact the function of microbial communities. The results present the link between soil functional microbial communities and the forest state. Functional guilds are used to determine a larger specific reaction of microbial communities due to the presence of a decline in a forest site. The more specific reaction, as functional groups, is used to determine the indicator potential of these microbiomes for a specific forest state. Mycorrhizal parameters are good indicators of soil symbiotic potential and provide useful information regarding the fungal inoculums and their colonization performance. The intricate interplay between soil chemical parameters, forest decline, and substrate utilization by functional microbial communities provides a better understanding of ecosystem health.

2. Materials and Methods

2.1. Site Description and Sample Processing

The research took place in Vârciorog, an old Romanian settlement that is located in the center of Bihor County. In the north-west of the Pădurea Craiului Mountains, there is a small depression in their contact with the Şerghișului and Tașnadului Hills, called the Vârciorog Depression.

The Vârciorog commune, with its three component villages, Vârciorog, Fâșca, and Şerghiş, covers an area of 8081 ha of land, of which 4300 ha are forests and pastures, and has 2430 inhabitants. It consists of a series of grey clays and silts that belong to the Sarmatian Corniţel Formation [16–18]. It is located on the western slopes of the Apuseni Mountains in the Borod Basin, close to the city of Oradea/Nagyvárad on the eastern margin of the Pannonian Basin. The invertebrate fauna of the section was intensively studied by Filipescu et al. [18]. They conclude that the microfossil assemblages from Vârciorog are referenceable to the early Sarmatian Elphidium reginum zone. But the thickness of this section they reported is by far smaller than the one measured in the field. They recognize a shallow marine palaeoenvironment with continental influence and repeated sea level changes, as well as a progradation trend, suggesting an uplift in the source area. The fish fauna (based on otoliths) of the section and some other nearby exposures were studied by Reichenbacher et al. [19]. They postulated a heterogenous nearshore environment along a rocky coast. The co-occurrence of brackish water taxa and shallow marine elements indicates a brackish to marginal marine habitat with freshwater influx from rivers draining the adjacent rising Apuseni Mountains Hír, János, and Vlad, Codrea, and Prieto, Jerome [20]. Two new early Sarmatian s. str. (latest middle Miocene) rodent faunas from the Carpathian Basin. Palaeobiodiversity and palaeoenvironments [21,22].

Climatically, the area is subject to the influence of western air masses, with corresponding consequences in temperature, precipitation, vegetation, soils, degree of humanization, etc. The average annual temperature is 6–8 °C in the mountainous area, rising to 8–9 °C in the depressed area. Average precipitation is 900 mm/year. Periods of high humidity, such as generally the end of May and the first half of June, alternate with periods of very poor rainfall. The stratification of the vegetation is not very evident; the extensive deciduous forests, in which there are rare clumps of spruces, are interrupted by wide meadows, installed especially on the karst plateaus. In the deciduous forests, the beech is dominant; in

small clumps, the gorun and the wild chestnut appear. The subsoil resources specific to the area are white clay/kaolin at Aştileu, Vadu Crişului, and Şuncuiuş, lignite at Vadu Crişului, Şuncuiuş, bauxite at Vadu Crişului, Bratca, Şuncuiuş, Bratca dolomites, quartz sand, thermal water, sand and gravel from the river bed, and limestone for construction [23–25].

2.2. Soil Sampling

The soil samples were collected in March 2023 from 3 experimental variants (Figure 1). Site 1 is a declined mixed forest (coniferous and deciduous trees). In this area, the trees do not grow, and they dry up. The forest does not regenerate. The planted spruce is introduced outside the area. The age of the trees is 50 years.

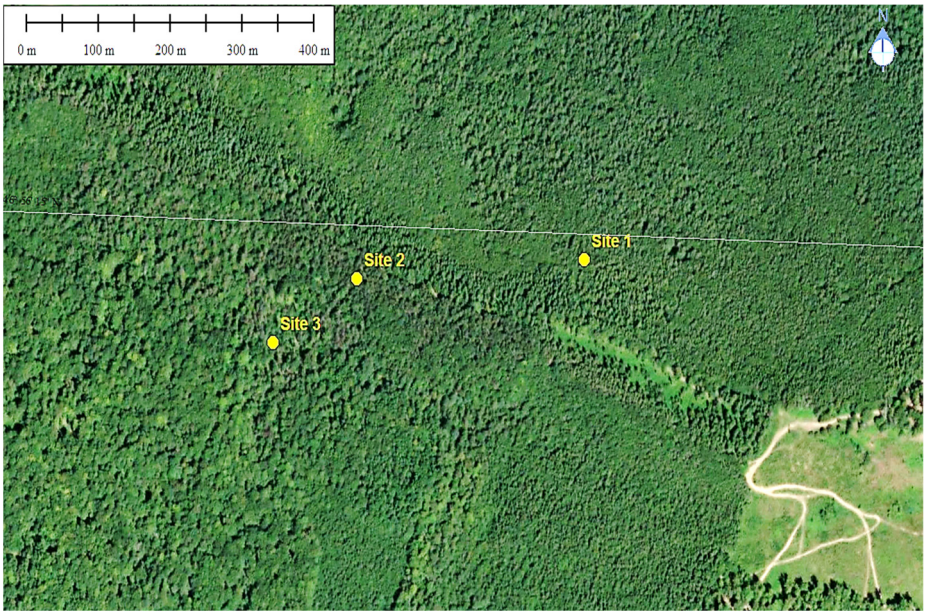

Figure 1. Location of research area (https://earth.google.com/web/, accessed on 17 May 2023).

In Site 2, the chestnut forest is in the stage of complete drying. The age is 80 years. The relief is represented by a slope with river stones. Sites 1 and 2 have been in decline for the last 15 years. The control site (CTRL), which is Site 3, is a functional forest cultivated with beech in a proportion of 90% and the rest with chestnut. It presents a rare alluvial deposition. The trees are healthy. The litter layer is uniform. The age of the tree is 80 years. We selected these sites to compare soil quality parameters (expressed by chemical and biological properties) in a functional forest (CTRL) with the parameters determined in declined forests (Site 1 and Site 2) to find out what are the stress factors that cause forest degradation and which stage of soil quality.

In the studied areas, there are no landslides, but there are bauxite mines 2 kilometers away. Soil samples were taken from 0–20 cm depth. For sampling, the square method (100 m^2 surface of each area) was used, and the depth of sample collection was 0–20 cm.

The foreign materials (plant debris, calcareous concretions, and other impurities) were removed, and the soil was sifted through a sieve with a diameter of <2 mm. The analytical sample (10 g) was extracted from the sifted soil by the method of quarters (this method of tillage was applied for each variant of soil separately, starting in the case of each variant from 10 g of soil).

2.3. Methods

2.3.1. Soil Chemical Parameters

Some chemical properties of the test soil were determined, such as pH value (pH), organic matter (humus), mobile phosphorus (mobP), mobile potassium (mobK), exchangeable aluminum (Al3), exchangeable base (SB), hydrolytic acidity (HA), cationic exchange

capacity (T), and base saturation (V). These soil chemical properties were determined by OSPA Bihor using a standard procedure (SR 7184/2001 [26]).

2.3.2. Abundance of Bacteria

The total number of aerobic mesophilic bacteria (C.F.U./g soil) in soil samples was analyzed with the plate count method [27–29] on a soil extract agar medium (pH 6.8 ± 0.2), which is suitable for the extraction of soil microorganisms. Two temperatures were set for the incubation procedure (25 °C and 37 °C), with the counting of colonies after 5 days in the case of the lowest temperature and 3 days for the highest one. Plates were inoculated with a suspension obtained through the serial dilution method (dilution factor10^7 for 37 °C and 10^6 for 25 °C) and incubated in a Pol-Eko ILW 53 incubator with a Biobase BC-50 colony counter for the assessment of visible colonies. Bacterial numbers were used as an indicator of microbial abundance due to their rapid reaction to any type of perturbation, especially in the declined forests where the organic matter cycles suffer from gaps.

2.3.3. Ecoplates (BIOLOG™) Method

For each soil sample, Ecoplates (BIOLOG™) were used to analyze the community-level physiological profile [30,31]. The method has the advantage of both the high number of substrates (31 + 1) and the continuous recording of microbial activity up to a plateau phase [32]. The use of Ecoplates was based on multiple rationales. The first is that the same sets of substrates were analyzed for each type of forest, and the results enable multiple comparisons from functional guilds to functional groups. Based on values recorded at the end of incubation, it is possible to identify the peaks (as functional groups), which are indicators of a specific state of the forest or of a change between two sites (which indicates a decline potential). In each well, a quantity of 150 µL of 10^{-4} soil suspension was inoculated and incubated in the dark at 25°C. The plate reading was performed continuously from 24 h until 120 h with a BioTek Epoch plate reader and an optical density of 590 nm. Average well color development (AWCD) and both Shannon and Simpson diversity indices were calculated [31–33] for each plate.

2.3.4. Determination of Arbuscular Mycorrhizal Potential

The arbuscular mycorrhizal potential was calculated to identify the native symbiotic potential of each soil [34–36]. *Trifolium repens* was chosen as a mycorrhizal tester for its adaptability and rapid growth in any type of soil condition. Also, it is a species that harvests mycorrhizas, produces a well-developed root system, and has a good natural presence in ecosystems. Seeds of *Trifolium repens* were placed in pots containing each type of soil and maintained for a period of 6 weeks. Prior to microscopic analysis, roots were stained through the modified ink-vinegar method [37]. The entire process was done at room temperature, with roots cleared with an NaOH 10% solution for 48 h, followed by a staining procedure for another 48 h with an ink-vinegar solution (5:5%). Stained roots were gently crushed and analyzed with an Optika C-P8 camera mounted on an Optika microscope B-388 PL at 10× and 40× magnification. Mycorrhizal parameters were represented by frequency (F%) and intensity (I%) of colonization, the percent of roots where arbuscules (A%) and vesicles (V%) are visible, the report between mycorrhized/non-mycorrhized areas, and the report between arbuscules/vesicles [38]. The frequency of colonization was assessed as root parts where mycorrhizas were present, while the intensity was used to score the fungal development in colonized areas. The presence of arbuscules indicates an increased transfer between the fungal partner and its host, while the presence of vesicles is associated with a higher nutrient reserve quantity in the mycorrhizal system that can be stored in these structures. Both F% and I% were used to calculate the colonization degree (%) to which the assessment of mycorrhizal strategy was performed [39,40].

2.4. Statistical Analysis

The data were statistically processed with a one-way ANOVA ($p = 0.05$) test, followed by the Duncan ($p = 0.05$) post-hoc test for pairwise comparisons of sample means. The results are presented as means and standard deviations. The means are accompanied by letters; different letters describe statistically significant means ($p = 0.05$). The statistical tests were done in triplicate (N = 3) for each site (CTRL, Site 1, and Site 2). The statistical analysis and graphical representations were performed with MATLAB 2023a v9.14 CWL (MathWorks, 1 Apple Hill Dr, Natick, MA, USA).

3. Results

The results of the comparison of aerobic mesophilic bacteria number and soil chemical properties between the studied areas (Table 1 revealed some significant differences. The number of aerobic mesophilic bacteria and pH values were higher at the CTRL site compared with Site 1 and Site 2. Different situations were recorded concerning the humus and exchangeable aluminum (Al_3), which registered lower values at the CTRL site in comparison with Site 1 and Site 2.

Table 1. Results of the number of aerobic mesophilic bacteria and soil chemical properties for the site factor.

Site	AMB (C.F.U./g Soil)	pH	Humus (%)	mobP (ppm)	mobK (ppm)
CTRL	40.80 a ± 4.32	4.98 a ± 0.07	3.95 b ± 0.36	43.70 a ± 12.96	90.80 a ± 6.82
Site 1	24.00 b ± 3.27	4.57 b ± 0.10	5.47 a ± 0.87	42.89 a ± 16.40	97.20 a ± 17.92
Site 2	17.83 b ± 3.45	4.58 b ± 0.10	5.35 a ± 0.57	38.02 a ± 23.77	112.80 a ± 4.16
Site	**Al_3 (me/100 g soil)**	**SB (me/100 g soil)**	**HA (me/100 g soil)**	**T (me/100 g soil)**	**V (%)**
CTRL	1.39 b ± 0.09	1.40 a ± 0.69	9.26 a ± 0.34	10.66 a ± 0.49	12.97 a ± 5.82
Site 1	2.25 a ± 0.48	1.20 a ± 0.20	11.52 a ± 1.65	12.72 a ± 1.69	9.52 a ± 1.80
Site 2	2.08 a ± 0.11	0.67 a ± 0.46	11.08 a ± 1.21	11.75 a ± 1.67	5.40 a ± 2.93

Note: The letters that accompanies the mean values are provided from ANOVA ($p = 0.05$) post-hoc Duncan test ($p = 0.05$) for pairwise comparisons of samples means. Different letters across the columns designates significant statistically significant different mean values.

Table 2 presents the results of the soil texture at the studied sites. Significant differences were registered in the coarse sand (%) values, fine sand (%), and dust (%) values. In the CTRL site, the coarse sand (%) values were lower than those in Site 1 and Site 2. Moreover, fine sand (%) values and dust (%) values were higher in the CTRL site compared with Site 1. Also, in Site 1, the fine sand (%) values were lower in comparison with Site 2, and the dust (%) values were higher in the CTRL site compared with Site 2.

Table 2. Results of the soil texture in the studied sites.

Sample	Coarse Sand (%)	Fine Sand (%)	Dust (%)	Colloidal Clay (%)	Physical Clay
CTRL	25.47 b ± 0.75	32.30 a ± 2.13	31.50 a ± 1.11	10.73 a ± 0.96	26.83 a ± 1.19
Site 1	33.60 a ± 3.70	27.30 b ± 3.41	23.60 b ± 2.01	15.50 a ± 3.42	30.07 a ± 3.82
Site 2	33.53 a ± 0.51	30.63 a ± 1.62	23.63 b ± 1.63	12.20 a ± 3.08	26.70 a ± 1.81

Note: The letters that accompanies the mean values are provided from ANOVA ($p = 0.05$) post-hoc Duncan test ($p = 0.05$) for pairwise comparisons of samples means. Different letters across the columns designates significant statistically significant different mean values.

As can be seen in Table 3, the aerobic mesophilic bacteria parameter was strongly and positively correlated with pH (0.8322) and strongly negatively correlated with humus (−0.7295) and Al_3 (−0.8018). Negative and strong correlations were found between pH and humus (−0.8629), Al_3 (−0.8879), HA (−0.8541), and T (−0.6756). Humus values were negatively and strongly correlated with the number of bacteria (−0.7295) and pH (−0.8629).

A strong positive correlation was determined between humus and Al_3 (0.7295), HA (0.8911), and T (0.7981). Strong and negative correlations were determined between Al_3 and bacteria (−0.8018) and between Al_3 and pH (−0.8879), respectively. Positive and strong correlations were found between Al_3 and humus (0.7295), HA (0.8386), and T (0.7243). Also, between SB and T, there was a strong positive correlation. HA was strongly negatively correlated with pH (−0.8541) and positively correlated with humus (0.8911), Al_3 (0.8386), and T (0.9343). T was averagely negatively correlated with pH (−0.6756) and strongly positively correlated with humus (0.7981), Al_3 (0.7243), and HA (0.9343).

Table 3. Correlations between bacterial and chemical parameters for all sample groups.

All Sample Groups										
Variables	**AMB**	**pH**	**Humus**	**mobP**	**mobK**	**Al_3**	**SB**	**HA**	**T**	**V**
AMB	**1**	**0.8322**	**−0.7295**	0.3129	−0.4746	**−0.8018**	0.4702	−0.6148	−0.4305	0.6031
pH	**0.8322**	**1**	**−0.8629**	−0.0917	−0.3210	**−0.8879**	0.4367	**−0.8541**	**−0.6756**	0.6221
Humus	**−0.7295**	**−0.8629**	**1**	−0.1220	0.0982	**0.7295**	−0.1961	**0.8911**	**0.7981**	−0.4166
mobP	0.3129	−0.0917	−0.1220	**1**	−0.0078	−0.0548	0.0784	0.0669	0.0933	0.0824
mobK	−0.4746	−0.3210	0.0982	−0.0078	**1**	0.0425	−0.5141	−0.0927	−0.2748	−0.4692
Al_3	**−0.8018**	**−0.8879**	**0.7295**	−0.0548	0.0425	**1**	−0.2591	**0.8386**	**0.7243**	−0.4475
SB	0.4702	0.4367	−0.1961	0.0784	−0.5141	−0.2591	**1**	−0.1122	0.2495	**0.9679**
HA	−0.6148	**−0.8541**	**0.8911**	0.0669	−0.0927	**0.8386**	−0.1122	**1**	**0.9343**	−0.3562
T	−0.4305	**−0.6756**	**0.7981**	0.0933	−0.2748	**0.7243**	0.2495	**0.9343**	**1**	0.0001
V	0.6031	0.6221	−0.4166	0.0824	−0.4692	−0.4475	**0.9679**	−0.3562	0.0001	**1**

Note: values in bold are different from 0 with a significance level $p = 0.05$.

The results of the correlations between bacterial and chemical parameters for the CTRL site, presented in Table 4, revealed a perfect correlation between the number of AMB and HA values (correlation coefficient = 1). The same situation was found between Al_3 and T. Ph values were strongly negatively correlated with mobP (−0.9974) and strongly positively correlated with SB (0.9976) and V (0.9994). Strong positive correlations were also determined between V and pH (0.9994) and between V and SB (0.9994).

Table 4. Correlations between bacterial and chemical parameters for the CTRL site.

Group Sample—CTRL										
Variables	**AMB**	**pH**	**Humus**	**mobP**	**mobK**	**Al_3**	**SB**	**HA**	**T**	**V**
AMB	**1**	−0.8046	−0.3007	0.8455	0.8201	−0.3952	−0.7616	**1.0000**	−0.3904	−0.7834
pH	−0.8046	**1**	−0.3244	**−0.9974**	−0.3201	0.8635	**0.9976**	−0.7994	0.8608	**0.9994**
Humus	−0.3007	−0.3244	1	0.2550	−0.7923	−0.7572	−0.3890	−0.3090	−0.7606	−0.3571
mobP	0.8455	**−0.9974**	0.2550	**1**	0.3879	−0.8246	−0.9900	0.8408	−0.8217	−0.9942
mobK	0.8201	−0.3201	−0.7923	0.3879	**1**	0.2015	−0.2538	0.8251	0.2066	−0.2869
Al_3	−0.3952	0.8635	−0.7572	−0.8246	0.2015	**1**	0.8963	−0.3871	**1.0000**	0.8805
SB	−0.7616	**0.9976**	−0.3890	−0.9900	−0.2538	0.8963	**1**	−0.7559	0.8939	**0.9994**
HA	**1.0000**	−0.7994	−0.3090	0.8408	0.8251	−0.3871	−0.7559	**1**	−0.3823	−0.7780
T	−0.3904	0.8608	−0.7606	−0.8217	0.2066	**1.0000**	0.8939	−0.3823	**1**	0.8780
V	−0.7834	**0.9994**	−0.3571	−0.9942	−0.2869	0.8805	**0.9994**	−0.7780	0.8780	**1**

Note: values in bold are different from 0 with a significance level $p = 0.05$.

The results presented in Table 5 highlight that in Site 1, there were no correlations between the studied parameters besides the strong positive correlation between AMB and mobK (0.9995).

In Site 2 (Table 6), a negative perfect correlation was found between mobK and SB (−1). Strong and negative correlations were also determined between mobK and T (−0.9978) and K and V (−0.9999). SB was positively and strongly correlated with T (0.9978) and V (0.9999). HA was also strong and positively correlated with T (0.9997). Moreover, positive and strong correlations were found between T and SB (0.9978) and HA (0.9997), respectively.

Table 5. Correlations between bacterial and chemical parameters for Site 1.

Group Sample—Site 1										
Variables	**AMB**	**pH**	**Humus**	**mobP**	**mobK**	**Al_3**	**SB**	**HA**	**T**	**V**
AMB	**1**	0.9574	−0.6345	0.5069	**0.9995**	−0.9133	0.1988	−0.9458	−0.9011	0.8653
pH	0.9574	**1**	−0.3841	0.2363	0.9476	−0.9920	0.4735	−0.8116	−0.7374	0.9732
Humus	−0.6345	−0.3841	**1**	−0.9879	−0.6588	0.2647	0.6314	0.8511	0.9069	−0.1615
mobP	0.5069	0.2363	−0.9879	**1**	0.5342	−0.1120	−0.7440	−0.7594	−0.8305	0.0065
mobK	**0.9995**	0.9476	−0.6588	0.5342	**1**	−0.8999	0.1674	−0.9557	−0.9145	0.8488
Al_3	−0.9133	−0.9920	0.2647	−0.1120	−0.8999	**1**	−0.5807	0.7316	0.6465	−0.9944
SB	0.1988	0.4735	0.6314	−0.7440	0.1674	−0.5807	**1**	0.1302	0.2458	0.6633
HA	−0.9458	−0.8116	0.8511	−0.7594	−0.9557	0.7316	0.1302	**1**	0.9931	−0.6556
T	−0.9011	−0.7374	0.9069	−0.8305	−0.9145	0.6465	0.2458	0.9931	**1**	−0.5623
V	0.8653	0.9732	−0.1615	0.0065	0.8488	−0.9944	0.6633	−0.6556	−0.5623	**1**

Note: values in bold are different from 0 with a significance level $p = 0.05$.

Table 6. Correlations between bacterial and chemical parameters for Site 2.

Group Sample—Site 2										
Variables	**AMB**	**pH**	**Humus**	**mobP**	**mobK**	**Al_3**	**SB**	**HA**	**T**	**V**
AMB	1	−0.9386	0.9942	0.6708	−0.8444	0.1478	0.8444	0.7920	0.8072	0.8516
pH	−0.9386	**1**	−0.9704	−0.8854	0.9774	−0.4799	−0.9774	−0.9540	−0.9613	−0.9802
Humus	0.9942	−0.9704	**1**	0.7469	−0.8973	0.2536	0.8973	0.8532	0.8662	0.9032
mobP	0.6708	−0.8854	0.7469	**1**	−0.9637	0.8327	0.9637	0.9841	0.9792	0.9600
mobK	−0.8444	0.9774	−0.8973	−0.9637	**1**	−0.6547	**−1.0000**	−0.9958	**−0.9978**	**−0.9999**
Al_3	0.1478	−0.4799	0.2536	0.8327	−0.6547	**1**	0.6547	0.7209	0.7031	0.6443
SB	0.8444	−0.9774	0.8973	0.9637	**−1.0000**	0.6547	**1**	0.9958	**0.9978**	**0.9999**
HA	0.7920	−0.9540	0.8532	0.9841	−0.9958	0.7209	0.9958	**1**	**0.9997**	0.9945
T	0.8072	−0.9613	0.8662	0.9792	**−0.9978**	0.7031	**0.9978**	**0.9997**	**1**	0.9968
V	0.8516	−0.9802	0.9032	0.9600	**−0.9999**	0.6443	**0.9999**	0.9945	0.9968	**1**

Note: values in bold are different from 0 with a significance level $p = 0.05$.

The Ecoplates approach to the soil functional microbiome (Table 7) revealed numerous differences between the microbial communities of the three tested sites. Basal community (water) has the lowest values in the CTRL variant, significantly lower than the other two sites. Site 1 shows a double basal activity higher than the control and 20% higher than Site 2.

Table 7. Results of the Ecoplates (BIOLOG™) method for the site factor.

Site	**Water**	**Pyruvic Acid Methyl Ester**	**Tween 40**	**Tween 80**	**α-Cyclodextrin**	**Glycogen**
CTRL	0.33 c ± 0.01	1.35 a ± 0.16 a	1.00 b ± 0.08	1.07 b ± 0.15	0.52 b ± 0.26	0.77 a ± 0.41
Site 1	0.62 a ± 0.06	1.54 a ± 0.30 a	1.38 a ± 0.24	1.41 a ± 0.06	0.92 ab ± 0.34	1.11 a ± 0.32
Site 2	0.51 b ± 0.04	1.58 a ± 0.21 a	1.36 a ± 0.08	1.18 b ± 0.08	1.16 a ± 0.19	1.67 a ± 0.61
Site	**d-Cellobiose**	**α-d-Lactose**	**β-Methyl-d-glucoside**	**d-Xylose**	**i-Erythritol**	**d-Mannitol**
CTRL	1.16 a ± 0.32	0.36 b ± 0.04	0.97 a ± 0.94	0.60 a ± 0.173	0.35 b ± 0.05	1.82 a ± 0.17
Site 1	1.19 a ± 0.34	0.89 a ± 0.37	1.70 a ± 0.92	1.09 a ± 0.475	0.66 a ± 0.11	2.06 a ± 0.09
Site 2	1.17 a ± 0.05	0.57 ab ± 0.16	1.05 a ± 0.85	0.85 a ± 0.405	0.59 a ± 0.14	1.90 a ± 0.31
Site	**N-Acetyl-d-glucosamine**	**d-Glucosaminic acid**	**Glucose-1-phosphate**	**d,l-α-Glycerol phosphate**	**d-Galactonic acidγ-lactone**	**d-Galacturonic acid**
CTRL	1.27 a ± 0.59	1.02 a ± 0.01	0.38 b ± 0.12	0.39 b ± 0.05	1.09 c ± 0.11	1.76 a ± 0.68
Site 1	2.04 a ± 0.78	1.16 a ± 0.27	1.14 a ± 0.43	0.78 a ± 0.17	1.40 b ± 0.17	2.45 a ± 0.05
Site 2	2.01 a ± 0.74	1.35 a ± 0.07	0.70 ab ± 0.41	0.67 ab ± 0.18	1.73 a ± 0.21	1.69 a ± 0.21

Table 7. *Cont.*

Site	2-Hydroxy benzoic acid	4-Hydroxy benzoic acid	γ-Hydroxy butyric acid	Itaconic acid	α-Keto butyric acid	d-Malic acid
CTRL	0.35 b ± 0.04	2.07 a ± 0.03	2.16 a ± 0.49	2.44 a ± 0.18	0.35 b ± 0.01	0.94 a ± 0.85
Site 1	0.74 a ± 0.20	2.32 a ± 0.16	2.75 a ± 0.38	2.15 a ± 0.44	0.75 a ± 0.09	1.23 a ± 0.20
Site 2	0.49 b ± 0.07	2.27 a ± 0.21	2.56 a ± 0.37	2.25 a ± 0.16	0.51 b ± 0.15	1.17 a ± 0.10
Site	**l-Arginine**	**l-Asparagine**	**l-Phenylalanine**	**l-Serine**	**l-Threonine**	**Glycyl-l-Glutamicacid**
CTRL	1.85 a ± 0.36	2.74 b ± 0.10	0.83 b ± 0.05	1.87 a ± 0.20	0.84 a ± 0.84	0.44 b ± 0.15
Site 1	2.23 a ± 0.35	2.98 a ± 0.16	1.07 a ± 0.15	1.86 a ± 0.26	0.77 a ± 0.16	0.79 a ± 0.16
Site 2	2.29 a ± 0.32	2.72 b ± 0.06	0.90 ab ± 0.12	1.90 a ± 0.46	0.58 a ± 0.17	0.66 ab ± 0.18
Site	**Phenylethylamine**	**Putrescine**	**Sum**	**AWCD**	**Polymers**	**Carbohydrates**
CTRL	1.47 a ± 0.46	1.08 a ± 0.36	35.30 b ± 3.35	0.81 a ± 0.11	3.36 b ± 0.54	8.66 b ± 0.71
Site 1	1.53 a ± 0.54	1.37 a ± 0.20	45.38 a ± 4.00	0.85 a ± 0.12	4.81 ab ± 0.88	13.02 a ± 2.54
Site 2	1.61 a ± 0.24	1.52 a ± 0.39	42.67 a ± 0.88	0.87 a ± 0.06	5.36 a ± 0.73	11.09 ab ± 1.43
Site	**Carboxylicand aceticacids**	**Amino acids**	**Amines/ amides**	**Shannon**	**Simpson**	
CTRL	12.18 b ± 1.68	8.56 a ± 0.91	2.55 a ± 0.72	3.23 b ± 0.03	0.96 a ± 0.01	
Site 1	14.96 a ± 0.68	9.70 a ± 0.78	2.89 a ± 0.74	3.33 a ± 0.07	0.96 a ± 0.01	
Site 2	14.02 ab ± 0.25	9.05 a ± 0.83	3.14 a ± 0.37	3.31 a ± 0.02	0.96 a ± 0.01	

Note: The letters that accompanies the mean values are provided from ANOVA ($p = 0.05$) post-hoc Duncan test ($p = 0.05$) for pairwise comparisons of samples means. Different letters across the columns designates significant statistically significant different mean values.

The sum of activities recorded for each site related to the entire functional microbiome reached only 35.303 units in the control site, with 10 units significantly increasing for Site 1 and more than 7 units in Site 2, while the AWCD showed similar activities. The sum of activities is significantly higher in Site 1 and Site 2, compared to CTRL, while AWCD does not record significant differences among the three tested sites. Within the diversity indices, only Shannon recorded significantly higher values in Site 1 and Site 2, compared to CTRL, while Simpson showed no significant differences between sites.

Within functional guilds, a higher share is related to carbohydrate decomposition. This functional guild recorded the highest activity associated with Site 1, significantly higher than CTRL, but without significant differences compared to Site 2. This substrate activity shows a similar trend as the basal community, with the maximum recorded in Site 1. Within carbohydrates, the α-d-lactose group shows significantly different activity, with Site 1 as the most active functional microbiome. The functional group that is responsible for i-erythritol decomposition recorded the lowest activity in CTRL, while both sites showed significantly higher activity. Both functional groups represented by glucose-1-phosphate and d,l-α-glycerol phosphate have the highest activity oriented to Site 1, followed by Site 2, with no significant differences between these two sites. Compared to CTRL, both functional groups recorded significantly higher differences.

The polymer functional guild has a gradual increase in the direction CTRL–Site 1–Site 2 and is significantly different between CTRL and Site 2. Within this functional guild, Tween 40 has the lowest values in CTRL, while both affected sites showed a significant increase in the functional group related to this substrate activity. Another polymer, Tween 80, presents a significantly different activity in the P1 direction, which indicates a different use of available substrates by soil microbiomes. For the α-cyclodextrin functional group, a significant difference was observed between the CTRL recorded activity and the activity in Site 2.

Carboxylic and acetic acids are functional guilds with more than 12 units of activity and showed a significant distribution of functional groups. This functional guild is associated with Site 1, showing significantly higher activity compared to CTRL. Two of these groups (2-hydroxy benzoic acid and α-keto butyric acid) had activity in Site 1, almost double that

in the CTRL site. A third functional group (d-galactonic acid γ-lactone) had significantly higher activity in Site 2, with a gradient increase from CTRL to Site 1.

The amino acids functional guild recorded an activity near 10 units, slightly different in all three sites analyzed. One functional group, l-asparagine, has significantly higher values in Site 1 of activity compared with the other two sites. For the l-phenylalanine and glycyl-l-glutamic acid functional groups, the differences between Site 1 and Site 2 were non-significant; only when Site 1 and Site 2 were compared to the CTRL was the activity considered significantly higher. The amines/amides functional guild showed no significant differences between functional groups and sites.

Mycorrhizal colonization potential (Table 8) shows different potentials for symbiosis formation. The frequency of colonization varies between 33%–34% (CTRL, Site 2) and 43% (Site 1). This indicates that less than 50% of the root system presents AM colonization and a reduced inoculum presence. At the same time, the intensity of colonization is 24% in Site 1, which indicates that AM symbionts can have almost one-quarter of the colonized roots. This parameter is slightly above 15% for the other two sites. From the total number of hyphae developed by AM fungi, only 2.8%–3.6% (CTRL, Site 2) can develop arbuscules, while in Site 1, this parameter is higher than 11%. In terms of comparison, this parameter shows a higher value of transfer between host and AM fungi in Site 1. At opposite vesicles, storage structures are present in a higher share in CTRL and Site 2, which indicates a supplementary abundance of nutrients in these sites that cannot be transferred to the host due to the low presence of colonizing hyphae.

Table 8. Mycorrhizal colonization parameters.

Sample	Frequency (%)	Intensity (%)	Arbuscules (%)	Vesicles (%)
CTRL	34.67 a ± 9.98	16.51 a ± 9.33	3.64 b ± 3.03	1.38 a ± 0.83
Site 1	43.36 a ± 6.26	24.56 a ± 4.59	11.36 a ± 4.47	0.44 a ± 0.08
Site 2	33.04 a ± 4.56	17.51 a ± 5.07	2.80 b ± 2.43	1.44 a ± 2.01
Sample	**Non-mycorrhizal area (%)**	**Colonization degree (%)**	**Mycorrhizal/Non-mycorrhizal**	
CTRL	**83.49 a ± 9.33**	**12.28 a ± 7.98**	**0.30 a ± 0.25**	
Site 1	75.44 a ± 4.59	17.70 a ± 6.39	0.55 a ± 0.24	
Site 2	82.49 a ± 5.07	11.42 a ± 3.89	0.31 a ± 0.14	

Note: The letters that accompanies the mean values are provided from ANOVA ($p = 0.05$) post-hoc Duncan test ($p = 0.05$) for pairwise comparisons of samples means. Different letters across the columns designates significant statistically significant different mean values.

The colonization degree of all three sites is more than 17% in Site 1 and 11%–12% in CTRL and Site 2. Based on this parameter, the overall volume explored by mycorrhizae is lower than 20%, which indicates a low colonization affinity of the inoculum in soil. Non-mycorrhizal areas exceed 75% and are visible in the multiple large uncolonized areas along the root cortex. All mycorrhizal/non-mycorrhizal ratios are below 1 as an indicator of a restrictive colonization strategy visible in host roots.

4. Discussion

The community-level physiological profile as profiled by the BiologEcoplates approach is a good method for targeting specific microbial processes or treatments [41]. Thus, it can assess the perturbances present in soil microbiomes by increasing rates of consumption for specific substrates [42–45]. In the present experiment, microbial functional communities' analysis showed a variety of potential related to the analyzed sites, both in terms of functional guilds and functional groups. The analysis of functional guilds shows a combined response to site conditions in terms of similar functional activities, while the analysis of functional groups offers information related to a very specific function of the microbiome [31]. Both functional guilds and groups were analyzed for each site to detect significant differences through ANOVA and Duncan multiple comparison. The signifi-

cantly higher guilds and groups associated with one site were used as indicators for the site conditions, while the functional microbiomes associated with two sites were considered indicators for a shift between conditions. The analysis of significant trends and their direction is very useful for determining the capacity of a specific functional microbiome to indicate a forest condition.

The variation recorded for each substrate showed specificity oriented toward one or two of the sites analyzed, with an increased intensity of substrate utilization, which enables their use as indicator substrates. Even if the general effect on the total microbial functional community showed similarities between sites within communities, there were some specific patterns that showed increased activity oriented toward a site [46–48]. Polymers guild is more related to Site 2 and is presented as a guild that has the highest significant activity in decay forest and decreases as the site remains functional (from Site 1 to CTRL). From this guild, Tween 80 functional group is an indicator of declined forest (Site 1), while α-cyclodextrin is related to decay, both showing significantly higher activities than similar microbiomes in the other analyzed sites.

Carbohydrates guild is an indicator of declined forest (Site 1), and its peak in this site is significantly higher than both CTRL and Site 2. In terms of usage, this guild of functional groups can be used as a total activity related to the phenomenon of decline in forests. This functional guild has three functional groups that are related to decline: α-d-lactose, glucose-1-phosphate, and d,l-α-glycerol phosphate, all of which present the highest activity in Site 1. Another functional group, i-erythritol, is an indicator of both decline and decay, and this group can be used as a test for forest sites that present these phenomena.

Carboxylic and acetic acids is a companion functional guild for Carbohydrates in the detection of decline in a forest site. Within this guild, two functional groups are highly related to decline—2-hydroxy benzoic acid and α-keto butyric acid—and indicate this phenomenon well due to the significant functional activities recorded. A third functional group—d-galactonic acid γ-lactone—is an indicator of decay and can be used for the detection of microbial communities in these types of sites.

Within the amino acids functional guild, l-asparagine is a clear indicator of declined forests, with significantly higher activity in this site. The group of l-phenylalanine and glycyl-l-glutamic acid showed a gradual increase compared to both Site 1 and CTRL sites, with the differences being significant compared to the functional forest. These two last functional groups have low activity in the CTRL forest site. The phenomenon of decline amplifies their activity, but when the forest is shifting to decay, the activity will still be visible but with decreased values. As an indicator, all three functional groups can be used for the detection of decline in forests, and based on their combined values, the future installation of decay can be forecast.

The analysis of total functional microbial activity is a good indicator of the similarity between the ecological functions of each site, and the analysis of each functional group shows the performance of each microbiome using the substrate [49,50]. This is related to the vegetation present at each site, which affects the assemblage of microbial communities and their activity [51,52]. As an indicator of microbial general activity, the sum of recorded activities is higher in declined and decayed forests, while the diversity indices calculated on functional profiles Shannon are related to the same forests. Overall, the phenomenon of decay and decline in a forest amplifies the activity of the functional microbiome and activates multiple functional groups. As a comparison between decline and decay forests, the decline activates a higher diversity of functional groups, with a larger area of substrate decomposition capacity.

Arbuscular mycorrhizal (AM) fungi colonization is a weak indicator of the decline or decay presence in a forest. The presence/absence of arbuscules and vesicles, combined with the intensity of colonization, was used to determine the colonization strategy [36,39]. This approach enables the detection of resistance colonization (at intensities lower than 10%), proliferative strategies (intensities between 10 and 25%, but lack of arbuscules or vesicles), respectively, transfer strategies (intensities higher than 25% and arbuscules present), and

storage strategies (intensities higher than 25% and vesicles present). The colonization potential is reduced; frequencies are under 50%, and the colonization degree is under 20% of the root volume. Our study revealed a slightly higher colonization frequency in declined forests, while both the control and the complete dried forests had similar values. The intensity follows the same trend, with 7%–8% higher in declined forests for this parameter. The only significant colonization parameter is arbuscularity, which represents more than 10% of decline forest. For this parameter, the intensity in the same forest site is near 25%, the threshold where the colonization strategy changes from proliferative to a transfer one. The analysis of colonization intensity and vesicles indicates a minimal proliferative-storage colonization strategy in both control and decayed forests. Declined forests have a higher transfer capacity (more than 10% arbuscularity), while functional forests and completely dried ones have a higher share of vesicles compared to declined ones. In terms of overall colonization, the declined forests are more susceptible to establishing this type of association due to their higher rate of changes in nutrient flows. As two completely opposite ecosystems, the functional forest and the completely dried one present lower colonization potential, which is associated with a slower flow of nutrients. Weak colonization is a good indicator of forest decay, while higher colonization intensities indicate the presence of decline in these ecosystems. Both the presence and abundance of arbuscules and vesicles can be used as indicators of forest health. A higher abundance of arbuscules is related to the presence of decline in forests, while a higher share of vesicles is related to decay. This phenomenon is due to the continuous change in nutrient flow and rates in declined forests, where the necessity of higher rates of transfer is required. In terms of vesicles, their presence can be associated with decayed forests, where there is a gap in the use of supplementary amounts of nutrients and the necessity for their storage in mycorrhizal structures.

The Ecoplates method for studying perturbation in forest ecosystems is a useful approach for the detection of changes in functional microbiomes, even those that do not present linear relationships [53,54]. The method uses a set of substrates, which can be traced as functional groups, and their values indicate the impact of both biotic and abiotic factors on a specific microbial activity [49,55]. In this context, the method acts as an indicator species analysis, with the same set of substrates related to different soil and ecosystem conditions [56–58]. For site and ecosystem comparison, such an approach is useful to detect even the smallest changes produced by external pressures. Also, the rapid response of microbial communities is a good indicator of perturbations in functional metabolism [47,59–61]. Both the functional groups and their assemblage in larger functional guilds can be used as indicators of functional alterations in soil microbiomes [30] and thus as potential indicators in the forest degradation stage. This type of assessment is permissive for the early detection of a potential decline or even the emergence of decay. By tracing the same functional groups, this analysis can be achieved faster than analyzing the vegetation for a long period of time. As a reverse of the method, the same functional groups can be a good indicator of restoration success in forests or even the mitigation of decline due to the applied management. These types of applications should be monitored and used for the development of new microbial indicators.

5. Conclusions

The results of the present study highlight the nuanced relationships between microbial functional communities, substrate utilization, and vegetation in different ecological sites. The specificity of substrate utilization and the influence of vegetation underscore the complex interplay between microbial ecology and the surrounding environment.

There is significant variation in the potential of microbial functional communities across the analyzed sites. Different functional guilds and groups are observed, indicating diverse microbial activities at each site. Each substrate shows specificity toward one or two analyzed sites, suggesting unique microbial community dynamics. Some substrates exhibit a higher intensity of utilization, making them potential indicators for specific sites. The heightened substrate utilization can serve as an indicator of the unique microbial

characteristics of each site. The analysis of total functional microbial activity serves as a good indicator of the similarity in ecological functions among the different sites. This suggests that, despite variations, certain ecological functions are shared among the sites. The type of vegetation at each site influences the assemblage of microbial communities. The presence of vegetation affects the microbial activity in the soil, indicating a close relationship between plant life and microbial communities.

In the context of microbial activity in forests, there is an interesting relationship between forest health (specifically, decline and decay) and the functional microbiome. The total microbial activity is higher in forests that are in a state of decline and decay. The Shannon index, which measures the diversity of functional profiles, shows a connection to forests experiencing decline and decay. The overall phenomenon of decay and decline in a forest amplifies the activity of the functional microbiome. This amplification is associated with the activation of multiple functional groups within the microbial community. In comparison between decline and decay forests, the decline phase activates a higher diversity of functional groups. Additionally, the decline phase is associated with a larger area of substrate decomposition capacity, indicating a more extensive range of microbial functions related to breaking down organic matter. The decay is associated with significantly higher activity of the polymers functional guild, while the decline is associated with a significant metabolism of carbohydrates. Tween 80, 2-hydroxy benzoic acid, α-keto butyric acid, and l-asparagine are indicator functional groups for decline and α-cyclodextrin for decay, with significantly higher activities compared to the other sites. Tween 40, i-erythritol, l-phenylalanine, and glycyl-l-glutamic are good indicators of a shift between decline and decay in forests, with slightly different activities recorded at these sites.

The results suggest that the microbial community response to forest decline and decay involves increased overall activity and a higher diversity of functional groups, particularly in the case of forest decline. The larger substrate decomposition capacity in declined forests may indicate a more complex and dynamic microbial ecology in response to changes in the forest ecosystem. Heightened microbial activity in forests undergoing decline and decay is a consequence of ecological processes associated with disturbances or environmental stressors. As forests undergo decline, trees may experience diseases or stress factors that lead to the death of plant material. Dead and decaying plant material provides a substrate for microbial colonization and growth. As plants decline, they may release chemical signals or compounds that attract decomposer organisms. This creates a feedback loop where the decline of plant material stimulates microbial activity, which accelerates the decomposition process. Understanding the drivers of heightened microbial activity in declined forests is crucial for assessing ecosystem health and the potential impacts on biodiversity. In the context of disturbances, conservation and management strategies may consist of the implementation of reforestation programs to replant native tree species in areas affected by decline or strategies to reduce nutrient leaching. Moreover, the site's history is a very important factor in the dynamics of the microorganism's activity in the context of climate change. Overall, studying microbial communities in mountain ecosystems provides valuable insights into the complex interactions between environmental variables and microbial diversity. This knowledge enhances our understanding of ecosystem dynamics, contributes to conservation efforts, and informs management strategies to preserve the ecological integrity of mountain regions. Studying soil health and microbial activity in declined forests is crucial for several reasons, offering insights into the ecological processes, factors contributing to decline, and potential strategies for conservation and restoration. Effective forest conservation in the face of decline requires a holistic and adaptive approach, integrating ecological, social, and economic considerations. Collaboration among scientists, policymakers, local communities, and conservation organizations is crucial for the success of these strategies.

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