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MICROBIAL BIODIVERSITY AND MULTIFUNCTIONALITY IN TERRESTRIAL ECOSYSTEMS: ROLE IN ECOLOGICAL PROCESSES AND RESPONSE TO DISTURBANCE

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ABSTRACT

Soil performs essential functions in maintaining terrestrial ecosystems. The ability of soil to simultaneously perform these functions is defined as multifunctionality, which is strongly influenced by variations in the structure and activity of microbial communities, in turn modulated by land use and management practices.

This PhD thesis aimed to clarify how different land uses and related management practices influence soil chemical, physical, and biological properties, the edaphic microbial community, contributing to understanding the ecological mechanisms that regulate multifunctionality in forest, grassland, and cropland systems in the Mediterranean area.

To this end, an experiment was conducted at both field and mesocosm scales to evaluate the short-term effects of polyethylene (PE) and biodegradable (BIO) plastic mulching films, compared with an untreated control (CNT). In parallel, in the Monte Matese area (Southern Italy), adjacent sites characterized by different land uses—forest (FO), pasture (PA), and meadow (ME)—were selected. Soils were sampled four times throughout the year in relation to the main management practices.

A wide range of parameters was analyzed, including: (i) soil physicochemical properties, such as water content, pH, organic matter, and nutrient content; (ii) biological indicators, including enzymatic activities involved in the main biogeochemical cycles (C, N, P, and S); and (iii) molecular parameters related to microbial community structure and interactions among taxa, assessed through co-occurrence network analysis. Finally, soil multifunctionality was evaluated using an integrated approach combining chemical and biological indicators and quantified using both the averaging approach (through the calculation of a synthetic index, SMF index) and the threshold-based approach.

Regarding plastic mulching, the observed effects differed depending on the material used and the scale of analysis. In the field experiment, BIO led to a 2-fold increase in β-glucosidase activity (enzyme involved in the C cycle) relative to PE. In the mesocosm experiment, PE increased total soil carbon by approximately 1.2-fold compared to BIO and CNT treatments. Despite these specific effects, no significant short-term changes in overall soil multifunctionality were observed with either plastic film; in addition, BIO showed potential to enhance soil multifunctionality.

Concerning land use, the results showed that it was a key determinant of soil microbial biomass, community structure and functions. Co-occurrence network analyses showed that FO was characterized by more complex and highly interconnected microbial networks, suggesting a more stable and resilient community structure. In contrast, PA and ME showed a progressive simplification of microbial interactions, although ME maintained high levels of diversity.

Functional differences also emerged. Nutrient acquisition strategies, assessed through enzymatic stoichiometric, indicated a greater phosphorus demand in FO, whereas PA and ME showed a more balanced relationship between microbial nutrient demand and soil nutrient availability.

These structural and functional dynamics were reflected in soil multifunctionality. FO and PA exhibited overall higher levels of soil functions and multifunctionality compared to ME. In particular, in PA, after six months of grazing, C and S cycles, decomposition function, and soil multifunctionality increased compared to other times. In ME, mowing practice positively affected multifunctionality, which remained stable throughout the year, leading to an increase in the nitrogen cycle, as highlighted by the threshold approach.

Overall, this research demonstrates that soil multifunctionality can be maintained under low-intensity management, whether through controlled grazing, annual mowing, or sustainable mulching practices. The findings of

this thesis highlights that resilience in soil systems is embedded in microbial functional diversity and the emergent properties of their communities. Together, these practices represent key factors for conserving soil functions and associated ecosystem services in Mediterranean mountain systems.

CHAPTER 1

INTRODUCTION

Ecosystem Services: Soil Functions and Multifunctionality

Soil is a fundamental component of the Earth's natural capital (Costanza et al., 1997). Defining and quantifying the natural capital of soil is essential for guiding environmental policies and promoting sustainable management practices (Mcbratney et al., 2014; Robinson et al., 2012). The natural capital of soil is represented by the set of physical, chemical, and biological properties that allow the soil to generate measurable and evaluable soil functions and services (Hewitt et al., 2015). This relationship can be described using a "cascade model" (Evers et al., 2018), in which biophysical structures and processes —including biodiversity and soil properties — generate ecological functions that can translate into services or disservices that directly or indirectly benefit or harm society. Institutions, social values and individual preferences shape policies, decisions and behaviours that may, intentionally or unintentionally, lead to the creation of novel ecosystems (Figure 1).

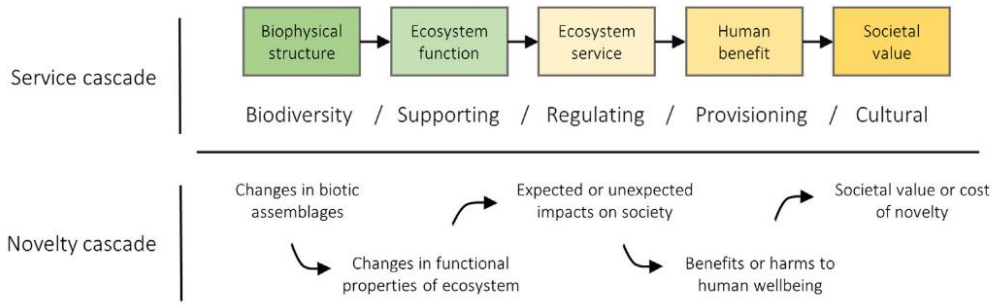

FIGURE 1 Service cascade illustrates the benefits society derives from the structure and functioning of ecosystems. Novel ecosystems contain assemblages of species that historically did not exist within a given biome and, as a result, can alter both ecosystem functioning and the services that result from it (Evers et al., 2018; (http://creativecommons.org/ licenses/by-nc-nd/4.0/).

The Millennium Ecosystem Assessment (MEA, 2005) defines ecosystem services as the set of benefits that humanity derives from the natural world and divides them into four main categories (Figure 2):

- Provisioning services that provide food, water, fibre, and fuel;
- Regulating services related to water purification, climate, and disease regulation;
- Cultural services, which include intangible benefits such as aesthetic, spiritual, and recreational values;
- Support services, which include fundamental ecological processes — such as soil formation, primary production and nutrient cycling — on which all other services depend.

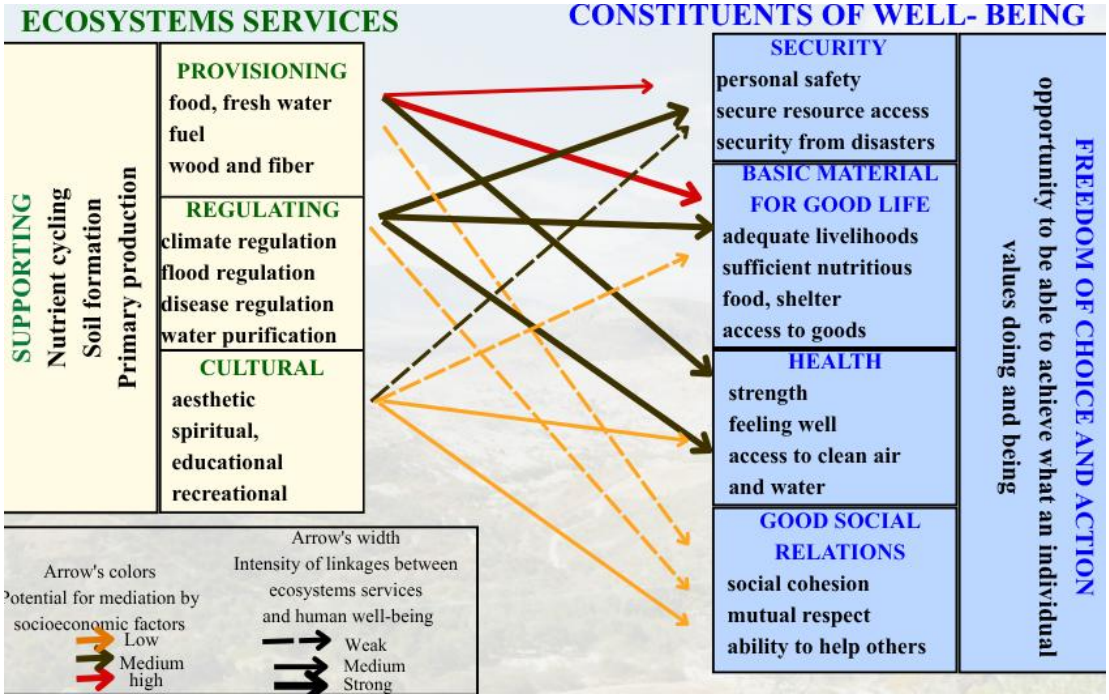

FIGURE 2 Intensity of the relationships between different categories of ecosystem services and the main dimensions of human well-being. The extent to which these relationships can be modified by socioeconomic factors (MEA, 2005, figure modified by me) are also highlighted.

Therefore, Ecosystem services form the basis of soil functioning and human well-being. Blum (2005) identified six main soil functions: (i) biomass

production; (ii) protection of humans and the environment; (iii) genetic reserve; (iv) physical basis for human activities; (v) source of raw materials; and (vi) geological and cultural heritage. Subsequently, the European Commission's soil protection strategy (EC, 2006) added a seventh function: the ability of soil to act as a carbon storage. The ability of soil to support these functions depends on its quality and health, which can be significantly altered by land use and management practices (Giuffrè et al., 2021). Forests, grasslands and croplands are the main types of land use, and the associated management practices, such as monocultures for timber production, tillage, grazing, and the use of pesticides, herbicides, and plastic mulching, can reduce ecosystem services, resulting in nutrient and biodiversity losses (Pereira et al., 2018). Studying these different types of land use is therefore essential to understand how biodiversity and soil properties influence soil functions in natural, semi-natural, and managed landscapes.

Soil biodiversity plays a crucial role in generating soil functions and providing ecosystem services that are essential for human well-being (MEA, 2005; Creamer et al., 2022). Soil organisms are historically divided into four groups based on size: (i) microflora (Archaea, bacteria and fungi); (ii) microfauna (protozoa and nematodes), with a diameter < 200 μm; (iii) mesofauna (mites, springtails and enchytraeids), with a diameter between 100 μm and 2 mm; (iv) macrofauna (earthworms, isopods and diplopods), with a diameter > 2 mm.

Furthermore, these organisms are divided into three large functional groups (Wurst et al., 2012): (i) Ecosystem engineers through bioturbation activities, modify soil structure, forming aggregates and pores that influence water retention capacity and nutrient availability; (ii) Chemical engineers involved in the degradation of organic matter, in carbon transformations and mineralization of major nutrients (N, P, S); (iii) Biological regulators that regulate population dynamics through predation, parasitism and competition, contributing to the natural control of pathogens and parasites.

These organisms are key to the stability and productivity of terrestrial ecosystems (Brussard, 1997; Creamer et al., 2022), contributing to carbon dynamics (Filser et al., 2016), through the formation of stable soil carbon pools; water purification and retention via bioturbation and the formation of aggregates; the nutrient cycling, by mineralizing organic matter and transforming nutrients (e.g., N, P, S) into available forms; the biological control of pathogens and pests, through antagonism, competition and predation, promoting overall plant health. Among soil organisms, microflora has the greatest influence on soil properties. Torsvik et al. (1996) estimated the presence of approximately 6,000 different bacterial genomes per gram of soil, highlighting their central role in carbon storage and their direct effects on climate change. The soil organic carbon pool derives directly from living soil organisms and necromass (Fu et al., 2025; Wang et al., 2021). Therefore, managing soil carbon content is one of the most effective tools for mitigating climate change.

Bacteria play a fundamental role in soil nutrient cycling, such as *Azotobacter* and *Rhizobium*, the nitrogen-fixing bacteria involved in nitrogen cycling, and numerous decomposer bacteria capable of degrading complex organic compounds, thus contributing to the release of nutrients such as nitrogen, phosphorus, sulfur, and carbon into the soil (Wani et al., 2015). Regarding fungi, they represent 70–80% of the total microbial biomass (Miller and Lodge, 1997) and establish mutualistic and non-mutualistic interactions. Among these, mycorrhizal fungi form mutualistic associations with plant roots, facilitating the exchange of nutrients. Arbuscular mycorrhizal fungi are the most widespread globally and colonize a wide range of plants, while ectomycorrhizal fungi show greater host specificity and often produce macroscopic fruiting bodies (Brussard, 1997).

Soil biodiversity provides the intrinsic capacity of soil to sustain multiple functions simultaneously, defined as multifunctionality (Allan et al., 2015; Wagg et al., 2014). The most recent studies have focused on soil

multifunctionality, also including the trophic functions sustained by the trophic guilds (Potapov, 2022), and how stress and management practices, by modifying soil properties and biological activity, can strongly influence it (Wang et al., 2024; Zhao et al., 2025; Jiao et al., 2022). Several tools, such as the Cornell Soil Health Test and Biofunctool, have been developed to assess multifunctionality by integrating physical, chemical and biological parameters through a minimum set of indicators (Creamer et al., 2022; Thoumazeau et al., 2019).

The most common approaches to quantify multifunctionality are the average approach (Maestre et al., 2012), which calculates the mean value of multiple functions, and the threshold approach (Gamfeldt et al., 2013; Zavaleta et al., 2010), which counts the number of functions exceeding the predefined thresholds, indicating whether several functions achieve high levels.

The soil microbial community is considered the main driver of multifunctionality due to its high structural and functional diversity (Bardgett and Van Der Putten, 2014; Delgado-Baquerizo et al., 2016). In particular in Mediterranean soils, where arid and semi-arid climate limits the availability of organic carbon is the activity of the microbial community rather than its structure to strongly regulate ecosystem multifunctionality (Bastida et al., 2016; Morales-Salmerón et al., 2025). Despite this, the relationships between microbial diversity and soil functionality are not yet fully understood. A key mechanism through which microbial communities support ecosystem multifunctionality is the balance among the acquisition of carbon (C), nitrogen (N), and phosphorus (P) (Sinsabaugh et al., 2008). Enzymatic activities associated with the C, P, and N cycles, respectively, are used to calculate the stoichiometric ratios.

The C:N:P ratios of soil enzyme activities provide information on nutrient acquisition strategies (Moorhead et al., 2016; Sinsabaugh et al., 2008). Soils with balanced C:N:P enzyme ratios generally show greater efficiency in mineralisation and nutrient cycling, along with faster organic matter

turnover, thus supporting a wider range of soil functions (Burns et al., 2013; Creamer et al., 2022).

A study by Wagg et al. (2014) demonstrated that soil biodiversity loss compromises soil multifunctionality. Progressive reductions in microbial diversity have led to a decline in plant productivity and floristic diversity. These results highlight a clear positive relationship between soil biodiversity and ecosystem multifunctionality. The influence of microbial communities on soil functions and multifunctionality is affected by environmental factors, soil properties, land use and management practices (Yu et al., 2024; Zheng et al., 2019). Indeed, several studies have demonstrated the impact of various land uses on biodiversity. In particular, in grasslands and croplands where disturbance levels are intermediate, microbial diversity can increase. At such levels of disturbance, biodiversity tends to reach maximum values, while it decreases both in highly stressed ecosystems —dominated by competitive selection— and in undisturbed ecosystems, characterized by competitive exclusion (Christel et al., 2024; Gleason et al., 2004). Also in Mediterranean area, land use change and management history profoundly influence the structure and functionality of microbial communities and, consequently, the overall functions of the soil and its multifunctionality (Lopezosa et al., 2024; Celis et al., 2026).

Soil Functions and Multifunctionality in Forest Ecosystems

Forests cover approximately 80% of the Earth's surface and represent a major reservoirs both of above- and below-ground biodiversity, and their multifunctionality emerges from the complex interactions between these components (Banerjee and van der Heijden, 2023). Soil microbial diversity is a key driver of forest multifunctionality, although the relative contribution of fungal and bacterial diversity remains poorly understood (Yuan et al., 2020). Multifunctionality also depends on interactions among functions that can lead to synergies or trade-offs (Hölting et al., 2019).

Forest management strongly affects soil multifunctionality by altering soil properties, resource availability, and biological activity. Understanding how soil microbial diversity and forest management interact to regulate forest multifunctionality is therefore critical for maintaining ecosystem stability under projected global climate change. While practices such as mixed-species stands, reduced harvesting intensity, and thinning generally promote multifunctionality, intensive management, including clear-cutting and soil disturbance, can impair multiple soil functions (Jangid et al., 2011; Delgado-Baquerizo et al., 2017). Achieving multifunctionality is often difficult because forest management prioritizes timber production over the provision of other ecosystem services (Peura et al., 2016), highlighting the need for integrated forest management to ensure true multifunctionality in forest ecosystems (Caicoya et al., 2023).

Among forest ecosystem services, soil carbon storage is particularly sensitive to management. Sustainable practices can increase carbon accumulation, while timber harvesting can cause a temporary reduction. Furthermore, the quality of litter inputs — often described by the C:N ratio — strongly influences stabilisation processes (Cotrufo et al., 2013; Pierson et al., 2021). A study conducted by Pierson et al. (2021) showed that twenty years of litter inputs with a high C:N ratio increase soil carbon content. These results confirm the importance of litter quality in carbon storage in forest soils.

Soil functions and multifunctionality in the grassland ecosystem

In Europe, grassland covers approximately 736,000 km² (https://ec.europa.eu/eu-grassland-watch/) and represents a key resource for livestock fodder production (Mayel et al., 2021; Richter et al., 2024). Management practices — particularly grazing in pastures and mowing in meadows — have a significant influence on the quantity and quality of the ecosystem services provided (Dubeux et al., 2007; Richter et al., 2024). Livestock farming practices are a major source of greenhouse gas emissions,

particularly carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O). Among these, methane produced by livestock digestive processes accounts for the largest share, followed by CH_4 emissions from manure management and N_2O emissions from the use of manure and fertilizers. One measure to reduce methane emissions is to improve forage quality and digestibility. Using legume-rich, more easily degradable forage species reduces CH_4 production (Sollenberger et al., 2019). Similarly, replacing soil chemical nitrogen fertilization with nitrogen-fixing legumes reduces N_2O emissions (Soussana et al., 2010), enriching the soil even in conditions of poor nutrient availability (Mencel et al., 2022).

Grasslands store large amounts of organic carbon in the soil (Bardgett and Wardle, 2003). However, the soil organic carbon sequestration is strongly influenced by climatic factors, soil properties, plant biodiversity and the intensity of management practices (Follett et al., 2001; Kucharik, 2007; Bai and Cotrufo, 2022). On the one hand, grazing—compared to mowing—can increase organic carbon levels in the topsoil thanks to the direct return of plant residues, roots, and livestock excrements (Mayel et al., 2021; Sollenberger et al., 2019). On the other hand, excessive livestock density or high grazing frequencies can cause soil compaction, resulting in a reduction in root biomass and an increase in organic matter mineralisation (Jair et al., 2014; Mayel et al., 2021). A study conducted by Hewins et al. (2018) demonstrated that moderate grazing can increase organic carbon compared to ungrazed pasture, improving the soil's ability to act as a carbon storage.

In grasslands, the distribution of livestock excrements is often uneven, creating a mosaic of microenvironments with varying fertility (Dubeux et al., 2007; Hirobe et al., 2013). Areas close to shade and watering holes tend to accumulate nutrients. As regards mowing, the use of manure as a soil amendment helps close the nutrient cycle, ensuring high productivity and preserving soil fertility (Mayel et al., 2021). Mowing once a year allows high production levels to be maintained and stimulates the natural regeneration of

grasslands. Conversely, excessive removal of plant biomass reduces the amount of litter on the soil, leading to a decrease in nutrient supply and a consequent compromise of microbial biomass (Gilmullina et al., 2020; Li et al., 2017).

A study conducted by Wang et al. (2020) demonstrated that moderate grazing is the most effective management strategy for maintaining high multifunctionality, promoting improved nutrient cycling, and enhancing plant productivity. Conversely, total grazing exclusion, often proposed for the recovery of degraded pastures, has proven to be the least effective practice for preserving soil biodiversity and ecosystem functionality. Furthermore, the study highlights that the effects of different management practices on ecosystem functioning depend on above-soil and below-soil biodiversity. Studies conducted by Richter et al. (2024) and Schils et al. (2022) have also shown that extensive management simultaneously improves several ecosystem services. Furthermore, Schils et al. (2022) compared the ecosystem services and multifunctionality of permanent grasslands with those of cropland and forests. Permanent grassland covers 34% of the agricultural area of the European Union (Eurostat, 2020). The EU defines permanent grassland as land used to grow grass or other herbaceous forage plants that have not been included in the crop rotation of the holding for at least five consecutive years. Permanent grassland provides a higher number of simultaneous functions than cropland, which, while ensuring higher yields, has lower biodiversity and lower carbon sequestration. When compared to forests, some trade-offs emerge: forests offer better hydrological regulation and erosion control services, while permanent grasslands have greater biodiversity and a higher presence of pollinators. In recent years, the EU Common Agricultural Policy (PAC) has recognised the importance of ecosystem services provided by permanent grassland through direct “green” payment, which aims to prevent its reduction by more than 5% and to protect the most sensitive areas from conversion to agricultural fields.

Soil functions and multifunctionality in plastic mulch-based cropping systems.

In recent years, the global area of cropland has been steadily increasing (FAO, 2021), causing soil erosion (Tilman et al., 2002), loss of biodiversity (Laurance et al., 2014), greenhouse gas emissions (DeFries et al., 2002), and a reduction in soil multifunctionality (Sünnemann et al., 2023). Appropriate management practices can mitigate negative impacts without compromising agricultural production (Lin et al., 2024). Mulching is a widely used practice in agriculture and consists of covering the soil with various materials (organic and synthetic) to limit weed growth and increase crop productivity. Polyethylene (PE) is the most widely used material in mulching practices (Iqbal et al., 2020; Kasirajan and Ngouajio, 2012) due to its strength and flexibility, and is often enriched with pigments and UV stabilisers. The colour of the film affects the amount of light radiation reaching the soil and, therefore, its temperature. Black and transparent films are the most effective in increasing soil temperature (Ham et al., 1993), promoting faster germination and greater microbial activity (Farias-Larios et al., 1999; Tindall et al., 1991). Although some studies have observed an increase in microbial activity under plastic mulch (Chen et al., 2017; Zhang et al., 2015), others have reported reductions, especially in warmer seasons, when temperatures can exceed the optimal limits for microorganisms (Moreno and Moreno, 2008). Furthermore, mulching with conventional plastic can influence microbial community structure, for example, by modifying the abundance of functional genes associated with ammonification processes (Dou et al., 2023).

Plastic mulching influences not only soil microclimatic and biological properties, but also key ecosystem services in cropland systems. Although it can substantially increase productivity -raising corn yield increases of 33% and water use efficiency by 32%, but also increases CO_2 emissions by 28% and reduces CH_4 uptake by 58% (Xu et al., 2024), highlighting a trade-off

between higher production and potential ecosystem disruption. The changes induced by the use of plastic films can therefore affect soil ecosystem functions and services, as well as soil multifunctionality. A study by Liu et al. (2024) demonstrated that conventional mulching can reduce soil multifunctionality due to reduced carbon content and microbial activity. The use of plastic films presents difficulties in removal and disposal. If the films are left in the soil, UV-induced photodegradation accelerates the formation of plastic fragments and microplastics (Li et al., 2020), which can accumulate in the soil, where can alter chemical and physical properties (Qiang et al., 2023) and affect the microbial community (Jiang et al., 2017), as well as overall soil functions. Fibers, in particular, can alter water retention capacity, thereby influencing plant growth and productivity (de Souza Machado et al., 2019). However, knowledge about the effects of microplastics on these processes remains limited and further studies are needed to fully understand them.

A recent study showed that, even under best management practices—which involve the careful collection of all plastic material after use—all the fields analysed were still contaminated with macro- and microplastics (Tiwari, 2024). Such contamination can compromise several key soil functions. In particular, the accumulation of macroplastics can alter soil structure, affecting aggregate formation and soil water retention. Moreover, the accumulation of macroplastics negatively influences nutrient cycling by reducing the phosphate; over time, this deficit may increase the need for additional fertilizer applications. The accumulation of plastic pollution may contribute to the total pool of soil organic carbon, but at the same time it can disrupt soil organic matter (SOM) cycling. In particular, at high macroplastic concentrations, a reduction is observed in both particulate organic matter (POM)-associated carbon and mineral-associated organic matter (MAOM)-associated carbon. Finally, these results suggest that the weathering of large plastic mulch residues contributes to the generation of microplastics in soil

and that visible, more easily observable plastic fragments can be quantified and used as indicators of microplastic contamination levels even in moderately polluted soils. Although the observed impacts were minor and primarily related to soil management history, plastic accumulation can still compromise some soil functions, with more pronounced effects at higher concentrations. As the amount of plastics increases over time with continued use, practices that limit their accumulation need to be adopted.

To reduce the environmental impact of conventional plastic mulching, biodegradable materials designed to degrade in the soil through microbial activity have been developed in recent years (Kasirajan and Ngouajio, 2012). Biodegradable plastic mulches can be obtained from polymers produced by microorganisms, from plant sources or from materials of fossil origin (Maréchal, 2003). Biodegradation occurs through extracellular depolymerisation (Luckachan and Pillai, 2011): several studies have found an increase in microbial biomass and enzymatic activity in soils mulched with bioplastics (Li et al., 2014; Yamamoto-Tamura et al., 2015), suggesting a possible contribution to soil functionality. The degradation of biodegradable mulches can also release micro-bioplastics, increasing organic carbon inputs and enhancing bacterial abundance, richness and diversity (Bandopadhyay et al., 2018). The release of easily assimilable organic compounds can also stimulate microbial groups involved in the nitrogen cycle, such as ammonium-oxidising bacteria, with potential effects on nitrification (Brust, 2019; Rillig, 2018). However, despite these potentially positive effects, significant gaps remain in knowledge about the long-term impacts of biodegradable mulches on soil ecosystems. Most studies focus on short-term effects, while effects on nutrient biogeochemistry, soil carbon, and microbial communities remain largely unexplored, necessitating long-term studies and direct comparisons with conventional plastic mulches.

CHAPTER 2

AIM OF THE STUDY

Soils play a central role in supporting ecosystem services, largely through the activity of soil organisms that regulate essential ecological functions. Disturbances that alter the physico-chemical properties of soil can, however, influence the structure and activity of microbial communities, with potential consequences for the processes they mediate.

The overarching aim of this doctoral research is to investigate how different management practices across various productive land systems affect soil multifunctionality, providing a knowledge base useful for the development of sustainable management strategies.

This work is guided by the hypothesis that disturbances deriving from grazing, mowing and the use of plastic mulch films (management practices investigated in this study) reduce the activity and biodiversity of the soil microbial community, thus impairing soil multifunctionality.

Chapter 3 evaluates the short-term effects of plastic mulch films at both field and mesocosm scales. The Chapter investigates the impacts of plastic mulch films on soil microbial community and physico-chemical properties, in order to assess whether their application constitutes a disturbance to soil multifunctionality in agricultural systems.

Chapters 4, 5 and 6 focus on soils under three different land uses: forest, pasture and meadow. They examine the effects of grassland management practices, specifically grazing in pastures and mowing in meadows, on soil microbial communities and multifunctionality.

Chapter 4 explores whether management-induced disturbances alter resource availability, thereby shaping microbial nutrient demand and nutrient allocation strategies.

Chapter 5 compares soils under different land-use types in terms of microbial network complexity, cohesion, and the nature of microbial interactions, providing insights into community organization and potential change in ecological relationships linked to management practices.

Chapter 6 quantifies soil multifunctionality by assessing multiple functions related to key biogeochemical cycles and organic matter decomposition, evaluating whether management practices in grasslands reduce soil multifunctionality through impacts on microbial processes.

Overall, this dissertation provides an integrated view of the ecological processes underlying soil multifunctionality, offering evidence-based guidance for sustainable management of croplands and grasslands. By linking microbial community dynamics to ecosystem functioning, this work contributes to the development of strategies that maintain soil health, resilience and ecosystem services in managed landscapes.

CHAPTER 3

BIODEGRADABLE PLASTIC FILMS AS ECO-SUSTAINABLE ALTERNATIVE TO POLYETHYLENE FILMS: EFFECTS ON SOIL MULTIFUNCTIONALITY[1]

Abstract

The use of plastic films is among the major sources of pollution in agricultural soils and can impact soil properties and ecological functions. This study aimed to assess the short-term effects of polyethylene (PE) and biodegradable (BIO) plastic films on soil quality and multifunctionality by comparing them with an untreated control (CNT). A comprehensive set of abiotic (texture, bulk density, pH, water content, organic and total carbon, and total nitrogen) and biotic (enzymatic activities, DNA yield, eubacterial and fungal biomass) soil properties was assessed 6 months after treatment application, both in mesocosm and field experiments. These parameters were used to: (1) calculate ecological indices (IBR, integrated biomarker response index; MAI, metabolic activity index; SQI, soil quality index) to assess soil quality; (2) derive soil functions (N and C storage and decomposition), then integrated into a soil multifunctionality index (SMF) and analyzed using a random forest approach to identify the most influential variables contributing to soil multifunctionality. In the field experiment, both BIO and PE plastic films increased soil water content compared to CNT, while BIO led to a 2-fold increase in β-glucosidase activity relative to PE and CNT. In the mesocosm experiment, PE increased total soil carbon by approximately 1.2-fold compared to BIO and CNT treatments. PE enhanced carbon storage capacity in mesocosm conditions, aligning with the results from the random

[1] Esposito A., Santini G., Santorufo L., Morra L., Maisto G., De Nicola F., Picariello E. Biodegradable plastic films as eco-sustainable alternative to polyethylene films: Effects on soil multifunctionality. *Ecosphere*. 16(9), e7038. https://doi.org/10.1002/ecs2.70388

forest analysis, which identified carbon storage as a key driver of soil multifunctionality. Despite these specific effects, no significant short-term changes in overall soil multifunctionality were observed for both plastic films. Among the indices tested, MAI highlighting differences between treatments emerged as the better integrative tool to monitor early functional changes in soil ecosystems.

Keywords

carbon storage; decomposition; nitrogen storage; non- and bio-degradable plastic films; random forest analyses, soil multifunctionality index

Introduction

In Europe, approximately 310,000 tonnes of plastic films are used in agriculture, with about 77,000 tonnes specifically attributed to mulching applications (APE Europe, 2019). If on the one hand, the use of plastic films offers numerous benefits, as suppressing pathogens and weeds and increasing crop productivity (Chakraborty et al., 2008; Shinde et al., 2023), on the other hand, it poses potential drawbacks. The degradation of plastic films can release microplastics, which may persist and accumulate in the soil, disrupting soil structure, altering key chemical and physical properties (Qiang et al., 2023) and consequently affecting the microbial community (Jiang et al., 2017). Globally, plastic contamination of agricultural soils from covering films is estimated to range between 0.5 and 2.3 million tonnes (Kedzierski et al., 2023) contributing to soil quality decline (Bone et al., 2010). This highlights the need for careful evaluation of plastic use in agricultural systems. Ecological concerns about soil degradation can be addressed by encouraging the use of biodegradable plastic films, considered a sustainable alternative (Sintim et al., 2021; Zhang et al., 2022). Biodegradable plastic films are a potential source of organic carbon, which can act as a trophic source for the soil microbial community, promoting

biomass and enzyme activity (Li et al., 2014; Yamamoto-Tamura et al., 2015). These biological responses, in turn, may enhance overall soil functioning.

In this context, a comprehensive assessment of soil quality is fundamental for sustainable soil management. However, since single physicochemical or biological parameters provide only a partial view (Allan et al., 2015; Nannipieri et al., 1990; Zhang et al., 2020), an integrative approach is needed. The concept of soil multifunctionality, that is, the ability of the soil to perform several functions simultaneously, offers a more holistic framework, encompassing multiple ecosystem functions and services to better capture the complexity of soil responses (Chen et al., 2019; Garland et al., 2021). Soil enzymes, involved in organic matter decomposition and nutrient cycles (Housman et al., 2021), are useful indicators to assess soil multifunctionality (Hu et al., 2023; Lucas-Borja and Delgado-Baquerizo, 2019).

Although several studies under controlled conditions have examined the effects of microplastic addition on soil physicochemical properties (Meng et al., 2023; Zhou et al., 2021), research on the impacts of realistic agricultural practices, such as the use of polyethylene and biodegradable plastic films, on physicochemical attributes and biological indicators relevant to soil quality, remains limited. Addressing this gap requires experiments in controlled conditions (mesocosms) and in the field (Carrieri et al., 2021; Hu et al., 2021).

Thus, this study aimed to evaluate, at both field and mesocosm scale, the short-term (after 6 months) effects of two plastic films (polyethylene and biodegradable) compared to untreated control, on soil physicochemical and biological properties, indicators of soil quality, as well as on derived functions that define soil multifunctionality. Soil quality was evaluated using integrated ecological indices: the metabolic activity index (MAI) that assesses the functions of the soil microbial community (Picariello et al., 2021), the soil quality index (SQI) that takes into account the chemical and

biological properties of the soil (Andrews et al., 2004) and the integrated biomarker response index (IBR) which is based on the integration of soil biological properties data (Beliaeff and Burgeot, 2002).

Soil multifunctionality was analyzed through the soil multifunctionality index (SMF) (Garland et al., 2021). Furthermore, given that the interactions between plastic films, soil biota, and ecosystem functions poorly understood, the study aimed to identify the overall direct and indirect effects of the soil properties and functions on SMF, applying a random forest approach, thereby addressing this critical knowledge gap. Given that biodegradable plastic films are usually considered a sustainable alternative, we hypothesize that their application would positively affect both soil quality and multifunctionality.

Materials And Methods

Experimental design

In an organic farm of Capua (41°06′07.3″ N, 14°14′21.8″ E, Caserta, Southern Italy), in autumn 2021 an experimental field was established; the field was divided in 12 plots with 3 treatments (4 plots for each treatment), according to a randomized block design: soil covered with low-density polyethylene plastic film (PE), soil covered with biodegradable plastic Mater-bi film 18 μm thickness (BIO), and soil not covered as control (CNT). Polyethylene and Mater-bi films were produced by Novamont S.p.A. Mater-bi EF04P (Polymer Name: Thermoplastic Starch, Density: 1270.0–1270.0 kg/m3) has characteristics and properties of use very similar to traditional plastics but is both biodegradable and compostable in accordance with European standard EN 13432.

The same treatments were set up for experimentation in mesocosms, with 12 pots of 1 m in diameter and 60 cm height (4 pots for each treatment: PE, BIO, and CNT). In autumn 2020, each mesocosm was filled to a height of 20 cm with limestone of different sizes (diameter = 1–4 cm) from a quarry (Caserta,

Italy). Fresh agricultural soil was placed (30 cm height) on the limestone. The mesocosms were left outdoors and exposed to weather conditions and not irrigated. Table 1 shows the soil physicochemical properties in field and mesocosm experiments before the experiment was set up.

TABLE 1. Soil physical–chemical characteristics: Soil bulk density (BD), pH, water content (WC), total C and N concentrations (TC and TN), and organic C content (Corg) in soil and texture type prior to experimental setup (mean ± SE; $n = 4$).

Soil physical–chemical characteristic	Field	Mesocosm
BD (g cm^{-3})	1.4 ± 0.10	0.19 ± 0.010
pH	7.8 ± 0.038	7.0 ± 0.11
WC (% D.M.)	16 ± 0.85	41 ± 1.0
TC (% D.M.)	2.0 ± 0.23	4.5 ± 0.23
TN (% D.M.)	0.54 ± 0.26	0.33 ± 0.014
Corg (% D.M.)	1.4 ± 0.14	3.2 ± 0.31
Texture type	Sandy-clay silt	Silty sand

Note: The texture classification is according to Shepard (1954).

Abbreviation: D.M., dry MASS.

Laboratory analyses

From each plot/mesocosm, after 6 months from the experiment setup, five soil samples were collected at a depth of 15 cm and pooled to obtain a homogenous composite sample. All laboratory analyses were carried out in triplicate.

On soil cores, the bulk density was measured by the undisturbed (intact) core method, according to FAO (2020).

Fresh sieved (<2 mm) soil samples were analyzed for: pH by potentiometric method in a soil:distilled water (1:2.5 = mass:mass) suspension; water content (WC) by gravimetric method.

Air-dried (105°C) soil samples were analyzed for: texture (according to Shepard, 1954) by wet sieving (>63 μm fraction) and sedimentation method (2–63 μm fraction), after treatment to remove organic material and aggregates (Gee and Dani, 2002); total C (TC) and N (TN) contents by CNS Analyzer (Thermo Finnigan) after pulverization (Fritsch Analysette Spartan 3 Pulverisette 0); organic carbon (Corg) content by CNS Analyzer (Thermo Finnigan) after treatment with HCl (10%) to exclude carbonates (Memoli et al., 2018).

TC and TN contents were used to calculate the stocks of soil total carbon and nitrogen (in grams per square meter):

$$\text{TC stock} = H \times BD \times TC \times 10 \quad (1)$$

$$\text{TN stock} = H \times BD \times TC \times 10 \quad (2)$$

Where *H* is soil depth (15 cm), BD is bulk density (in grams per cubic centimeter), TC is soil total carbon content (in grams per kilogram), and TN is soil total nitrogen content (in grams per kilogram; Zeng et al., 2021).

Fresh sieved (<2 mm) soil samples were analyzed for biological properties. The hydrolases (HA), dehydrogenase (DHA), β-glucosidase (BG), and urease (U) enzymatic activities were measured by spectrophotometric methods, respectively, according to Adam and Duncan (2001), Alef and Nannipieri (1995), Memoli et al. (2018), and Tabatabai (1982).

The soil total microbial biomass (eubacteria and fungi, MB) was determined by DNA extraction and subsequent amplification of a specific gene by qPCR (Nannipieri et al., 2003; Santini et al., 2022). According to modifications of Ceccherini et al. (2007), soil total DNA was extracted using a FastDNA SPIN Kit for soil (MP Biomedicals). DNA yield (nanogram per gram DNA soil dry

mass) and purity were evaluated using Nanodrop 2000, and DNA quality was assessed by agarose gel electrophoresis. Specific primers were used to quantify qPCR 16S rDNA (Muyzer et al., 1993; Simmons et al., 2007) and 18S rDNA (Chemidlin Prévost-Bouré et al., 2011) sequences (eubacteria and fungi, respectively) in each soil DNA sample. qPCR was performed with 2X SYBR Green qPCR mix (low rox) (GDSBio, Guangzhou, China), 10 μM each forward and reverse primers, 40 ng of template DNA, and sterile ddH_2O to reach the appropriate volume of reactions of 25 μL. Each sample was assayed by AriaMx Real-time PCR System (Agilent Research Laboratories, CA, USA); the melting curve conditions were performed from 55 to 95°C, with increments of 0.5°C every 5 s. Sterile water was used as a negative control. The run efficiencies ranged from 111% to 171%, with R^2 values ranging from 0.912 to 0.977. Nanograms of the target sequence were normalized to a gram of soil dry mass.

According to EPA (1996), phytotoxicological assays were carried out using *Sorghum saccharatum* L. and *Lepidium sativum* L. as test organisms and evaluated on fresh sieved (<2 mm) soil samples. Ten seeds were placed in petri dishes containing fresh soil equivalent to 10 g oven-dried soil (dry mass, DM); the soil was then saturated with water. Standard medium (OECD, 1984) was used as a negative control. After incubation in the dark (72 h, at 25°C), the number of germinated seeds and root elongation were measured as described by Santini et al. (2022).

Data treatment

Metabolic activity index

The MAI, useful for quantifying the impact of polyethylene and biodegradable plastic films on soil microbial community functions, is based on the ratio between enzymatic activities in the soils under PE and BIO treatments and those in the uncovered CNT soil. Enzymatic activities are normalized to soil organic carbon content (Picariello et al., 2021)

$$\text{MAI} = \frac{1}{n} \sum_{i=1}^{n} \frac{Pij}{Pcij} \quad (3)$$

where:

$$Pij = \frac{A_{ij}}{Ref_j} \quad (4)$$

$$Pcij = \frac{Ac_{ij}}{Refc_j} \quad (5)$$

and A_{ij} and Ref_j are, respectively, any value of enzymatic activity and organic carbon content, reference parameter, in soils of treatments PE and BIO; Ac_{ij} and $Refc_j$ are, respectively, each enzyme activity value and the reference parameter in CNT soil.

MAI values can vary from +1 to +∞, increasing with the increase in metabolic activity of the soil.

Soil quality index

The SQI, which provides reliable numerical data about soil functionality (Pulido et al., 2017), was calculated considering the soil chemical and biological properties, following the method described by Andrews et al. (2004). Data on chemical and biological properties have been classified from 0 to 1 according to Liebig et al. (2001) and scores have been assigned applying the function "more is better" or "less is better." The "more is better" function has been assigned to WC, Corg, TC and TN, enzymatic activities, and microbial biomass (Marzaioli et al., 2010). The maximum score for pH was assigned to 7 (Liebig et al., 2001). The SQI for each treatment (PE, BIO, and CNT) was calculated by summing the parameter scores S and dividing them by the number of parameters n.

$$SQI = \sum_{i=1}^{n} \frac{Si}{n} \quad (6)$$

An ideal soil would have an SQI value of 1 for the highest quality soil and 0 for the severely degraded soil.

Integrated biomarker response index

The IBR constitutes a robust tool to evaluate the integrated effects of plastic films on soil microbial communities. IBR is used in many field and laboratory research (Arzate-Cárdenas and Martínez-Jerónimo, 2011; Damiens et al., 2007; Serafim et al., 2012).

The IBR for each treatment (PE, BIO, and CNT) was calculated by summing triangular star plot areas Ai for each two neighboring biomarkers in a given data set following the method described by Beliaeff and Burgeot (2002).

$$IBR = \sum_{i=1}^{n} Ai \quad (7)$$

Soil multifunctionality index

The soil multifunctionality is an index that allows us to synthesize several soil properties and processes (Garland et al., 2021). For each treatment (PE, BIO, and CNT), the indicators TN stock, TC stock, HA, DHA, BG, U, and MB were normalized [log (1 + *x*)] and then standardized through the *Z* score transformation (Le Bagousse-Pinguet et al., 2019). More specifically (Figure 1), the *Z* score of TN stock was used to determine the function of N storage (Hu et al., 2023); the *Z* score of TC stock was used to determine the function of C storage; the average of the *Z* scores of the indicators DHA, BG, U, HA, and MB was used to calculate the function of decomposition (Wang et al., 2022). Then, the SMF value was calculated as the average of the three abovementioned soil functions (Jucker and Coomes, 2012).

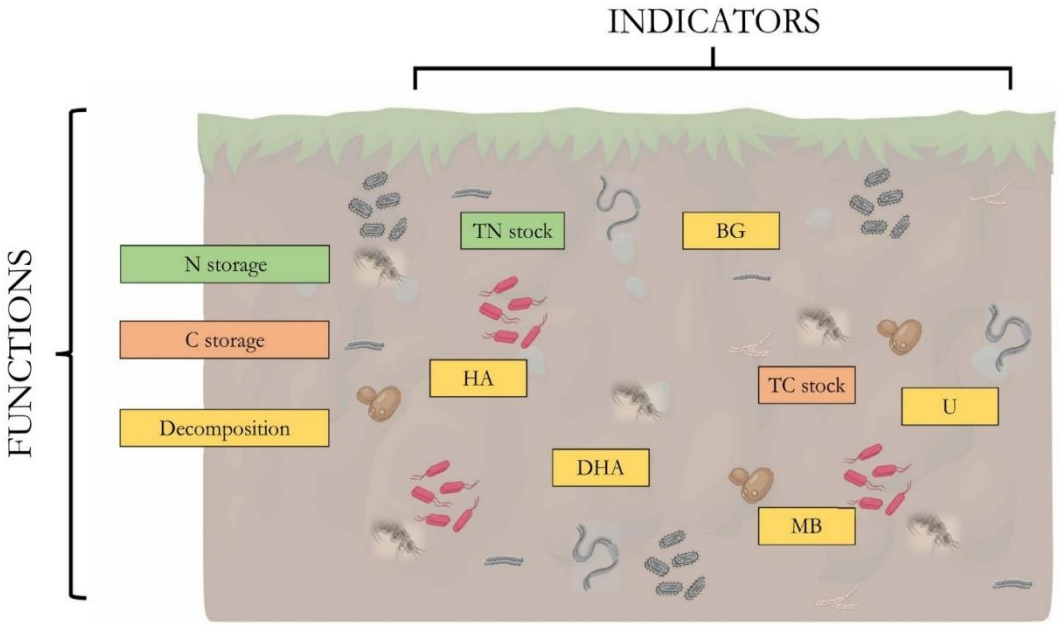

FIGURE 1 Individual soil functions and related indicators. Illustration credit: Alessia Esposito. BG, β-glucosidase; C, carbon; DHA, dehydrogenase; HA, hydrolase; MB, microbial biomass; N, nitrogen; TC, total C; TN, total N; U, urease.

Statistical analyses

All the analyses were performed in the R 4.1.2 programming environment (R Core Team, 2021).

PERMANOVA analyses (*vegan* package, *adonis2* function) were carried out to test the response of the physicochemical properties and all biotic properties of the soil against the two experimental factors: the treatments (PE, BIO, and CNT) and experiment scale (field and mesocosm). The p-values were calculated using the Monte Carlo permutation test (999 permutations).

Nonparametric Kruskal–Wallis test followed by Conover's multiple range test (*PMCMRplus* package, *kruskalTest*, *kwAllPairsConoverTest* functions) was applied to evaluate the significance of the differences in each parameter among treatments.

Random forest analysis with 500 permutations (*rfPermute* package, *rfPermute* function) was carried out to assess abiotic properties and soil functions affecting SMF. The overall model significance and cross-validation R^2 were evaluated (*A3* package, *a3* function). A partial least squares path

modeling (PLS-PM) was developed (*plspm* package, *plspm* function) to estimate the direct and indirect effects of the soil properties (pH and WC) and the soil functions (N storage, C storage and decomposition) on SMF.

Results And Discussion

PERMANOVA highlighted significant differences only for the factor experiment scale ($F = 41.3$, $p < 0.001$), and thus, the field and mesocosm results have been analyzed separately.

In the field experiment (Figure 2a), soil pH was higher ($p < 0.05$) in BIO (7.9) than in PE (7.7), according to Xue et al. (2023). However, as confirmed in the literature, an effect of biodegradable plastic film on soil pH was not found (Li et al., 2014; Sintim et al., 2019). The WC was higher ($p < 0.05$) in PE and BIO (19% and 18% dry mass, respectively) than in CNT (16% dry mass), according to the results of other authors (Di Mola et al., 2021; Zhao et al., 2014). Indeed, the presence of both plastic films on the soil surface could have created a barrier that has reduced water evaporation and gas exchanges, increasing temperature and soil moisture (Ding et al., 2021). In the mesocosm experiment (Figure 2b), a higher TC ($p < 0.05$) was found in PE (4.4% dry mass) than in BIO and CNT (both with values approximately of 3.7% dry mass). Since soil organic carbon did not show significant differences among treatments, the differences found in total carbon could be due to soil inorganic carbon. Likely, on a small scale (pots 1 m diameter) low-density polyethylene film can release carbonates that were added in thermoplastics to reduce production costs (Chiappero et al., 2021), increasing soil inorganic carbon. However, it cannot be ruled out that the different pH values among treatments can also be involved in soil carbonate equilibrium (Niu et al., 2023).

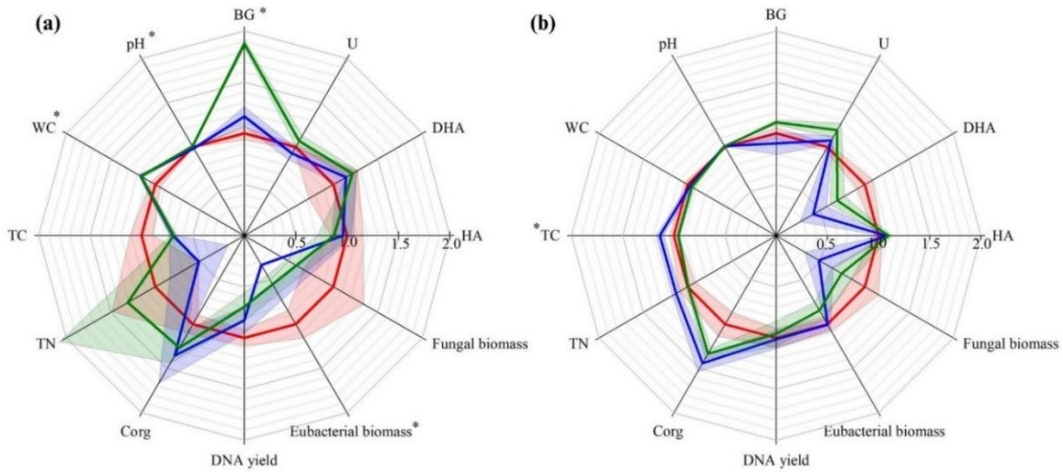

FIGURE 2 Radar plots of pH, water content (WC), total C and N concentrations (TC, TN), organic C content (Corg), hydrolase (HA), dehydrogenase (DHA), urease (U), β-glucosidase (BG), DNA yield, eubacterial and fungal biomass in (a) field and (b) mesocosm experiments. The plots show the means ± SE (filled area) of normalized values respect to the CNT. The values for eubacterial and fungal biomass, in PE and BIO treatments in the field experiment, are to be multiplied by 10. BIO, soil covered with biodegradable plastic film (green line); CNT, soil not covered (red line); PE, soil covered with low density polyethylene film (blue line). An asterisk indicates significant ($p < 0.05$) differences among treatments.

Regarding the phytotoxicity results (Appendix S1: Figure S1), in both field and mesocosm experiments, the test using *L. sativum* L. showed a biostimulation effect, whereas *S. saccharatum* L. exhibited an inhibition effect, likely due to the higher sensitivity of this species compared to *L. sativum* L. (Labella et al., 2024; Mamindy-Pajany et al., 2011; Tatuśko-Krygier and Jakubus, 2019). No significant differences were observed among treatments.

Although plastic film is reported to modify the soil environment, affecting the structure and functions of soil microbial communities (Bandopadhyay et al., 2018; Díaz-López et al., 2019; Liu et al., 2021), contrary to our expectation, the values of the enzymatic activities did not differ among treatments after 6 months from films placing. The only exception was in the field experiment (Figure 2a), for the β-glucosidase ($p < 0.05$), which showed

higher values in BIO (3.36 mmol PNP g^{-1} min^{-1} dry mass) than in PE (2.09 mmol PNP g^{-1} min^{-1} dry mass) and in CNT (1.79 mmol PNP g^{-1} min^{-1} dry mass). Likely, the degradation of the biodegradable film into fragments observed in the field releases carbon compounds into the soil (Zhou et al., 2021), affecting β-glucosidase enzyme involved in the degradation of C substrates, such as amylase and cellulose (Deng and Popova, 2011).

According to some studies, the effect of plastic films on the structure and function of the microbial community is minimal and insignificant (Bandopadhyay et al., 2020; Di Mola et al., 2021; Santini et al., 2023; Sintim et al., 2020, 2021). Similarly, total DNA yield did not show differences among treatments, both in field (Figure 2a) and mesocosm experiments (Figure 2b). However, in the field, eubacterial abundance (Figure 2a) showed significantly ($p < 0.05$) higher values in PE (106.8 ng g soil dry $mass^{-1}$) and BIO (101.4 ng g soil dry $mass^{-1}$) with respect to CNT (32.1 ng g soil dry $mass^{-1}$). Previous field studies also revealed that the use of biodegradable and non-biodegradable films led to a significant enrichment of bacteria in soil (Balakrishna et al., 2015; Bandopadhyay et al., 2020; Dong et al., 2017). Fungal abundance did not show significant differences (Figure 2a,b); the effect of plastic films on the fungal community was inconsistent among studies: some authors reported a reduction in the absolute fungal abundance under non-biodegradable plastic films (Shinde et al., 2023; Wan et al., 2023), while others attributed the use of plastic films to the formation of soil aggregates and improved soil conditions, with a higher reproduction and growth of fungi (Bo et al., 2024).

Among the calculated indices, the SQI and the IBR did not show statistically different values among treatments, in both field and mesocosm experiments (Table 2, Appendix S1: Figure S2), likely due to the short-time observation scale, whereas the MAI index was able to highlight brief-time effects of treatments after 6 months, differently from individual soil microbial

enzymatic activities. In fact, the MAI value in the mesocosm experiment was higher ($p < 0.05$) in BIO with respect to PE (Table 2b), in agreement with our initial hypothesis. These results confirm the sensitivity of this index developed to study the resistance and resilience of the microbial community to soil perturbation (Picariello et al., 2021).

TABLE 2 Indices in soil not covered and soil covered with polyethylene and biodegradable plastic films in (a) field and (b) mesocosm experiments (mean ± SE; $n = 4$).

Treatment	MAI	SQI	IBR
(a) Field			
CNT	…	0.46 ± 0.14^{a}	-2.5 ± 0.27^{a}
PE	3.5 ± 0.68^{a}	0.45 ± 0.072^{a}	-2.4 ± 0.29^{a}
BIO	4.2 ± 0.61^{a}	0.48 ± 0.040^{a}	-2.6 ± 0.16^{a}
(b) Mesocosm			
CNT	…	0.46 ± 0.067^{a}	-2.2 ± 0.23^{a}
PE	2.4 ± 0.18^{b}	0.46 ± 0.076^{a}	-2.0 ± 0.51^{a}
BIO	3.1 ± 0.16^{a}	0.52 ± 0.021^{a}	-2.2 ± 0.32^{a}

Note: Different letters indicate statistically significant differences ($p < 0.05$) among treatments.

Abbreviations: BIO, soil covered with biodegradable plastic film; CNT, soil not covered; IBR, integrated biomarker response index; MAI, metabolic activity index; PE, soil covered with low-density polyethylene film; SQI, soil quality index.

We focus on soil multifunctionality due to the role and the importance of multiple soil functions in providing ecosystem services. The SMF index did not differ among treatments in both experiments (Figure 3), although some differences among treatments have been detected in *Z* score values of single soil functions, as C storage function was higher ($p < 0.05$) in soil covered with PE with respect to BIO (Figure 4) in the mesocosm experiment. The decomposition function showed, both in the field and mesocosm experiments (Figures 3 and 4), lower values in PE with respect to BIO, as confirmed by the results of Moreno and Moreno (2008). The trend observed in the SMF

index suggests that the BIO treatment can enhance soil multifunctionality in the short term, thereby reinforcing our initial hypothesis that the treatment using biodegradable plastic will, by extension, prove to be the most sustainable in the long term.

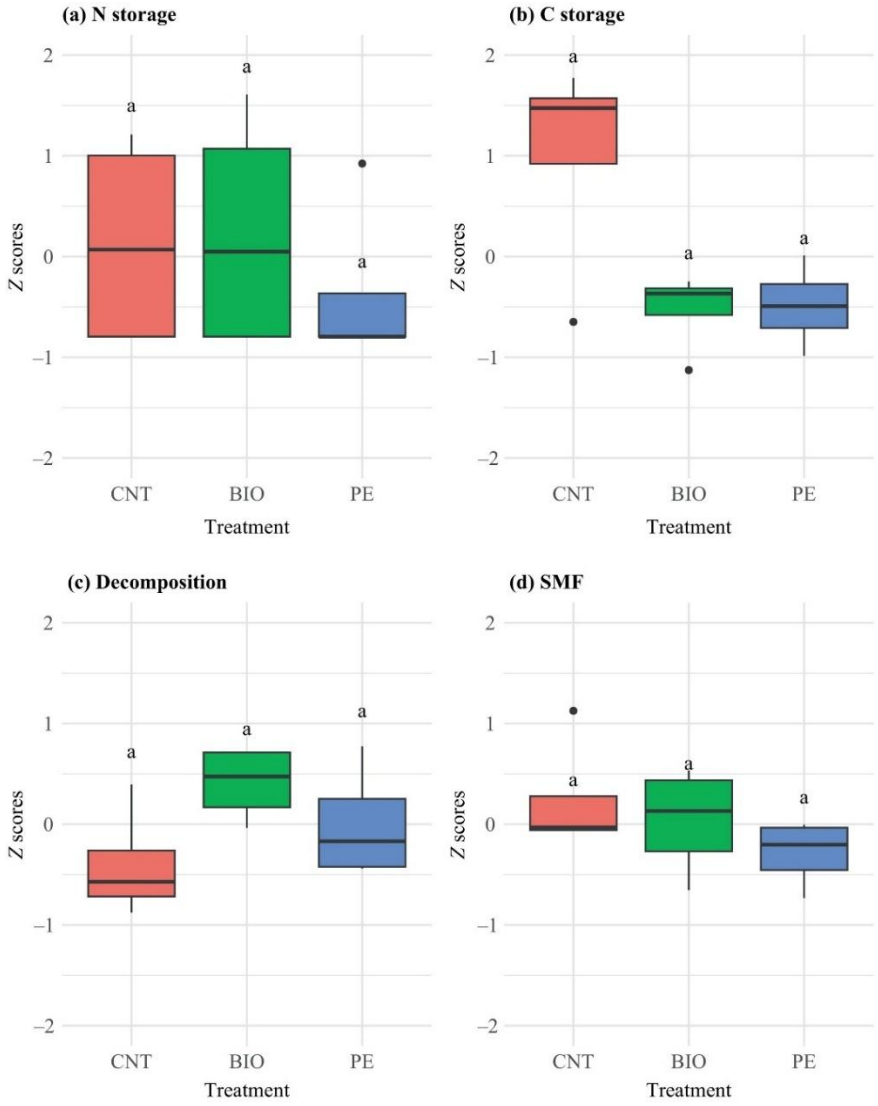

FIGURE 3 Box plots of (a–c) soil functions and (d) the soil multifunctionality (SMF) index in the field experiment. The box indicates the 25th and 75th percentiles, the continuous lines indicate the median values. The indicators of soil functions were transformed [log $(1 + x)$] and then standardized through the Z-score transformation. BIO, soil covered with biodegradable plastic film (green); CNT, soil not covered (red); PE, soil covered with low density polyethylene film (blue). Different letters indicate significant ($p < 0.05$) differences among treatments.

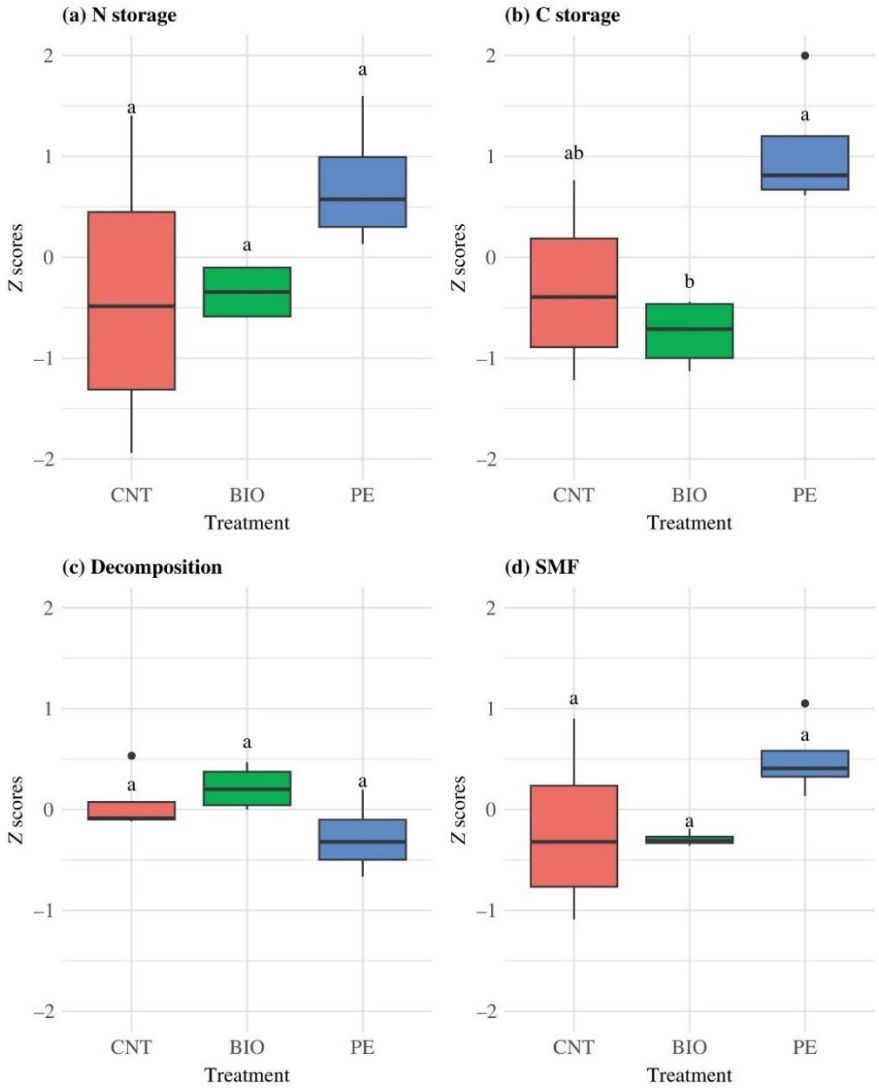

FIGURE 4 Box plots of (a–c) soil functions and (d) soil multifunctionality (SMF) index in mesocosm experiment. The box indicates the 25th and 75th percentiles, the continuous lines indicate the median values. The indicators of soil functions were transformed [log $(1 + x)$] and then standardized through the Z score transformation. CNT, soil not covered (red); BIO, soil covered with biodegradable plastic film (green); PE, soil covered with low density polyethylene film (blue). Different letters indicate significant ($p < 0.05$) differences among treatments.

A random forest model was constructed to evaluate the predictive capability of the studied variables, that is, pH, WC, and soil functions, on SMF (Figure 5a). The results showed that N and C storage were the most prominent predictors ($p < 0.05$) of soil multifunctionality. The PLS-PM model allowed us to explore the positive and negative relationships between soil

properties/functions and SMF (Figure 5b,c) in agricultural soil, regardless of the experimental spatial scale.

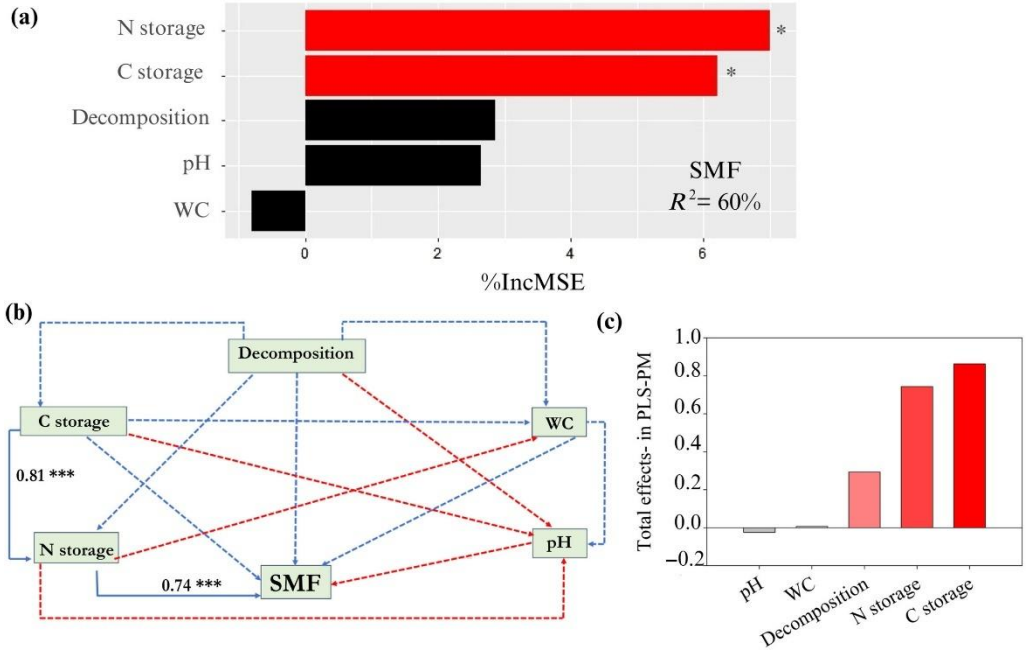

FIGURE 5 (a) The importance, expressed as percentage increase of mean square error (%IncMSE), of different soil abiotic properties and soil functions as predictors of soil multifunctionality index (SMF) in random forest modeling. An asterisk indicates significant predictors (*$p < 0.05$); R^2 represents the percentage of variance explained by the full model. (b) The direct and indirect impacts of soil abiotic properties and soil functions on SMF by partial least squares path modeling (PLS-PM). Red lines indicate negative impacts, while blue lines show positive impacts. A solid line and asterisks represent significant (***$p < 0.001$) effects, while a dotted line represents nonsignificant effects. (c) The total effects of different soil abiotic properties and soil functions calculated by direct and indirect impacts from PLS-PM. WC, water content.

The PLS-PM (Figure 5b) with good fitness of 54% highlights the central role of nitrogen (N) and carbon (C) storage in sustaining soil multifunctionality: it showed that C storage had a positive and direct effect on N storage (0.81, $p < 0.001$); in addition, C storage and decomposition showed a positive total effect on SMF (Figure 5c). N storage positively and directly regulated SMF (0.74, $p < 0.001$) and showed a positive total effect on SMF (Figure 5b,c), as also found by Hu et al. (2023).

This supports the concept that targeted management can simultaneously optimize various soil functions critical for agroecosystem sustainability.

Conclusions

Biodegradable plastic films have been considered a great alternative to avoid plastic pollution in the soil. In this study, we attempt to quantify the impact of polyethylene and biodegradable plastic films after 6 months from application on soil quality and multifunctionality, by field and mesocosm experiments.

Although at short term no significant effects on overall soil multifunctionality were observed for either plastic film, biodegradable films (BIO) highlighted potential for enhancing soil multifunctionality. This effect is particularly important when considering their use, especially over extended periods.

The positive influence of polyethylene (PE) film on carbon storage in the mesocosm experiment suggests that different plastic types may modulate distinct soil functions, even in the short term. However, the persistence of microplastics and potential long-term risks associated with conventional plastics raise concerns about their continued use.

From a practical perspective, the MAI emerges as a useful integrative tool for detecting early functional changes in soil ecosystems, offering a valuable metric for assessing the ecological sustainability of agricultural soil management practices. N and C storage functions were revealed as key drivers of soil multifunctionality regardless of the experimental approach, and their monitoring could be of crucial importance to preserve soil and related ecosystem services provided.

While the findings are not comprehensive, they provide a crucial first step in understanding the short-term impacts of plastic film application on ecosystem functions and soil multifunctionality. These insights are critical for developing sustainable soil management strategies aimed at mitigating

long-term soil degradation, especially considering the extensive use of plastic films over extended periods.

Acknowledgments

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Data Availability Statement

Data (Santini, 2025) are available from Mendeley Data: https://doi.org/10.17632/snst3vptgh.

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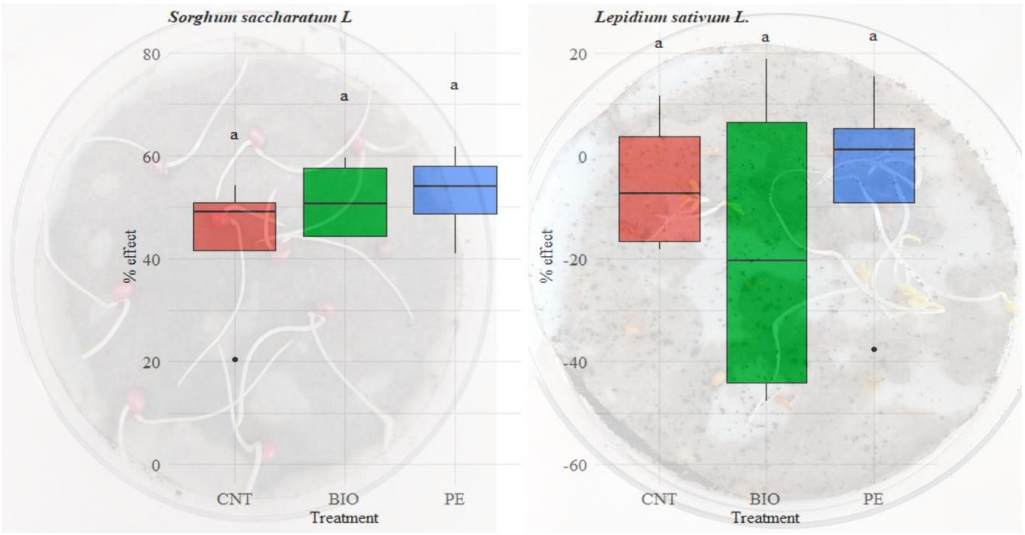
Supplementary material

a)



b)

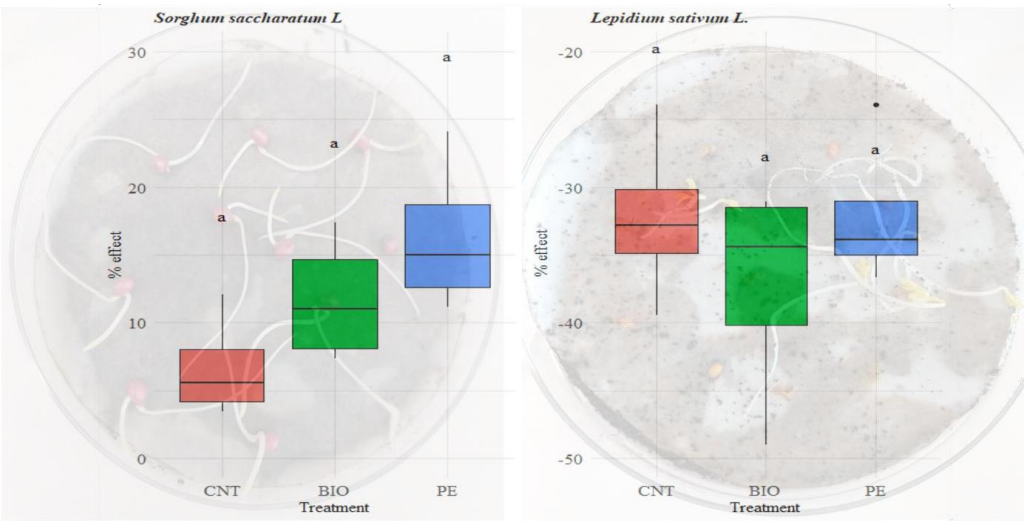


FIGURE S1 Box plots of the percentages effect of phytotoxicity tests in: (a) field and (b) mesocosm experiment. The box indicates the 25th and 75th percentiles, the continuous lines indicate the median values. CNT = soil not covered (red), BIO = soil covered with biodegradable plastic film (green), PE = soil covered with low density polyethylene film (blue)

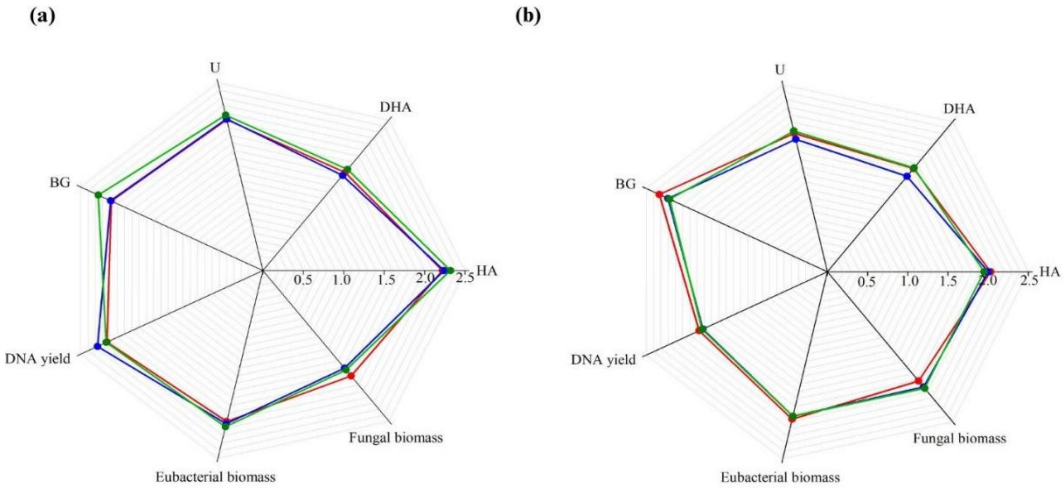


FIGURE S2 IBR star plot of the hydrolase (HA), dehydrogenase (DHA), urease (U) and ß-glucosidase (BG), DNA yield, Eubacterial and fungal biomass in (a) field and (b) mesocosm experiment. CNT = soil not covered (red), BIO = soil covered with biodegradable plastic film (green), PE = soil covered with low density polyethylene film (blue)

CHAPTER 4

SOIL ENZYME ACTIVITY AND STOICHIOMETRY IN RELATION TO DIFFERENT LAND USES IN THE MEDITERRANEAN AREA[2]

Abstract

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

[2] Esposito A., Picariello E., Gómez-Brandón M., Fornasier F., Baldi V., Napoletano M., Baldantoni D., De Nicola F. Soil enzyme activity and stoichiometry in relation to different land uses in the Mediterranean area. Ecosystems. Under review in *Ecosystems*.

highlight that practices, such as annual mowing and low grazing pressure, have helped preserve the balance of soil microbial nutrients.

Keywords

Enzyme stoichiometric ratios; land use; grazing; mowing; grassland; nutrient acquisition

Introduction

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

community, being unaffected by fluctuations in overall enzymatic activity (Moorhead et al., 2016). In general, enzymatic stoichiometry is affected by environmental factors (Feyissa et al., 2022), among which the land use (de Medeiros et al., 2023) and the associated management practices (He et al., 2020; Reinhart et al., 2022).

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

With the aim to fill the described gaps, this study evaluated the impact of different land uses - pastures, meadows and forests - on microbial soil

nutrient acquisition strategies. To achieve this purpose, extracellular enzymatic activities involved in carbon, nitrogen and phosphorus acquisition by the soil microbial community were analysed; their enzymatic stoichiometric ratios (C:N, C:P, N:P) and vector analysis were also investigated to assess microbial nutrient demand (Moorhead et al., 2013). We hypothesize that grazing in pasture and mowing in meadow, alter the metabolic strategies of soil microorganisms, leading to a nutritional imbalance, differently from forest soil, where management practices were absent.

Materials And Methods

Study area and soil sample collections

In Mount Matese (southern Italy), three adjacent sites characterized by different land uses were selected: pasture – PA (41°24'56.3" N 14°25'11.3" E), meadow – ME (41°24'8.4" N 14°26'56.5" E) and forest – FO (41°25'00.5"N 14°25'53.2"E). All the sites are located at an elevation of approximately 1010 m a.s.l. and are developed on an Andosol Pachy-Eutrisilic substrate (FAO, 1976).

The area is characterized by an average annual temperature of about 17 °C and a total annual precipitation of about 1385 mm (https://agricoltura.regione.campania.it/meteo/archivio_meteo.html). The PA site has been used for mixed grazing of cattle, sheep and horses since 1982. Grazing typically lasts six months per year, from early summer to early winter, followed by a five-month spring-rest grazing. The ME site is managed as a forage meadow since 1982, fertilized with manure (applied annually at a rate of approximately 30 t ha^{-1}) and sown with a seed mixture including *Lolium perenne* L., *Lolium multiflorum* L., *Trifolium pratense* L., *Dactylis glomerata* L., *Festuca arundinacea* L., *Phleum pratense* L., *Lotus corniculatus* L. and *Trifolium repens* L. The FO site, dominated by *Fagus*

sylvatica L., represents an undisturbed forest ecosystem that has remained unmanaged since the beginning of the last century.

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

Table 1 Sampling dates for all land use (T1–T4) and the corresponding management phases for PA = pasture and ME = meadow.

Sampling time	date	PA	ME
T1	4 June 2023	beginning of the grazing	sowing
T2	24 July 2023	after one month of grazing	mowing
T3	4 December 2023	after six months of grazing	five months after mowing
T4	6 May 2024	after the five-months resting grazing	one year after sowing

Soil laboratory analyses

Each composite soil sample was sieved (<2 mm) and analysed in triplicate. On fresh soil samples, twelve enzymatic activities were quantified by fluorometric assay using a Synergy HT microplate reader (BIO-TEK) after applying a heteromolecular exchange procedure, as described in Bardelli et al. (2017). Enzymatic activities were expressed as nmol of product released h^{-1} g^{-1} soil dry weight (d.w.). The functions and the substrates associated with each activity are shown in detail in Table 2. On oven-dried (105 °C) and grounded soil samples, total C and N concentrations were determined according to Colombo & Teodoro (2015) using a CHNS elemental analyzer (Carlo Erba 1500). Total macro- (P, Ca, K, Mg) and micronutrient (Fe, Zn, Mn, Cu) concentrations were measured by optical emission spectrometry

with inductively coupled plasma (Thermo Scientific TM iCAP PRO XP Duo) after microwave-assisted (Anton Paar GmbH Multiwave-3000) acid digestion using concentrated HNO_3 (USEPA, 1996).

Table 2 Soil enzyme groups (Hy-C, Hy-N, Hy-P, Ox-C) and their individual activities, with acronyms, functions, and substrates

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

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

Data treatment

The measured soil enzymatic activities were grouped and summed to calculate the soil enzymes involved in the acquisition of C, N and P (Liu et al., 2020; Yang et al., 2022). The hydrolytic activities related to carbon (Hy-C) acquisition were obtained by summing β-glucosidase (BG), cellobiohydrolase (Cell), xilosidase (Xilo) and β-galactosidase (BGAL), those related to nitrogen (Hy-N) by summing chitinase (NAG), leucine-

aminopeptidase (LAP) and serin – like – protease (CBZ); while for phosphorous (Hy-P), acid phosphomonoesterase (AP), pyrophosphate - phosphodiesterase (pyroP) and phosphodiesterase (bisP) were considered. For oxidative enzymes (Ox-C), which catalyse the oxidation of complex molecules such as lignin (Yang et al., 2022), the activities of phenol oxidase (OX) and peroxidase (PEROX) were summed.

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

$$E_{CN} = \ln BG \,/\ln (NAG + \text{LAP}) \quad (1)$$

$$E_{CP} = \ln BG \,/\ln (AP) \quad (2)$$

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

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

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

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

$$Vector\ angle = Degrees\left(ATAN2(X, Y)\right) \quad (5)$$

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

Statistical analysis

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

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

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

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

Results

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

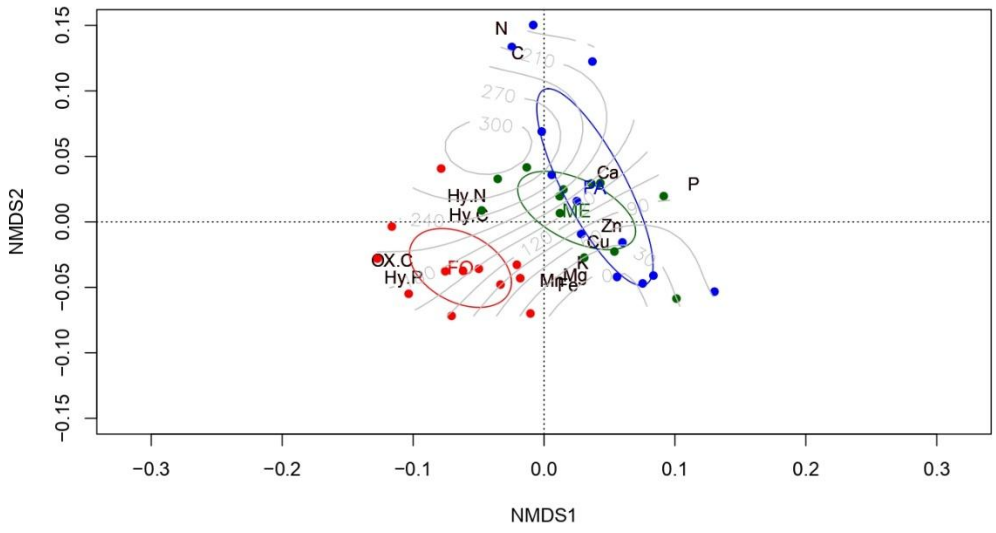


FIGURE 1 Non-metric multidimensional scaling (NMDS) biplot, with the superimposition of the temporal gradient (0 -T1; 30 – T2; 180 – T3; 365 – T4) and of the confidence ellipses ($\alpha = 0.05$), showing the differentiation among land uses (forest, pasture and meadow) in relation to soil total nutrient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

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

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

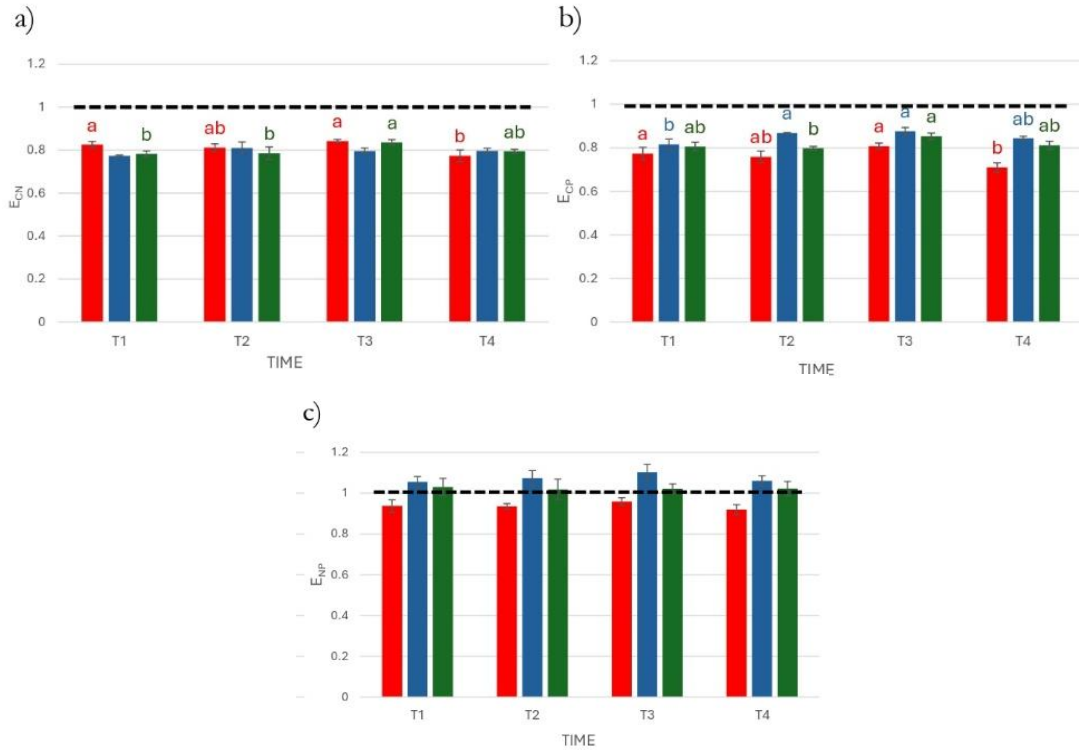


FIGURE 2 Bar plots (mean values ± s.e.) of the soil stoichiometric activities – E_{CN} (a), E_{CP} (b) and E_{NP} (c) – in FO = forest (red), PA = pasture (blue), ME = meadow (green), at each sampling time (T1, T2, T3 and T4). Different letters indicate significant differences among sampling times in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

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

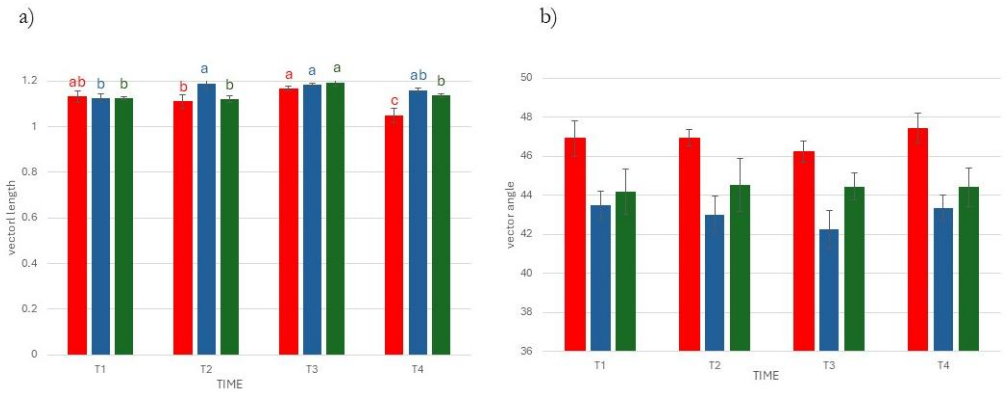


FIGURE 3 Bar plots (mean values ± s.e.) of the vector length (a) and vector angle (b) in FO = forest (red), PA = pasture (blue), ME = meadow (green), at each sampling time (T1, T2, T3 and T4). Different letters indicate significant differences among sampling times in each land use ($p < 0.05$).

TABLE 3 E_{CN}, E_{CP}, E_{NP}, vector length and angle (mean ± s.e) in forest (FO), pasture (PA) and meadow (ME) across the year. Significant differences ($\alpha = 0.05$) among land uses are indicated by different letters; n = 12

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

Note: enzymes used for the calculation of ECN, ECP, ENP, vector length, and angle are given in Table 2 and described in the Materials and Methods section

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

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

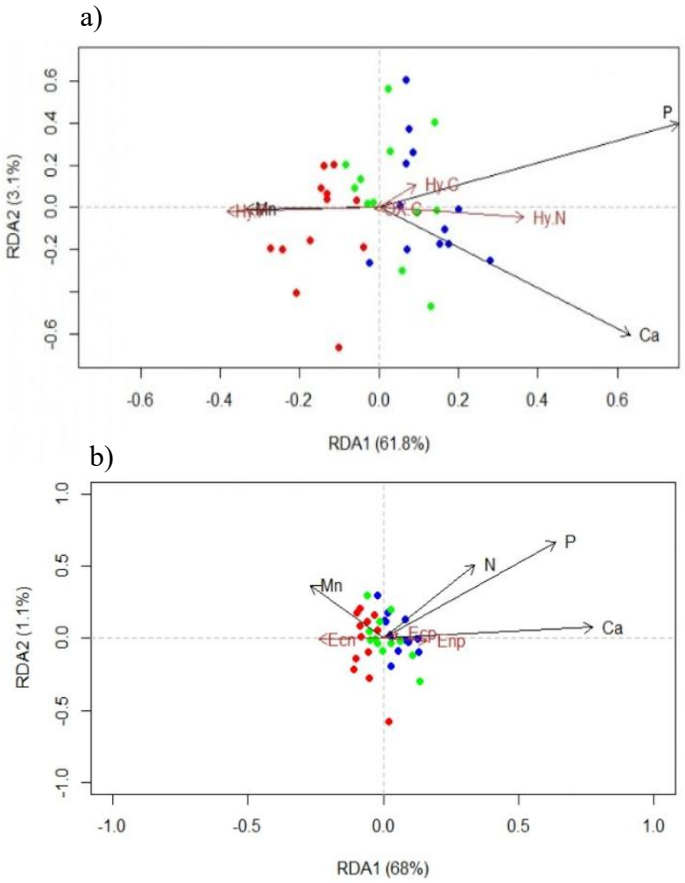


FIGURE 4 Redundancy analysis of the correlations between total nutrient concentrations selected from the model and (a) enzyme activity or (b) stoichiometric enzyme activity ratios in each land use. FO = forest (red), PA = pasture (blue) and ME = meadow (green)

Discussion

The studied forest soil showed a greater microbial demand for phosphorus relative to nitrogen compared to the pasture and meadow soils, as indicated by the vector angle. This result suggests a possible limitation of phosphorus, also confirmed by the high soil enzyme activity of acid phosphomonoesterase

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

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

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

In our study, we found a greater microbial demand for nitrogen relative to phosphorus in pasture soils compared to forest soils, as indicated by the vector angle. Moreover, microorganisms appeared to invest more in carbon acquisition than in nutrient acquisition, particularly after one and six months of grazing, as highlighted by vector length. These results suggest that, although the total nitrogen concentration in pasture soils was higher than in forest and meadow soils — with values within the typical range for pastures with moderate or light grazing (Han et al., 2008) - the microbial community may be affected a relatively low labile organic matter compared to meadow, especially after six months of grazing (Table S7). Such a condition may lead microorganisms to invest more resources in carbon acquisition and, consequently, to increase their demand for nitrogen more than for phosphorus under limited carbon availability, consistent with the findings of as also reported by Zhao et al. (2025). Grazing may have resulted in reduced availability of labile carbon, possibly due to reduced inputs of litter to the soil

and continued removal of vegetation cover, which resulted in decreased carbon flux from plants to soil through lower production of root exudates (Stark & Kytöviita, 2006). The increased microbial investment in carbon acquisition at T3 may also be partly due to climatic conditions, as a similar pattern has been observed in forest and meadow. This result is likely related to temperatures in previous months (mean temperature ~18 °C), which increased enzyme activity, as reported by Li et al. (2022). Overall, the results suggest that grazing and temperature can alter the availability of labile carbon, thereby modifying microbial nutrient demand and affecting both extracellular enzymatic activity and soil enzyme stoichiometry. Furthermore, the high concentration of nutrients such as calcium, phosphorus, zinc and copper found in pasture compared to forest and meadow soils could be attributed to the continuous presence of livestock excreta, which are an important source of micro- and macronutrients and tend to accumulate in the soil (Garcia et al., 2008; Assmann et al., 2017; Indraratne et al., 2021).

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

Calcium can affect enzymatic activity by acting as a cofactor, directly activating enzymes involved in the decomposition of organic matter (He et

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

Contrary to our hypothesis, pastures and meadows did not show strong nutritional imbalance, a condition particularly evident in the meadow. The low enzymatic activities found in these ecosystems compared to the forest confirm this condition and suggest that nutrients were not strongly limiting. This can be explained by a considerable energy cost associated with enzyme synthesis, making it advantageous only when nutrients represent a truly scarce resource for the microbial community (Burns et al., 2013). In meadow, five months after mowing, less microbial investment in nitrogen and phosphorus acquisition than carbon was observed, as indicated by the ECN and ECP ratios. When the stoichiometric ratios of soil enzymes approach the theoretical equilibrium value of 1:1:1 defined by Sinsabaugh et al. (2008), the enzymatic activities associated with the carbon, nitrogen and phosphorus cycles are balanced and stable, indicating the ability of soil enzymes to adapt

to environmental changes and maintain a functional balance between the main biogeochemical cycles. In the meadow, the microbial community did not show a marked nutrient demand to stimulate intense microbial activity; this is probably due to mowing, which is carried out only once a year, may not have drastically reduced nutrient availability. In fact, well-calibrated mowing can even increase the carbon and nitrogen content of the soil (Mayel et al., 2021). Conversely, increasing the frequency of mowing reduces plant species diversity and the number of soil microorganisms, consequently decreasing soil carbon and nitrogen turnover (Li et al., 2017; Mayel et al., 2021).

Conclusions

In forest soils, we observed an increased demand for nutrients (P). In meadow and pastures, no strong nutrient demand was detected, except in pastures, where a greater microbial acquisition of nitrogen compared to phosphorus was observed. Our results show that practices, such as annual mowing and low grazing pressure, thanks to reduced disturbance and maintenance of organic inputs to the soil, have helped to preserve soil microbial nutrient balance and can therefore be considered sustainable management practices in the Mediterranean area.

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Supplementary material

TABLE S1 Soil total micro- (Cu, Fe, Mn and Zn) and macronutrient (C, N, P, Ca, Mg and K) concentrations (mean ± s.e), in forest (FO), pasture (PA) and meadow (ME) across the year. When present, different letters indicate significant differences among sampling times in each land use; n = 3

Land use	Time	C % d.w.	N % d.w.	P g kg^{-1} d.w.	Cu g kg^{-1} d.w.	Fe g kg^{-1} d.w.	Mn g kg^{-1} d.w.	Zn g kg^{-1} d.w.	Mg g kg^{-1} d.w.	K g kg^{-1} d.w.	Ca g kg^{-1} d.w.
FO	T1	15.2 ± 1.47	3.31 ± 0.277	2.22 ± 0.0497 a	0.0311 ± 0.000287 a	30.7 ± 0.267 a	1.06 ± 0.0154 a	0.112 ± 0.00153 a	6.16 ± 0.0386 a	6.27 ± 0.406 a	5.87 ± 1.18
	T2	17.0 ± 1.17	3.87 ± 0.308	2.21 ± 0.0513 a	0.0304 ± 0.000558 a	29.7 ± 0.296 ab	1.07 ± 0.0254 a	0.109 ± 0.00167 a	6.03 ± 0.0521 a	5.86 ± 0.174 a	6.12 ± 0.977
	T3	15.9 ± 0.157	4.41 ± 0.124	1.95 ± 0.0689 a	0.0275 ± 0.000887 a	26.0 ± 1.12 b	0.972 ± 0.0409 a	0.100 ± 0.00305 a	5.32 ± 0.162 a	5.20 ± 0.277 a	6.27 ± 1.05
	T4	16.2 ± 1.47	4.60 ± 0.409	0.993 ± 0.214 b	0.0173 ± 0.00252 b	17.8 ± 1.85 c	0.601 ± 0.0755 b	0.060 ± 0.00938 b	3.34 ± 0.370 b	3.44 ± 0.560 b	3.11 ± 0.397
PA	T1	14.0 ± 0.990 b	3.41 ± 0.191 c	4.88 ± 0.252 a	0.0338 ± 0.00233 a	28.3 ± 2.56 a	0.943 ± 0.0358 a	0.142 ± 0.0436 a	5.95 ± 0.494 a	6.96 ± 0.787 a	7.70 ± 0.977 ab
	T2	13.5 ± 0.315 b	2.52 ± 0.0671 c	4.66 ± 0.284 a	0.0352 ± 0.00119 a	30.1 ± 0.717 a	0.997 ± 0.0441 a	0.138 ± 0.0821 a	6.31 ± 0.0850 a	6.92 ± 0.156 a	7.21 ± 1.32 ab
	T3	68.7 ± 2.93 a	14.6 ± 0.839 a	4.81 ± 0.0327 a	0.0320 ± 0.000494 ab	21.9 ± 0.520 b	0.754 ± 0.0107 b	0.139 ± 0.00278 a	4.95 ± 0.0803 b	5.81 ± 0.118 a	10.5 ± 1.49 a
	T4	15.2 ± 0.501 b	6.59 ± 0.613 b	4.00 ± 0.0257 b	0.0277 ± 0.000245 b	20.1 ± 0.986 b	0.716 ± 0.0116 b	0.116 ± 0.00178 b	4.23 ± 0.144 b	5.30 ± 0.207 b	6.85 ± 0.605 b
ME	T1	14.5 ± 2.43	2.97 ± 0.765 b	3.16 ± 0.326 a	0.0322 ± 0.00181 a	26.7 ± 1.13 a	0.770 ± 0.00723 a	0.117 ± 0.00586 a	5.52 ± 0.257 a	5.09 ± 0.301 a	9.29 ± 3.12 a
	T2	16.1 ± 1.11	5.42 ± 1.26 a	3.02 ± 0.320 a	0.0293 ± 0.00133 a	25.7 ± 1.10 a	0.753 ± 0.0452 a	0.113 ± 0.00717 a	5.19 ± 0.260 ab	4.75 ± 0.406 a	6.80 ± 2.42 ab
	T3	18. 2 ± 0.328	7.21 ± 0.761 a	2.64 ± 0.263 a	0.0275 ± 0.000741 a	22.2 ± 1.13 ab	0.659 ± 0.0130 a	0.103 ± 0.00359 a	4.50 ± 0.257 bc	4.77 ± 0.319 a	4.45 ± 0.268 b
	T3	15.5 ± 1.02	6.08 ± 0.647 a	1.96 ± 0.154 b	0.0199 ± 0.00103 b	19.2 ± 0.788 b	0.520 ± 0.0262 b	0.0769 ± 0.00530 b	3.84 ± 0.0795 c	4.08 ± 0.851 b	3.45 ± 0.510 b

TABLE S2 Soil Ox-C enzyme and Hy-C, N and P enzyme activities (mean ± s.e), in forest (FO), pasture (PA) and meadow (ME) across the year. When present, different letters indicate significant differences among sampling times in each land use; n = 3

Land use	Time	OX-C	Hy-C	Hy-N	Hy-P
		nmol h^{-1} g^{-1} d.w.	nmol h^{-1} g^{-1} d.w	nmol h^{-1} g^{-1} d.w	nmol h^{-1} g^{-1} d.w
FO	T1	0.807 ± 0.109	176 ± 40.7 a	381 ± 122 ab	609 ± 110 a
	T2	0.658 ± 0.0679	163 ± 17.1 a	337 ± 22.7 ab	600 ± 43.7 a
	T3	0.944 ± 0.0848	213 ± 18.0 a	412 ± 51.3 a	641 ± 42.7 a
	T4	0.496 ± 0.0232	89.0 ± 9.54 b	222 ± 29.7 b	397 ± 13.8 b
PA	T1	0.424 ± 0.0404	124 ± 10.2 b	335 ± 32.4 ab	342 ± 7.02 ab
	T2	0.305 ± 0.0562	126 ± 21.4 b	241 ± 7.02 b	242 ± 25.9 b
	T3	0.534 ± 0.0851	192 ± 17.7 a	499 ± 91.6 a	381 ± 30.6 a
	T4	0.439 ± 0.0339	145 ± 9.82 ab	345 ± 41.8 ab	333 ± 10.0 ab
ME	T1	0.340 ± 0.0289	87.0 ± 9.07	205 ± 16.2	247 ± 24.4
	T2	0.454 ± 0.0493	118 ± 9.54	273 ± 36.8	329 ± 19.1
	T3	0.318 ± 0.0199	120 ± 8.09	214 ± 11.3	235 ± 11.0
	T3	0.379 ± 0.0413	109 ± 9.83	253 ± 35.9	266 ± 19.6

TABLE S3 Soil total micro- (Cu, Fe, Mn and Zn) and macronutrient (C, N, P, Ca, Mg and K) concentrations, Ox-C enzyme and Hy-C, -N and -P enzyme activities (mean ± s.e), in forest (FO), pasture (PA) and meadow (ME). When present, different letters indicate significant differences among land use; n = 12

	FO	**PA**	**ME**
C **% d.w.**	16.0 ± 0.544 b	27.9 ± 7.15 a	16.1 ± 0.737 b
N **% d.w.**	3.98 ± 0.190 c	6.77 ± 1.45 a	5.42 ± 0.603 b
P **g kg^{-1} d.w.**	1.84 ± 0.160 c	4.58 ± 0.133 a	2.70 ± 0.182 b
Cu **g kg^{-1} d.w.**	0.0266 ± 0.00177 b	0.0322 ± 0.00102 a	0.0272 ± 0.00148 b
Fe **g kg^{-1} d.w.**	26.0 ± 1.60 a	25.1 ± 1.41 ab	23.5 ± 1.00 b
Mn **g kg^{-1} d.w.**	0.926 ± 0.0610 a	0.853 ± 0.0383 a	0.675 ± 0.0321 b
Zn **g kg^{-1} d.w.**	0.0954 ± 0.00664 b	0.134 ± 0.00375 a	0.102 ± 0.00527 b
Mg **g kg^{-1} d.w.**	5.21 ± 0.350 a	5.36 ± 0.271 a	4.76 ± 0.217 b
K **g kg^{-1} d.w.**	5.19 ± 0.364 b	6.25 ± 0.279 a	4.68 ± 0.170 b
Ca **g kg^{-1} d.w.**	5.34 ± 0.563	8.08 ± 0.659	5.99 ± 1.09
OX-C **nmol h^{-1} g^{-1} d.w.**	0.727 ± 0.0603 a	0.426 ± 0.0346 b	0.373 ± 0.0221 b
Hy-C **nmol h^{-1} g^{-1} d.w**	160 ± 17.1 a	146 ± 10.6 ab	108 ± 5.54 b
Hy-N **nmol h^{-1} g^{-1} d.w**	338 ± 36.5	355 ± 35.9	236 ± 14.4
Hy-P **nmol h^{-1} g^{-1} d.w**	561 ± 39.7 a	324 ± 17.8 b	269 ± 13.6 b

Table S4 Soil potential soil enzymatic activities (mean ± s.e), in forest (FO), pasture (PA) and meadow (ME) across the year. When present, different letters indicate significant differences (a) among sampling times in each land use (n = 3), and (b) among land use (n = 12)

Land use	Time	BG nmol h^{-1} g^{-1}d.w.	BGAL nmol h^{-1} g^{-1}d.w.	Cell nmol h^{-1} g^{-1}d.w.	Xilo nmol h^{-1} g^{-1}d.w.	CBZ nmol h^{-1} g^{-1}d.w.	NAG nmol h^{-1} g^{-1}d.w.	LAP nmol h^{-1} g^{-1}d.w.	AP nmol h^{-1} g^{-1}d.w.	BisP nmol h^{-1} g^{-1}d.w.	PiroP nmol h^{-1} g^{-1}d.w.	OX nmol h^{-1} g^{-1}d.w.	PEROX nmol h^{-1} g^{-1}d.w.
a)													
FO	T1	129 ± 30.4 ab	20.3 ± 4.37 b	8.67 ± 2.73 bc	17.7 ± 3.28 a	8.00 ± 2.08 b	59.0 ± 21.0	314 ± 99.5 ab	510 ± 74.1 a	39.7 ± 13.8	59.0 ± 26.7 ab	0.290 ± 0.0533 b	0.517 ± 0.0579 ab
	T2	110 ± 12.7 b	25.7 ± 2.19 ab	8.67 ± 0.882 ab	18.3 ± 2.19 a	12.0 ± 1.53 ab	70.0 ± 5.77	255 ± 27.5 ab	488 ± 41.2 a	42.7 ± 10.9	69.3 ± 20.1 a	0.249 ± 0.0477 b	0.409 ± 0.0231 b
	T3	153 ± 11.7 a	27.7 ± 2.73 a	11.0 ± 1.53 a	20.7 ± 2.19 a	15.0 ± 3.06 a	58.0 ± 2.65	338 ± 45.8 a	508 ± 22.2 a	49.3 ± 14.3	83.7 ± 28.1 a	0.384 ± 0.632 a	0.560 ± 0.0248 a
	T4	62.7 ± 7.22 c	13.3 ± 1.33 c	3.67 ± 0.333 c	9.33 ± 0.882 b	8.33 ± 0.88 b	28.7 ± 2.19	185 ± 28.4 b	336 ± 7.22 b	26.3 ± 7.36	34.7 ± 12.7 b	0.234 ± 0.0163 b	0.262 ± 0.008762 c
PA	T1	87.3 ± 8.41 b	18.0 ± 0.577 b	5.67 ± 0.882 bc	13.0 ± 1.15 b	12.3 ± 0.333 b	43.3 ± 4.70	279 ± 28.6	237 ± 9.29	56.0 ± 3.46 ab	49.0 ± 1.00	0.121 ± 0.00383	0.303 ± 0.0365 ab
	T2	85.7 ± 13.9 b	17.7 ± 2.60 b	9.67 ± 2.60 ab	12.7 ± 2.33 b	5.7 ± 0.333 c	26.3 ± 5.24	209 ± 11.2	169 ± 29.4	45.0 ± 4.04 b	28.0 ± 0.577	0.0711 ± 0.0168	0.234 ± 0.0424 b
	T3	131 ± 12.9 a	28.7 ± 2.73 a	12.3 ± 1.20 a	19.7 ± 1.86 a	21.7 ± 3.84 a	57.7 ± 3.38	419 ± 91.8	259 ± 6.43	73.3 ± 18.6 a	48.7 ± 8.88	0.1321 ± 0.0193	0.402 ± 0.0658 a
	T4	101 ± 6.03 ab	22.3 ± 1.76 b	6.67 ± 0.882 c	15.3 ± 1.20 ab	12.3 ± 1.33 b	45.0 ± 2.00	288 ± 39.2	237 ± 10.5	54.0 ± 8.54 ab	42.0 ± 2.00	0.106 ± 0.0063	0.333 ± 0.0276 ab
ME	T1	61.0 ± 5.51	14.3 ± 2.03	2.67 ± 0.667 bc	9.00 ± 1.15	15.0 ± 1.15 a	24.7 ± 3.71	165 ± 12.7 ab	171 ± 32.7	43.0 ± 9.07	33.7 ± 7.45	0.121 ± 0.0121	0.219 ± 0.0182
	T2	79.3 ± 5.55	19.7 ± 1.76	6.33 ± 1.20 ab	12.7 ± 1.20	6.00 ± 0.577 b	41.0 ± 3.61	226 ± 32.6 b	244 ± 32.8	39.0 ± 8.54	46.0 ± 7.02	0.147 ± 0.0445	0.307 ± 0.0174
	T3	83.3 ± 5.55	19.7 ± 1.45	7.00 ± 1.15 a	10.7 ± 0.333	15.0 ± 0.577 a	34.0 ± 3.61	165 ± 8.74 a	180 ± 13.8	30.0 ± 1.73	25.3 ± 1.20	0.107 ± 0.00517	0.212 ± 0.0153
	T4	77.9 ± 6.66	16.3 ± 1.86	4.33 ± 0.882 c	11.7 ± 0.882	12.0 ± 0.577 a	52.3 ± 28.4	189 ± 22.0 ab	212 ± 21.2	28.7 ± 3.48	25.3 ± 2.33	0.140 ± 0.0184	0.239 ± 0.0242
b)													
FO		114 ± 11.9 a	21.7 ± 1.97	8.00 ± 1.05 a	16.5 ± 1.49 a	10.8 ± 1.24	53.9 ± 6.62	273 ± 31.7	460 ± 28.3 a	39.5 ± 5.59	61.7 ± 10.9	0.289 ± 0.0277 a	0.437 ± 0.0376 a
PA		101 ± 7.51 ab	21.7 ± 1.66	8.58 ± 1.08 a	15.2 ± 1.15 ab	13.0 ± 2.01	43.1 ± 3.94	298 ± 33.4	225 ± 12.6 b	57.1 ± 5.60	41.9 ± 3.38	0.108 ± 0.00938 b	0.318 ± 0.0265 b
ME		75.2 ± 3.64 b	17.5 ± 1.02	5.08 ± 0.67 b	11.0 ± 0.598 b	12.0 ± 1.21	38.0 ± 7.08	186 ± 11.8	202 ± 14.9 b	35.2 ± 3.29	32.6 ± 3.39	0.129 ± 0.0122 b	0.244 ± 0.0138 c

TABLE S5a Variance contribution of soil total P, Mn and Ca concentrations in the RDA of soil enzyme activities. Asterisks indicate significance levels: * $p < 0.001$.

	Df	Variance %
P	1	36 *
Mn	1	17 *
Ca	1	12 *
Residual	32	35

TABLE S5b Variance contribution of soil total Ca, Mn, P and N concentrations in the RDA of soil enzyme activities stoichiometry ratio. Asterisks indicate significance levels: * $p < 0.01$; ** $p < 0.001$.

	Df	**Variance %**
Ca	1	41 **
Mn	1	16 *
P	1	9.3 *
Residual	31	31

Table S6 Soil biomass-specific enzymatic activities (mean ± s.e), in forest (FO), pasture (PA) and meadow (ME). When present, different letters indicate significant differences (a) among sampling times in each land use (n = 3), and (b) among land use (n = 12)

Land use	Time	BG_biomass nmol h^{-1} µg $dsDNA^{-1}$d.w.	BGAL_biomass nmol h^{-1} µg $dsDNA^{-1}$d.w.	Cell_biomass nmol h^{-1} µg $dsDNA^{-1}$d.w.	Xilo_biomass nmol h^{-1} µg $dsDNA^{-1}$d.w.-	CBZ_biomass nmol h^{-1} µg $dsDNA^{-1}$d.w.	NAG_biomass nmol h^{-1} µg $dsDNA^{-1}$d.w.	LAP_biomass nmol h^{-1} µg $dsDNA^{-1}$d.w.	AP_biomass nmol h^{-1} µg $dsDNA^{-1}$d.w.	BisP_biomass nmol h^{-1} µg $dsDNA^{-1}$d.w.	PiroP_biomass nmol h^{-1} µg $dsDNA^{-1}$d.w.	OX_biomass nmol h^{-1} µg $dsDNA^{-1}$d.w.	PEROX_biomass nmol h^{-1} µg $dsDNA^{-1}$d.w.
a)													
FO	T1	0.72 ± 0.14 a	0.11 ± 0.020 a	0.048 ± 0.013	0.099 ± 0.015 a	0.045 ± 0.010	0.032 ± 0.100	1.7 ± 0.47	2.8 ± 0.26 a	0.22 ± 0.066	0.33 ± 0.13	0.0016 ± 0.00026	0.0029 ± 0.00022 a
	T2	0.52 ± 0.072 ab	0.12 ± 0.013 a	0.041 ± 0.0041	0.087 ± 0.011 a	0.057 ± 0.0087	0.033 ± 0.020	1.2 ± 0.16	2.3 ± 0.19 b	0.20 ± 0.057	0.33 ± 0.10	0.0012 ± 0.00026	0.0019 ± 0.00016 bc
	T3	0.58 ± 0.011 a	0.10 ± 0.0042 ab	0.041 ± 0.0038	0.078 ± 0.0038 ab	0.056 ± 0.0080	0.21 ± 0.0094	1.3 ± 0.11	1.9 ± 0.17 bc	0.18 ± 0.042	0.30 ± 0.085	0.0014 ± 0.00016	0.0021 ± 0.000059 ab
	T4	0.31 ± 0.017 b	0.066 ± 0.027 b	0.018 ± 0.0018 b	0.046 ± 0.0017 b	0.041 ± 0.0020	0.14 ± 0.018	0.93 ± 0.16	1.7 ± 0.13 c	0.13 ± 0.028	0.17 ± 0.051	0.0012 ± 0.000029	0.0013 ± 0.000042 c
PA	T1	0.67 ± 0.045	0.14 ± 0.015	0.044 ± 0.0053 ab	0.10 ± 0.0054	0.097 ± 0.0090 a	0.34 ± 0.040	2.1 ± 0.13	1.9 ± 0.21 a	0.43 ± 0.020	0.38 ± 0.036 a	0.00094 ± 0.000056	0.0023 ± 0.00022
	T2	0.60 ± 0.11	0.12 ± 0.021	0.067 ± 0.020 a	0.089 ± 0.019	0.039 ± 0.0026 b	0.18 ± 0.039	1.5 ± 0.13	1.2 ± 0.22 b	0.31 ± 0.032	0.19 ± 0.011 b	0.00049 ± 0.00012	0.0016 ± 0.00035
	T3	0.53 ± 0.038	0.12 ± 0.012	0.050 ± 0.0034 ab	0.080 ± 0.0049	0.088 ± 0.014 a	0.24 ± 0.024	1.7 ± 0.31	1.1 ± 0.060 b	0.29 ± 0.069	0.20 ± 0.030 b	0.00053 ± 0.000064	0.0016 ± 0.00022
	T4	0.56 ± 0.044	0.12 ± 0.013	0.037 ± 0.0057 b	0.085 ± 0.0085	0.068 ± 0.0033 ab	0.25 ± 0.016	1.6 ± 0.13	1.3 ± 0.12 ab	0.29 ± 0.28	0.23 ± 0.0040 ab	0.00058 ± 0.000015	0.0018 ± 0.000062
ME	T1	0.69 ± 0.019	0.16 ± 0.014 a	0.030 ± 0.0054	0.102 ± 0.0082	0.17 ± 0.0076 a	0.30 ± 0.024	1.9 ± 0.16 ab	1.9 ± 0.26 a	0.50 ± 0.12 a	0.39 ± 0.099 a	0.0013 ± 0.000052	0.0025 ± 0.00011 ab
	T2	0.69 ± 0.022	0.17 ± 0.010 a	0.054 ± 0.0069	0.110 ± 0.0076	0.054 ± 0.0096 c	0.37 ± 0.065	2.0 ± 0.47 a	2.1 ± 0.14 a	0.36 ± 0.11 ab	0.42 ± 0.10 a	0.0014 ± 0.00054	0.0027 ± 0.00029 a
	T3	0.60 ± 0.051	0.14 ± 0.014 ab	0.051 ± 0.0089	0.077 ± 0.0043	0.11 ± 0.0061 b	0.25 ± 0.032	1.1 ± 0.091 b	1.3 ± 0.091 b	0.22 ± 0.016 b	0.18 ± 0.011 b	0.00077 ± 0.000057	0.0015 ± 0.00015 c
	T4	0.56 ± 0.021	0.12 ± 0.0043 b	0.031 ± 0.0024	0.086 ± 0.0077	0.089 ± 0.0091 b	0.34 ± 0.15	1.4 ± 0.17 ab	1.6 ± 0.22 ab	0.21 ± 0.027 b	0.18 ± 0.0068 b	0.0010 ± 0.00015	0.0017 ± 0.00016 bc
b)													
FO		0.53 ± 0.057	0.10 ± 0.0082 b	0.037 ± 0.0045	0.077± 0.0071	0.050 ± 0.0039 c	0.25 ± 0.032	1.3 ± 0.14	2.2 ± 0.16 a	0.18 ± 0.023	0.28 ± 0.047	0.0013 ± 0.00010 a	0.0021 ± 0.00018
PA		0.59 ± 0.032	0.13 ± 0.0071 ab	0.049 ± 0.0057	0.088 ± 0.0051	0.073 ± 0.0076 b	0.25 ± 0.021	1.7 ± 0.11	1.3 ± 0.11 b	0.33 ± 0.025	0.25 ± 0.026	0.00064 ± 0.000062 b	0.0019 ± 0.00013
ME		0.63 ± 0.022	0.15 ± 0.0078 a	0.041 ± 0.0043	0.093 ± 0.0050	0.105 ± 0.013 a	0.31 ± 0.038	1.6 ± 0.1	1.7 ± 0.12 ab	0.32 ± 0.051	0.29 ± 0.044	0.0011 ± 0.00014 ab	0.0021 ± 0.00017

According to the methods of soil chemical analyses (Colombo and Teodoro, 2015), pH was determined on sieved fresh soil samples by a potentiometric method (SenseION+PH3, Hach) in a soil suspension: distilled water (1:2.5 = v: v). On oven-dried sieved soil samples (105 °C for 48 h; MMM Group Ecocell), soil organic matter (SOM) was measured by calcination in muffle (550 ◦C for 4 h, Nabertherm GmbH, Controller B 170); labile and recalcitrant C were determined using a two-step acid hydrolysis procedure, as described by Rovira and others (2007). The labile fraction mainly consists of polysaccharides of plant and microbial origin, whereas the recalcitrant fraction includes cellulose-containing compounds that are more resistant to degradation than the labile fraction. Labile and recalcitrant C concentrations were measured by spectrophotometric analysis at 254 nm (UV–VIS Jenway 6715 Spectrophotometer), adapting the Standard Methods 5910 B (SM, 2000). The stable C residue was analysed with a CHNS elemental analyser (Carlo Erba 1500).

Soil total microbial biomass (MB) was measured from the double-stranded DNA (dsDNA) concentration using 0.25 g of fresh sieved in 0.12 M sodium phosphate solution at pH 7.8 as an extraction buffer (Fornasier and others 2014). PicoGreen reagent (Life Technologies, Carlsbad, CA, USA) was then used to quantify dsDNA, expressed as micrograms of dsDNA g^{-1} soil d.w. after being corrected for soil moisture content.

* Colombo C, Teodoro M (2015) Metodi di Analisi Chimica del Suolo. 978-88-940679-0-3, Pubblicità & Stampa, Modugno (BA), Italy

*Rovira, P., and Vallejo, V. R. (2007). Labile, recalcitrant, and inert organic matter in Mediterranean forest soils. Soil Biology and Biochemistry, 39, 202–215. https://doi.org/10.1016/j.soilbio.2006.07.021

*SM (2000) Standard methods for the examination of water and waste water. Method 5910 UV-Absorbing Organic Constituents

*Fornasier, F., Ascher, J., Ceccherini, M.T., Tomat, E., Pietramellara, G., 2014. A simplified rapid, low-cost and versatile DNA-based assessment of soil microbial biomass. Ecological Indicator. 45, 75–82

Table S7 Soil pH, soil organic matter (SOM), labile, recalcitrant and stabile organic matter fractions (C labile, recalcitrant and stabile) and microbial biomass (MB) (mean ± s.e), in forest (FO), pasture (PA) and meadow (ME). When present, different letters indicate significant differences (a) among sampling times in each land use (n = 3) and (b) among land use (n = 12)

Land use	Time	pH	SOM % soil d.w.	C LABILE mg/g d.w.	C RECALCITRANT mg/g d.w.	STABILE C mg/g d.w.	MB µg dsDNA g^{-1} d.w.
a)							
FO	T1	6.52 ± 0.228	25.6 ± 0.371 ab	22.8 ± 0.886 b	4.69 ± 0.375 a	133 ± 4.30 ab	177 ± 9.91 b
	T2	6.10 ± 0.0814	24.6 ± 0.130 b	22.7 ± 0.745 b	3.16 ± 0.600 a	119 ± 4.36 ab	212 ± 5.04 b
	T3	6.45 ± 0.154	28.0 ± 0.340 a	19.4 ± 0.462 b	2.79 ± 0.278 a	145 ± 8.14 a	265 ± 15.7 a
	T4	5.96 ± 0.0976	24.3 ± 0.777 b	33.6 ± 2.27 a	2.34 ± 0.361 b	110 ± 7.03 b	201 ± 11.9 b
PA	T1	6.31 ± 0.0705	22.5 ± 1.42 c	23.3 ± 1.38 b	5.37 ± 0.484 a	93.8 ± 9.74 c	130 ± 10.7 c
	T2	6.24 ± 0.0441	19.7 ± 1.96 d	23.9 ± 1.16 b	4.87 ± 0.349 a	103 ± 12.1 bc	144 ± 7.88 c
	T3	6.65 ± 0.148	30.8 ± 1.12 a	16.8 ± 0.212 c	4.48 ± 0.380 a	165 ± 5.67 a	245 ± 11.4 a
	T4	6.34 ± 0.0407	25.8 ± 1.16 b	29.4 ± 2.07 a	3.06 ± 0.394 b	130 ± 9.29 b	182 ± 10.9 b
ME	T1	6.64 ± 0.210	24.2 ± 1.30	30.4 ± 1.37 b	4.93 ± 0.488	110 ± 5.79	88.0 ± 5.51 b
	T2	6.40 ± 0.350	23.8 ± 0.836	30.8 ± 1.14 b	5.21 ± 0.844	118 ± 4.40	115 ± 9.02 ab
	T3	6.14 ± 0.0814	25.6 ± 0.299	30.0 ± 0.995 b	3.90 ± 0.131	115 ± 4.99	139 ± 3.76 a
	T4	6.54 ± 0.133	25.5 ± 0.173	42.2 ± 2.33 a	3.28 ± 0.893	113 ± 4.58	139 ± 17.1 a
b)							
FO		6.26 ± 0.0937	25.6 ± 0.445	24.6 ± 1.73 b	3.25 ± 0.315 b	127 ± 4.84 a	214 ± 11.0 a
PA		6.38 ± 0.0622	24.7 ± 1.45	23.4 ± 1.53 b	4.44 ± 0.255 a	123 ± 9.30 ab	175 ± 14.8 b
ME		6.43 ± 0.114	24.8 ± 0.432	33.4 ± 1.54 a	4.33 ± 0.315 a	113 ± 2.29 b	120 ± 8.03 c

CHAPTER 5

LAND USE DRIVES SOIL MICROBIAL NETWORK AND FUNCTIONS IN MEDITERRANEAN MOUNTAIN ECOSYSTEMS[3]

Abstract

Land use and management practices, such as grazing and mowing, can strongly influence soil microbial communities. This study assessed the impact of different land uses—pasture (PA), meadow (ME), and forest (FO)—on microbial structural and functional diversity in the Matese mountain area (Mediterranean region). Soil physico-chemical properties (water content, organic matter, and pH), enzymatic activities related to the C, N, and P cycles, and microbial biomass were analyzed. In addition, microbial diversity and microbial co-occurrence networks were investigated. Results showed that land use is a major driver of microbial biomass. Co-occurrence network analysis of interactions among microbial taxa showed that FO exhibited more complex microbial networks, reflecting a stable ecosystem. In contrast, PA and ME soils showed a progressive simplification of microbial network, although ME maintained high levels of microbial diversity. Differences in bacterial and fungal community composition reflected the influence of management practices and land use. Overall, this study highlights that sustainable grassland management is crucial for preserving soil microbial biodiversity and maintaining ecosystem functional stability.

[3] Esposito A., Picariello E., Tripied J., Terrat S., De Nicola F. Land Use Drives Soil Microbial Network And Functions In Mediterranean Mountain Ecosystems. Submitted in *Applied Soil Ecology*

Keywords

Microbial biomass; Co-occurrence network analysis; soil enzyme activity; land use; grazing, mowing

Introduction

Soil represents a reservoir of biodiversity, containing a 1/3 of the biodiversity present on Earth (FAO, 2020), with a huge quantity and diversity of microorganisms. The soil microbial community is also known to play key roles in maintaining ecological functions (Creamer et al., 2022; Huang et al., 2022), such as the nutrient cycling regulation (Creamer et al., 2022). Microorganisms, through the production of extracellular enzymes, promote the mineralization of organic matter and the transformation of nutrients (e.g. N, P and S) in bioavailable forms, essential for soil fertility and ecosystem productivity (Creamer et al., 2022; Luo et al., 2017).

Soil functions are closely linked to the microbial structure and activity, which respond to various factors including land use, soil physicochemical properties, and climatic conditions (Christel et al., 2024; Smith et al., 2021). These factors influence microbial structure and co-occurrence networks, thereby directly affecting the overall functioning of the soil. In particular, several studies have shown that organic carbon content is the main driver of microbial biomass, whereas soil pH and texture influence soil microbial diversity (Christel et al., 2024; Karimi et al., 2018). Land use also has a significant influence on microbial biomass and interaction networks: forests and grasslands are generally characterised by higher microbial biomass (Gschwend et al., 2021), and in forests greater co-occurrence network complexity has been observed compared to grasslands and croplands, likely due to less intensive management practices (Christel et al., 2024; Karimi et al., 2019).

Among the different types of land use, grasslands are among the most widespread ecosystems in Europe, covering 736,000 km^2

(https://ec.europa.eu/eu-grassland-watch/), and are the result of centuries of anthropogenic land use, constituting an important component of the cultural landscape (Hejcman et al., 2013). They also represent a fundamental resource for the production of fodder for livestock feed (Mayel et al., 2021; Richter et al., 2024). Depending on how they are used, grasslands can be classified as meadows, managed by mowing, or as pastures, characterised by direct livestock grazing (Wezel et al., 2021). Currently, increasing population and food demand is leading to intensive grassland management practices, with intensification of grazing and mowing practices (Mayel et al., 2021).

Mowing and grazing can reduce the amount of organic matter (Mayel et al., 2021; Wang et al., 2024), leading to a reduction in microbial biomass and diversity (Mencel et al., 2022; Wang et al., 2024), as microorganisms depend on organic inputs as a source of energy (Bastida et al., 2021). On the other hand, the release of excrement during grazing and the use of manure as a soil amendment can promote high soil organic carbon levels, thereby increasing microbial biomass (Brummerloh and Kuka, 2023). In addition, mowing increases soil sensitivity to daily temperature fluctuations, thereby further affecting microbial activity, and diversity (Wang and Liu, 2020).

Furthermore, the frequency and intensity of these practices can influence the soil microbial community (Mencel et al., 2022). Indeed, pastures characterized by spring rest grazing can improve plant diversity, contributing to the restoration of soil chemical-physical properties and, consequently, to an increase in both the amount and diversity of soil microorganisms (Khatri-Chhetri et al., 2022; Zhang et al., 2025). In recent years, numerous studies have analyzed the influence of different land uses, such as grasslands and forests, on the abundance and diversity of microbial communities (Karimi et al., 2018; Siles et al., 2023). In contrast, only a few studies have explored the specific effects of grazing and mowing on microbial communities in Mediterranean soils (Bonanomi et al., 2022; Gavrichkova et al., 2008). It is therefore necessary to further investigate the impacts of management

practices and land use on soil microbial communities to identify sustainable management strategies that promote soil resilience to disturbances. This study aims to evaluate the effects of three land uses (forest, pasture, and meadow) on soil activity, structure, and interaction within the soil microbial community. To achieve this goal, we evaluated: (i) several enzyme activities related to the biogeochemical cycles of C, N, and P; (ii) microbial biodiversity, using Hill numbers to determine microbial richness, exponential Shannon diversity, and inverse Simpson index for both bacterial and fungal communities; (iii) the composition of both bacterial and fungal communities. Finally, we analyzed microbial networks at the genus level, quantifying the complexity of microbial interactions for each land use using co-occurrence network analysis. This approach allows us not to consider taxa as isolated units, but to build an integrated picture of microbial communities and their surrounding environment, thus representing a promising tool for assessing the stability and functioning of ecosystems (Guseva et al., 2022; Karimi et al., 2017).

Materials and methods

Study area and soil sample collections

Within the Matese Mountains (Southern Italy), three adjacent sites differing in land use, forest (FO), pasture (PA), and meadow (ME), were selected at 1010 m a.s.l., on a homogeneous Pachi-Eutrisilic Andosol substrate (FAO, 1976). FO, dominated by European beech (*Fagus sylvatica* L.), is an unmanaged stand that has remained undisturbed since the early 20th century. PA has been used continuously for mixed grazing of cattle, sheep, and horses. Grazing occurs for approximately six months, from early summer to early winter, followed by a five-month spring rest-grazing. ME is managed as a forage production system. It was amended with manure and sown in June 2023 with a mixture of forage species. The meadow was mowed in July 2023. Samplings were conducted simultaneously at each site four times between

June 2023 and May 2024. The experimental design is therefore two-factorial, with the factors “land use” and “sampling time”. A completely randomized design was adopted, with three plots (5 × 5 m) established for each land use type. Within each plot, eight soil cores were collected at a depth of 0–10 cm and combined to obtain one composite sample per plot. Each composite sample was analysed independently. Before analysis, soils were sieved (< 2 mm) and stored at −20 °C for molecular analyses.

Soil chemical-physical and biological analysis

According to the methods of soil chemical analyses (Colombo and Teodoro, 2015), on fresh sieved soil samples, pH by a potentiometric method (SenseION+PH3, Hach) in a soil: distilled water (1:2.5 = v: v) suspension, and soil water content (WC) by oven-drying method (at 105 °C until constant weight), were measured.

On oven-dried sieved soil organic matter (SOM) was measured by calcination in muffle (550 °C for 4 h; Nabertherm GmbH, Controller B 170).

On fresh sieved soil samples, seven enzymatic activities were analysed by spectrophotometric method, as described in Picariello and De Nicola (2024). The enzymatic activities analysed were: arylsulfatase activity (AryS) linked to S cycle, β-glucosidase (BG) and laccase (Lacc) activity linked to C cycle, phosphatase activity (Phos) linked to P cycle, β-glucosaminidase activity (NAG) linked to N cycle and hydrolase activity (FDA) as potential total enzymatic activity.

On freeze-dried sieved soil samples, DNA was extracted and quantified following the standard protocol of the GenoSol platform (INRAe, France; Djemiel et al., 2024), which includes cell lysis, deproteinization, alcohol precipitation, and washing of the extracted nucleic acids. The amount of crude DNA, estimated by agarose gel electrophoresis, was used as an indicator of microbial biomass (BMM), as described by Dequiedt et al.

(2011). Bacterial and Fungal diversity was analyzed by metabarcoding of the 16S rRNA (V3–V4 regions) and 18S rRNA (V7–V8 regions) genes, following the methodology described by Cottin et al. (2025). The sequencing was performed using an Illumina MiSeq instrument (Illumina Inc., San Diego, CA, USA).

Bioinformatic analyses

Bioinformatic analyses were performed using BIOCOM-PIPE pipeline as described by Djemiel et al. (2020). In addition, to ensure homogeneous comparisons between samples, the data sets were normalized by randomly selecting 10,000 readings for each sample. The sequences thus obtained were then used to generate global OTU matrices with ReClustOR (Terrat et al., 2020) and to make taxonomic assignments by comparison with the SILVA r132 database (Quast et al., 2013).

Alpha diversity was estimated through Hill numbers, described by Alberdi and Gilbert. (2019), which represent the actual number of equiabundant OTUs and allow samples to be compared on a linear scale. The q parameter (order of diversity) regulates the weight given to OTUs as a function of their relative abundance. Hill numbers with q = 0 diversity corresponds to bacterial and fungal richness (B_ and F_ richness); giving equal weight to all taxa regardless of their abundance and thus emphasizing rare OTUs, q = 1, diversity corresponds to Shannon exponential (B_ and F_Shannon diversity) corresponding to common bacterial and fungal OTUs; When q = 2, diversity corresponds to the inverse Simpson index (B_ and F_Inverse Simpson), giving greater weight to the dominant bacterial and fungal OTUs.

The microbial co-occurrence networks analysis was performed, following the methodology described by Karimi et al. (2019), following two main steps: (i) standardization of the number of soil samples, fixed at 11 samples per land use, to avoid effects related to differences in sample size; (ii) generation of network replicates (12 in total) aimed at representing the natural

heterogeneity of soils within each land-use type. For each replicate, network construction was based on a contingency matrix containing the relative abundances of microbial genera in the 11 randomly selected soil samples. The networks were derived by calculating Spearman correlations among the abundance levels of microbial genera across samples, considering only correlations with coefficients greater than 0.60. Positive correlations were interpreted as co-occurrence (facilitation) relationships, while negative correlations were interpreted as co-exclusion (antagonistic) relationships.

The topology of the resulting networks was described using a set of metrics, including: the number of nodes, the total number of links, the number of bacterial links, the number of fungal links, the number of bacterial–fungal links, the connectance, and the ratio of positive to negative links (P:N).

Statistical analysis

The permutation analysis of variance (PERMANOVA) was applied to test the overall response of pH, WC, SOM, AryS, BG, Lacc, Phos, NAG, FDA, BMM, B_ and F_ richness, B_ and F_Shannon diversity, and B_ and F_Inverse Simpson against land use and sampling time (fixed factors). P-values were calculated using the Monte Carlo permutation test (999 permutations) together with the permutation of residuals under an unrestricted permutation model. To visualize the variation of the above-mentioned parameters among different land uses, we performed principal component analysis (PCA).

Since PERMANOVA did not show significant differences among sampling time, significant differences among land use in the aforementioned parameters, relative abundances of bacterial and fungal communities, and co-occurrence network metrics were evaluated using one-way ANOVA. When necessary, data were transformed by Box–Cox to meet ANOVA assumptions. If these assumptions were respected, a post hoc LSD test (least significant difference) was applied with p-values corrected according to the

Bonferroni method ($\alpha = 0.05$) for multiple comparisons; otherwise, the Kruskal–Wallis test was used, again with Bonferroni correction for multiple comparisons among land use.

Variance partitioning analysis was used to identify the main drivers of the shift in microbial community (BMM and bacterial and fungal diversity indices; explained variables). Physico-chemical properties (pH, WC, and SOM) and land use were selected as explanatory variables of the shift in microbial community. To reduce collinearity, explanatory variables with VIF > 4 were eliminated and the most relevant ones selected based on correct BIC and R^2. Finally, the variation explained by the explanatory variables was modelled through redundancy analysis, with progressive selection of variables and significance tests based on 10,000 permutations.

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Results

The PERMANOVA performed on soil chemical-physical properties, enzyme activities, BMM, B_ and F_richness, B_ and F_Shannon diversity, and B_ and F_Inverse Simpson highlighted overall significant differences only for the land use factor ($F = 15$; $p < 0.001$). The first two PCA components explained 61% of the variance, highlighting a clear separation between FO and ME (Figure 1). The FO soil was characterized by high values of BMM and enzymatic activities AryS and Lacc, whereas ME showed higher values of B_richness, B_Shannon diversity and B_Inverse Simpson index. The PA ellipses were partially overlapped with those of FO and ME, suggesting an intermediate pattern between the two land uses.

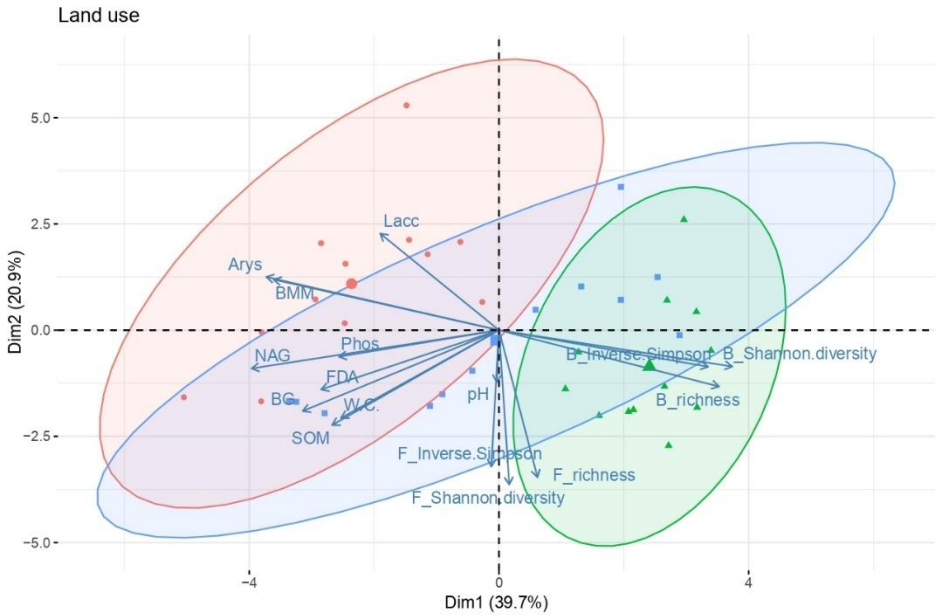

FIGURE 1 Biplot of the principal component analysis (PCA) with the superimposition of the confidence ellipses showing the differentiation among land uses. The graphs show the first two principal components (PC1 and PC2) and the variance explained by them. Forest (red), pasture (blue), meadow (green)

Analyzing individual parameters (Table 1), BMM and AryS enzyme activity differed significantly among all land uses ($p < 0.001$), following the trend FO > PA > ME; Lacc, Phos and NAG activities were higher in FO and PA compared to ME (at least $p < 0.05$), whereas BG was higher in PA than ME ($p < 0.05$). Also B_richness and B_Shannon diversity showed significant differences among all land uses ($p < 0.001$), with a different trend (ME > PA > FO) respect BMM and AryS; also B_Inverse Simpson index showed the lowest values in FO ($p < 0.001$) than ME and PA.

TABLE 1 pH, WC, SOM, AryS, Phos, Lacc, BG, NAG, FDA, BMM, B_ and F_richness, B_ and F_Shannon diversity, B_ and F_Inverse Simpson index (mean ± s.e) in forest (FO), pasture (PA) and meadow (ME). Significant differences among land use are indicated by different letters; n = 12

	FO	**ME**	**PA**
pH	6.257 ± 0.09620	6.430 ± 0.1091	6.380 ± 0.06015
WC **% soil d.w.**	71.41 ± 4.228	61.55 ± 5.835	62.40 ± 8.687
SOM **% soil d.w.**	25.62 ± 0.4843	24.80 ± 0.4152	24.69 ± 1.389
AryS **mg PNP g^{-1} soil d.w. h^{-1}**	2.117 ± 0.05154 a	0.6321 ± 0.04980 c	0.9970 ± 0.05841 b
Phos **mg PNP g^{-1} soil d.w. h^{-1}**	3.214 ± 0.4025 a	1.848 ± 0.2345 b	3.049 ± 0.4038 a
Lacc **µg ABTS g^{-1} soil d.w. h^{-1}**	39.28 ± 5.241 a	17.56 ± 1.415 b	32.63 ± 2.841 a
BG **mg PNP g^{-1} soil d.w. h^{-1}**	1.050 ± 0.05731 ab	0.7739 ± 0.06571 b	1.214 ± 0.1599 a
NAG **mg PNP g^{-1} soil d.w. h^{-1}**	0.4685 ± 0.05840 a	0.1627 ± 0.01775 b	0.3395 ± 0.05729 a
FDA **mg FDA g^{-1} soil d.w. h^{-1}**	0.4638 ± 0.05429 a	0.2987 ± 0.03802 ab	0.3533 ± 0.04755 b
BMM **µg soil g^{-1}**	283.0 ± 12.34 a	111.6 ± 8.769 c	220.9 ± 11.59 b
B_richness	909.3 ± 9.351 c	1002 ± 14.00 a	959.2 ± 11.97 b
B_Shannon diversity	298.5 ± 10.90 c	384.2 ± 8.881 a	342.3 ± 8.937 b
B_Inverse Simpson	111.0 ± 7.562 b	165.2 ± 5.623 a	144.0 ± 6.378 a
F_richness	527.4 ± 26.87	591.6 ± 20.81	584.4 ± 14.46
F_Shannon diversity	74.90 ± 6.870	88.24 ± 3.916	82.66 ± 3.303
F_Inverse Simpson	18.23 ± 0.7925	20.17 ± 1.369	19.86 ± 0.9801

Overall, the variance partitioning model explained 87% of the total variability for the explained variable BMM. A small proportion of the explained variance was attributable to interactions among the variables (5.5%). Among the main explanatory variables, land use accounted for the largest percentage, explaining 73% of the modelled variance. SOM explained smaller but significant proportions (8.0%).

Land use and pH contributed to the overall variance of the model for the explained variables B_richness (54 and 6.4 % respectively), B_Shannon diversity (60 and 5.4 % respectively), and B_Inverse Simpson (57 and 6.1 % respectively). In the model, no variable was found to be significant for the explained variables fungal diversity indices (Table 2).

TABLE 2 Explained variance (%) of the soil microbial biomass (BMM) and biodiversity indices according to soil physico-chemical and biological proprieties (pH, WC and SOM,) and land uses. Missing values indicate that the variable was not retained in the model. Significance level is indicated as follows: *p < 0.05. **p < 0.01. ***p < 0.001.

	BMM		**B_richness**		**B_Shannon**		**B_Inverse Simpson**	
	Explained variance (%)	F	Explained variance (%)	F	Explained variance (%)	F	Explained variance (%)	F
Land use	73	90 ***	54	19***	60	24***	57	21***
pH			6.4	4.5*	5.4	4.3*	6.1	4.6*
WC								
SOM	8.0	20***						
Residual	13		45		40		42	
Interactions	5.5		-5.6		-5.04		-5.6	
Model	87	72 ***	54	13***	60	16***	57	14***

Regarding the composition of the microbial community, *Proteobacteria, Bacteroidetes, Actinobacteria-p, Acidobacteria, and Planctomycetes* were

the most abundant bacterial phyla. Other phyla, such as *Chloroflexi, Gemmatimonadetes-p, Firmicutes, Dependentiae*, and *Latescibacteria-p*, showed relative abundances less than 5 %.

Relative abundances varied among land uses (Figure 2): *Proteobacteria* was more abundant in FO (37%; $p < 0.001$) than in ME and PA (32%); *Bacteroidetes* was more abundant in PA (31%; $p < 0.001$) than in FO and ME (19%); *Actinobacteria-p* was more abundant in ME (14 %; $p < 0.001$) compared to PA (9.9 %), whereas *Acidobacteria* was more abundant in FO and ME (7.5%; $p < 0.001$) compared to PA (5.4%).

Also, less abundant phyla showed significant differences among land use: *Chloroflexi, Firmicutes* and *Gemmatimonadetes-p* were more abundant in ME; *Dependentiae* in PA, whereas *Latescibacteria-p* in FO (Figure 2).

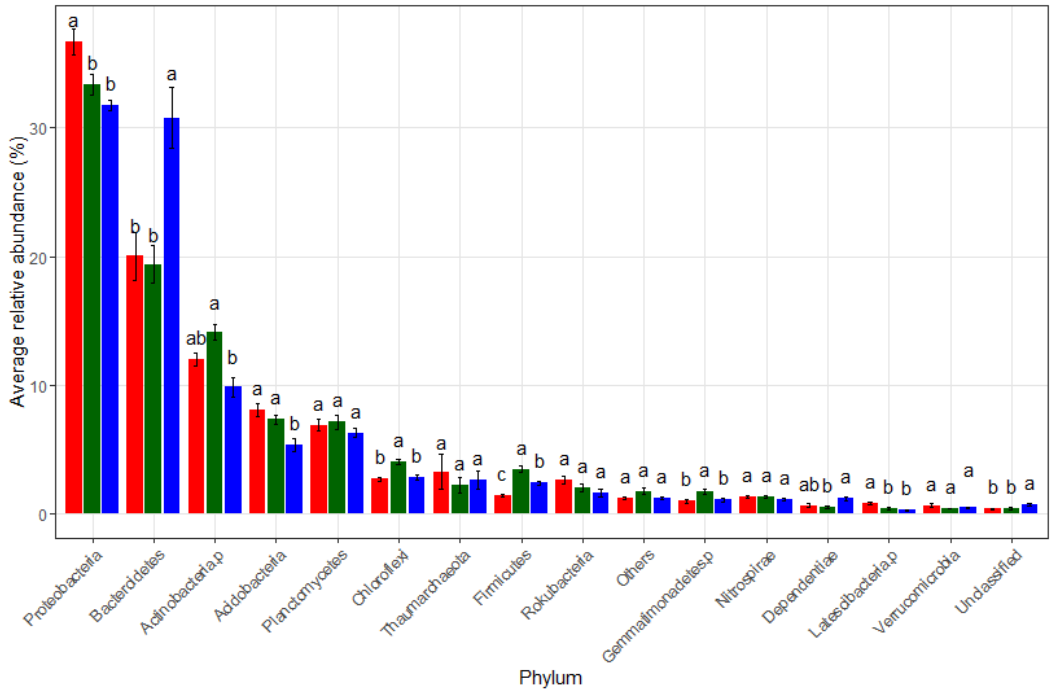

FIGURE 2 Bar plots (mean values ± SE) of the relative abundance of bacterial phyla in forest (red), pasture (blue), and meadow (green).“Others” represents the sum of all bacterial phyla representing fewer than 0.5% of sequences. Different letters indicate significant differences among land use.

Regarding the fungal community, the phyla *Ascomycota, Basidiomycota, Mucoromycota*, and *Cryptomycota* were the most abundant. Relative abundances significantly differed among land uses (Figure 3), for *Basidiomycota* ($p < 0.001$), with abundance in FO (44%) > PA (33%) >ME (27%), and *Ascomycota* ($p < 0.001$) with abundance in ME (42%) and PA (35%) higher than FO (27%). Finally, *Cryptomycota* showed higher ($p < 0.001$) relative abundances in ME (5.9%) and PA (5.4%) compared to FO (2.4%); *Chytridiomycota* showed higher ($p < 0.05$) relative abundances in PA (4.6%) than in FO (2.7%).

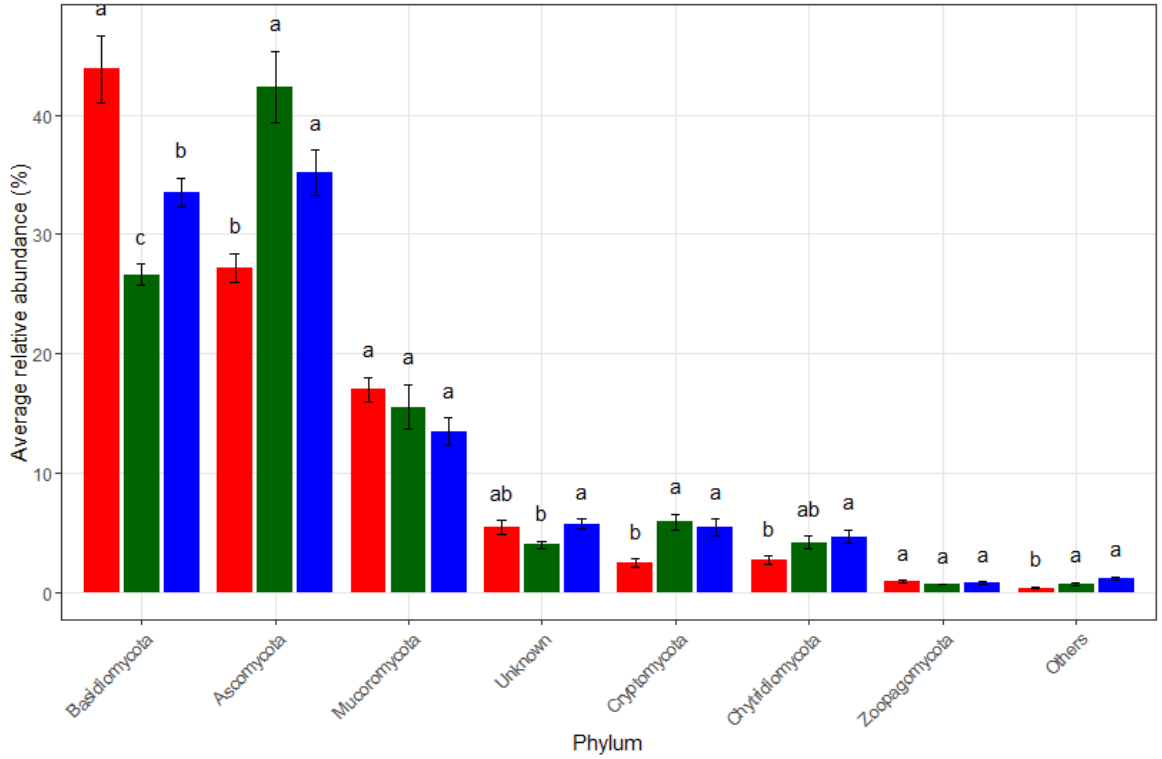

FIGURE 3 Bar plots (mean values ± SE) of the relative abundance of fungal phyla in forest (red), pasture (blue), and meadow (green). Different letters indicate significant differences among land use. "Others" corresponds to the sum of all fungal phyla representing fewer than 0.5% of sequences.

Also, microbial co-occurrence network metrics showed significant differences ($p < 0.001$) among the three land uses: the highest number of links, overall connectance, fungal links, and links between bacteria and fungi,

were found in FO, followed by ME and PA (FO > ME > PA). In contrast, the P:N ratio was highest in ME, exceeding FO and PA (ME > FO > PA). The number of nodes was higher in ME ($p < 0.001$) than FO and PA. The links between bacteria were also higher in FO ($p < 0.001$) than in ME and PA (Table 3; Figure 4a-c).

TABLE 3 Microbial networks metrics – Number of links and nodes, connectance, P:N ratio, links between bacteria, links between fungi and links between bacteria and fungi (mean ± s.e) – among three land use: Forest (FO), Meadow (ME) and Pasture (PA). Values with different letters differ significantly. The number of replicates used to evaluate the range of variation within and among land uses was set at 12. The samples were drawn independently for each of the 12 network replicates analyzed at genus level.

	Land use		
	FO	ME	PA
Number of links	5742 ± 250.8 a	2680 ± 70.57 b	2038 ± 62.28 c
Number of nodes	701.5 ± 0.3589 b	723.2 ± 0.3658 a	702.8 ± 0.5050 b
Connectance	0.02337 ± 0.001015 a	0.01026 ± 0.0002676 b	0.008267 ± 0.0002552 c
P:N ratio	1.309 ± 0.01201 b	1.451 ± 0.009671 a	1.235 ± 0.008949 c
Links between bacteria	2723 ± 100.9 a	1426 ± 43.71 b	1256 ± 37.27 b
Links between fungi	922.4 ± 36.59 a	366.1 ± 7.568 b	234.3 ± 8.340 c
Links between bacteria and fungi	2108 ± 115.5 a	888.5 ± 24.30 b	547.8 ± 18.78 c

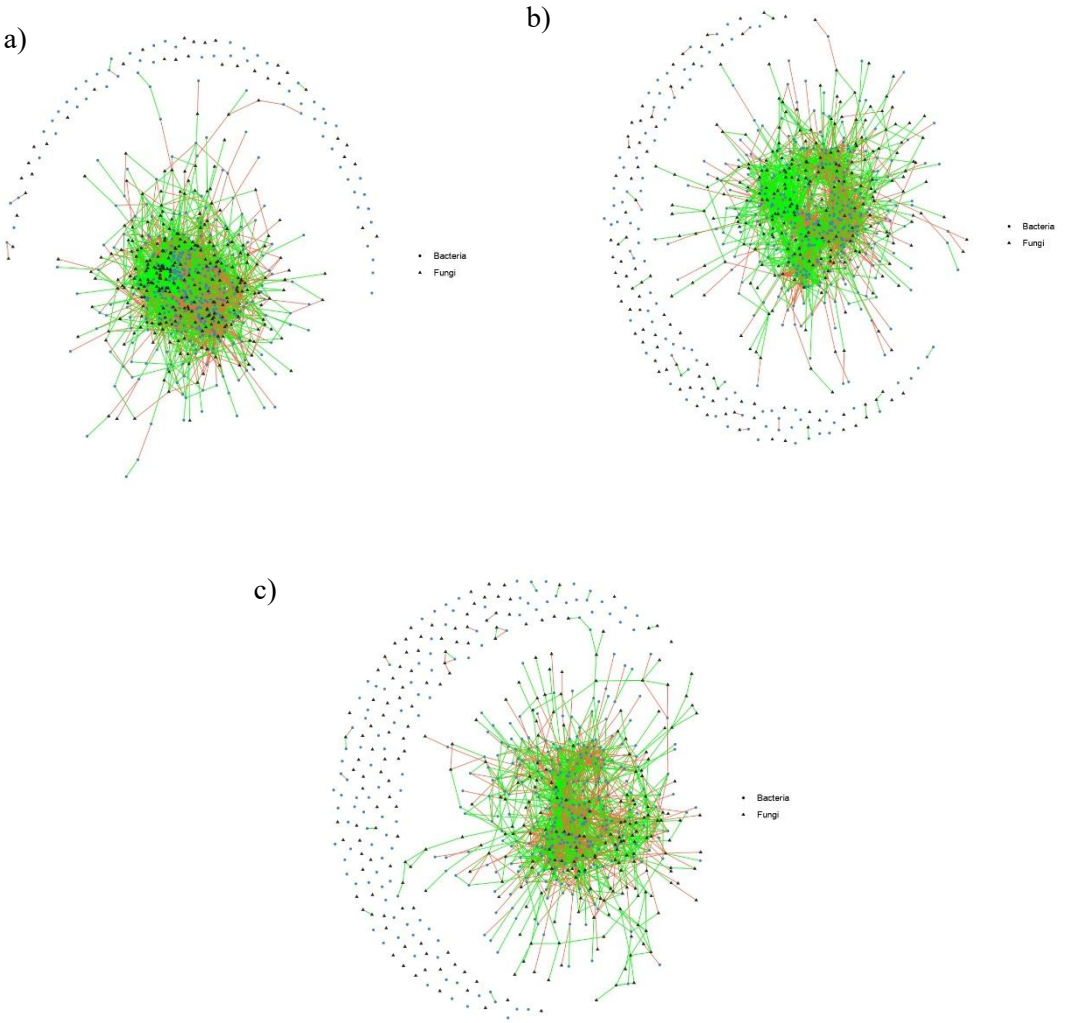

FIGURE 4 Visualization of the most complex microbial co-occurrence networks (with the most links and the highest connectance) among the twelve network replicates for each land use: Forest (a), Meadow (b) and Pasture (c). The red edges represent the negative links, and the green edges represent the positive links. Bacteria are represented by circles, whereas fungi are represented by triangles.

Discussion

In this study, the results demonstrate that land use is a key determinant of soil microbial biomass, functions, and structure. Forest soils exhibited significantly higher microbial biomass than pastures and meadow soils, as well as greater enzymatic activities, particularly when compared to meadows. Consistently with the variance partitioning analysis, land use was identified as the main driver of microbial biomass, followed by the soil organic matter content and pH.

The enhanced microbial biomass and enzyme activity in forest soil can be attributed to the denser vegetation cover and the continuous litter inputs, which increase soil organic matter availability and provide a source of carbon and energy for soil microorganisms. These findings are in agreement with a previous study showing that forest ecosystems support more active and functionally diverse microbial communities than managed grasslands (Wang et al., 2012).

In pasture soil, microbial biomass and activity were lower than in the forest but higher than in meadow, likely due to the organic input derived from livestock excreta, root exudates, and rhizodeposition (Schmidt et al., 2011; Cotrufo et al., 2024), may can partially compensate for the reduced plant litter input and support microbial processes (Mencel et al., 2022).

Overall, these findings confirm that land use acts as the main driver of soil microbial biomass, primarily through its control on organic matter inputs (Christel et al., 2024; Liu et al., 2022; Wallenius et al., 2011).

In our study, land use affected the structural complexity of microbial co-occurrence networks, revealing a progressive loss of connections with increasing management intensity. Network metrics showed that forest soils hosted highly interconnected microbial communities, characterized by numerous intra- and inter-group (bacteria–fungi) interactions, a pattern typically associated with stable ecosystems. Several studies have shown that in low-disturbance environments, such as forests, the availability of diverse resources and stable microclimatic conditions promotes cohesion, interaction strength, and resilience within microbial communities (Karimi et al., 2018).

Conversely, meadow and pasture soils exhibited a marked reduction in connectivity and interaction number, indicating a structural simplification of the microbial networks. This pattern is likely related to management practices such as mowing and grazing, which alter resource inputs, soil structure, and microbial habitats. These findings are consistent with previous research linking anthropogenic management practices to a reduction in microbial

network complexity and stability (Christel et al., 2024; Karimi et al., 2019). Despite the reduced network complexity, meadow soils showed high bacterial diversity, as indicated by the indices calculated. This apparent decoupling between diversity and network complexity is consistent with the intermediate disturbance hypothesis, whereby moderate disturbance levels promote species coexistence by limiting competitive exclusion (Christel et al., 2024; Tardy et al., 2015). In this context, the management regime of the meadow, characterized by annual mowing and manure application, likely creates heterogeneous and transient resource conditions that sustain high taxonomic diversity.

However, the low network complexity in meadow and pasture soils can suggest a dominance of opportunistic species, capable of growing independently and establishing a limited number of ecological interactions with other taxa (Karimi et al., 2019; Labouyrie et al., 2023).

Overall, these findings indicate that forest soils function as highly organized ecosystems, consisting of a mosaic of interconnected ecological niches occupied by stable, non-opportunistic taxa. In contrast, meadow and pasture soils exhibit a progressive loss of microbial cohesion and a simplification of ecological networks, while maintaining high biodiversity dominated by opportunistic species typical of moderately disturbed environments. Regarding the bacterial community composition, *Proteobacteria* dominate across all land-use types, with higher relative abundances in forest compared to pasture and meadow. This pattern is consistent with their copiotrophic strategy and preference for carbon-rich environments (Jenkins et al., 2010; Lienhard et al., 2014). However, among *Proteobacteria,* only the *Gammaproteobacteria* class was abundant in meadow soils (Figure S1), likely reflecting the higher labile carbon content at this site (Table S1), derived from manure inputs used as amendments, and mowing residues. Organic amendments have been shown to promote this bacterial group by

increasing easily accessible carbon pools (Cui et al., 2023; Piazza et al., 2019).

Filamentous *Actinobacteria-p* are more sensitive to soil structural degradation compared to other bacterial groups, as confirmed by the lowest value in pasture soil, compacted by livestock trampling (Lienhard et al., 2014). The prevalence of this group in mowed grasslands is reported in previous studies (Liu et al., 2022; Mencel et al., 2022). Conversely, the high abundance of *Bacteroidetes* in pasture reflected its higher soil phosphorus content (data not shown). A positive relationship between *Bacteroidetes* and soil phosphorus content was also reported by Hermans et al. (2017).

Also the fungal community composition, as bacteria, differed among land uses. *Ascomycota* dominated in meadow and pasture soils, whereas the *Basidiomycota* were more abundant in forest soils, consistent with previous studies in grasslands and forest ecosystems, respectively (Chen et al., 2017; Navarro-noya et al., 2021). Finally, *Chytridiomycota* were more abundant in pasture soils, where their resilience to physical stress allows for a rapid recovery following grazing disturbance (Gleason et al., 2004; Mayel et al., 2021).

Conclusions

Overall, the findings of this study showed that land use is a key driver of soil microbial biomass, activity and network organization. Forest soils supported higher microbial biomass, enzymatic activity, and a more complex and interconnected microbial network, reflecting a stable and functionally integrated ecosystem sustained by a high content of organic matter. In contrast, meadow and pasture soils showed a progressive structural simplification of microbial network and a reduced connectivity, although meadows maintained high levels of diversity, consistent with the intermediate disturbance hypothesis.

At the taxonomic level, differences in microbial composition reflected the influence of management disturbance and soil nutrient (C and P) pool. These results highlight that soil management not only regulates the biomass and activity of microbial community, but also the complexity of ecological interactions. Maintaining a balance between disturbance intensity and organic matter conservation therefore emerges as a crucial strategy for preserving soil microbial biodiversity and stability in Mediterranean mountain ecosystems.

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Supplementary material

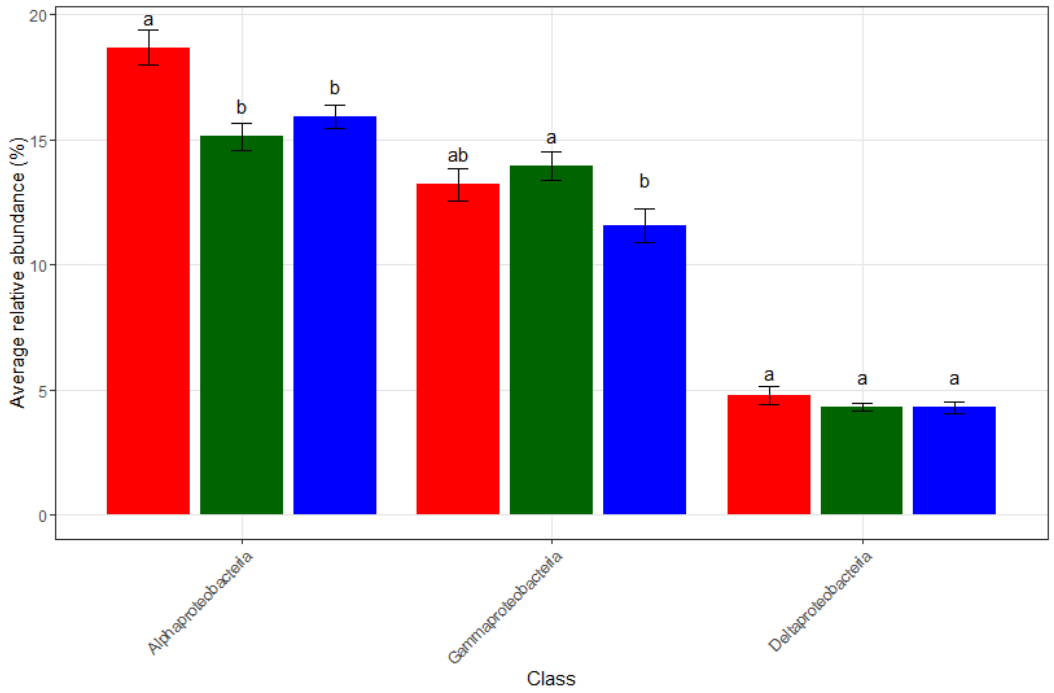

FIGURE S1 Bar plots (mean values ± SE) of the relative abundance of *Proteobacteria* class in forest (red), pasture (blue), and meadow (green). Different letters indicate significant differences among land use.

On air-dried and sieved soil samples, labile and recalcitrant C were determined using a two-step acid hydrolysis procedure, as described by Rovira et al. (2007). The labile fraction mainly consists of polysaccharides of plant and microbial origin, whereas the recalcitrant fraction includes cellulose-containing compounds that are more resistant to degradation than the labile fraction.

Labile and recalcitrant C concentrations were measured by spectrophotometric analysis at 254 nm (UV–VIS Jenway 6715 Spectrophotometer), adapting the Standard Methods 5910 B (SM, 2000). The stable C residue was analysed with a CHNS elemental analyser (Carlo Erba 1500).

TABLE S1 Labile C, recalcitrant C, and stable C (mean ± s.e) in forest (FO), pasture (PA) and meadow (ME) across the year. Significant differences among land use are indicated by different letters; n = 12

Land use	Labile C mg/g d.w.	Recalcitrant C mg/g d.w.	Stabile C mg/g d.w.
FO	24.6 ± 1.70 b	3.25 ± 0.321 b	127 ± 4.84 a
PA	23.4 ± 1.47 b	4.44 ± 0.311 a	123 ± 9.30 ab
ME	33.4 ± 1.68 a	4.33 ± 0.368 ab	113 ± 2.29 b

*Rovira, P., and Vallejo, V. R. (2007). Labile, recalcitrant, and inert organic matter in Mediterranean forest soils. Soil Biology and Biochemistry, 39, 202–215. https://doi.org/10.1016/j.soilbio.2006.07.021

*SM (2000) Standard methods for the examination of water and waste water. Method 5910 UV-Absorbing Organic Constituents

CHAPTER 6

MAINTAINING BALANCE IN MANAGED GRASSLANDS: THE ECOLOGICAL IMPACT OF MOWING AND GRAZING ON SOIL MULTIFUNCTIONALITY[4]

Abstract

Human management practices, such as grazing and mowing, can influence soil functions, as well as multifunctionality, defined as the ability of soil to perform multiple functions simultaneously. This study assessed the impact of different land uses and their associated management practices - pasture (PA) meadow (ME), and forest (FO) - on the soil multifunctionality throughout the year, in the Matese mountain area (Mediterranean region). Soil samples were collected across four times, relating to management practices in ME and PA areas: sowing, mowing, five months after mowing and one year after sowing in ME; at the beginning, after one and after six months of grazing, and after five months of not grazing in PA. To assess multifunctionality, several soil variables (8 enzymatic activities, soil microbial biomass, P and S content), were normalized and integrated into five soil functions (C, N, P and S cycles and decomposition), then used to quantify soil multifunctionality by the averaging and the threshold approaches. Results showed that the type and intensity of the disturbance influenced multifunctionality. PA and FO exhibited higher values of soil functions and multifunctionality compared to ME. In PA, after six months of grazing, C and S cycles, decomposition function and soil multifunctionality,

[4] Esposito A., Picariello E., Gómez-Brandón M., Fornasier F., De Nicola F. Maintaining Balance in Managed Grasslands: The Ecological Impact of Mowing and Grazing on Soil Multifunctionality. Under review in *Soil use and Management*

increased compared to other times. In ME, mowing practice positively affected multifunctionality, which remained stable throughout the year, leading to an increase in the nitrogen cycle, as highlighted by the threshold approach. Our findings highlight that this type of grazing pressure and the annual mowing contributed to maintaining soil multifunctionality.

Keywords

biogeochemical cycles; decomposition; grazing; land use; microbial biomass; mowing; soil multifunctionality

Introduction

Soils play a crucial role in delivering ecosystem services, such as food production, nutrient cycling, water retention, biodiversity conservation, and carbon sequestration (Guerra et al., 2022). The ability of soils to provide these services depends on their capacity to perform ecological functions, such as decomposition and nutrient cycling, which contribute to carbon storage and soil fertility. Soil biota play a key role in driving these functions, which together determine soil multifunctionality, defined as the ability of soil to perform multiple functions simultaneously (Creamer et al., 2022).

Soil multifunctionality in many terrestrial ecosystems is significantly affected by soil degradation (FAO, 2021), which results from practices like grazing, fertilization, and mowing. These practices place increasing pressure on soils, leading to a decline in microbial biodiversity (Labouyrie et al., 2023; Romero et al., 2024) and impairing the overall functionality of the soil (Zhu et al., 2021). Grasslands are among the largest terrestrial biomes, covering 736 000 km^2 in Europe (https://ec.europa.eu/eu-grassland-watch/), and most of them are maintained by human management, mainly grazing and mowing. Low- or moderate-intensity grazing regimes in pastures and mowing regimes in meadows increase biodiversity and productivity, enhancing a range of ecosystem services (Wezel et al., 2021; Schils et al., 2022). Pastures and

meadows soils are subjected to continuous changes in physical, chemical and biological properties due to livestock trampling and forage removal, which mutually impact soil function dynamics (Mayel et al., 2021). In pastures, livestock trampling causes soil compaction, leading to soil erosion (Dong et al., 2022), a decrease in microbial biomass (Proesmans et al., 2022), nutrient loss, and a decline in soil multifunctionality (Zhu et al., 2021). On the other hand, livestock excrement contribute to an increase in soil organic matter content and can influence nutrient cycle and release (Bielińska et al., 2017; Gilmullina et al., 2020). In contrast, mowing reduces the return of biomass to the soil (Li et al., 2017; Mayel et al., 2021), limiting the availability of substrate for soil microorganisms, which affects microbial biomass (Gilmullina et al., 2020) and matter cycle. Although some studies have simultaneously examined the effects of different land use and associated management practices, such as grazing and mowing, on grassland biodiversity (Delgado-Baquerizo et al., 2016; Tälle et al., 2016; Chen et al., 2021), to our knowledge, few researches have directly compared how these practices simultaneously influence soil multifunctionality (Wang et al., 2020; Zhu et al., 2021). Since the intensity and frequency of management practices, particularly during periods of active intervention, strongly influence the soil functionality by altering soil chemical properties, nutrient cycle and soil carbon stock (Mayel et al., 2021), it becomes crucial to provide a general recommendation on the most suitable management option. This is especially relevant in the context of future climate change scenarios, which are expected to decrease soil organic carbon stock in grasslands (Doblas-Rodrigo et al., 2022).

The aim of this study was to evaluate how soil multifunctionality varies across three types of land uses - forest, pasture and meadow - and how it changes over time in relation to their associated management practices within one year, and in response to seasonal dynamics. To achieve this objective, we considered five soil functions (related to the major element cycles and

decomposition) to evaluate the multifunctionality by both the averaging approach (Soil Multi Functionality index) and the threshold approach (Byrnes et al., 2014). The multifunctionality averaging method has some limitations, as it cannot distinguish between two functions with similar values or when a function with high values compensates another with low values. To address this, we also used the threshold approach, which counts functions exceeding critical thresholds simultaneously.

We hypothesise that (1) soil multifunctionality would be lower in managed systems, pasture and meadow, compared to the unmanaged forest; (2) the impacts on multifunctionality would be generally greater during periods of active management, thus when grazing and mowing practices occur.

Materials And Methods

Study area and soil sample collections

The study area is located on Matese mountain (Southern Italy) and characterised by an average annual temperature of 16 °C, with July as the hottest (average temperature 27 °C) and January as the coldest (average temperature 8 °C) month. The total amount of annual precipitation is 1218 mm, occurring predominantly in autumn and spring. The selected study area is located at an elevation of 1014 m above sea level (a.s.l.), on a lithological substrate classified as Pachi-Eutrisilic Andosol (FAO, 1976). Within a 1 km^2 area, three adjacent sites were selected, each characterized by a different land use: pasture (PA), meadow (ME) and forest (FO). Both PA and ME have been managed since 1982, although with different management practices. PA is a mixed grazing system for cattle, sheep and horses, continuously managed since 1982 with a stocking rate of 1.9 units of livestock/ha. Grazing occurs over a six-months period, from the beginning of summer until the beginning of winter. Followed by 5 months of spring rest. ME is a forage meadow, amended with manure and sown in 2023 with a species mixture consisting of 26% *Lolium perenne* L., 15% *Lolium multiflorum* L., 13% *Trifolium pratense*

L., 11% *Dactylis glomerata* L. and *Festuca Arundinacea* L., 10% *Phleum pratense* L. and 7% *Lotus corniculatus* L. and *Trifolium repens* L. FO is a forest dominated by the European beech (*Fagus sylvatica* L.). A completely randomized design was implemented, consisting of three adjacent plots (5 x 5 m) for each land use type. Samplings were carried out four times from June 2023 to May 2024 (June-T1; Jul -T2; December-T3 and May-T4) relating to specific management practices in ME (mowing) and PA (grazing) sites (Figure 1). The experimental design followed a two-factorial scheme, with the factors “Land use” and “Time”. At each plot, eight soil cores were collected at 0-10 cm depth (the most biologically active fraction) and pooled in order to obtain a homogeneous sample. Each soil sample was sieved (< 2 mm) and stored at -20°C for the soil biological analysis. Soil samples from each plot were separately analysed, with 3 laboratory replicates for each plot.

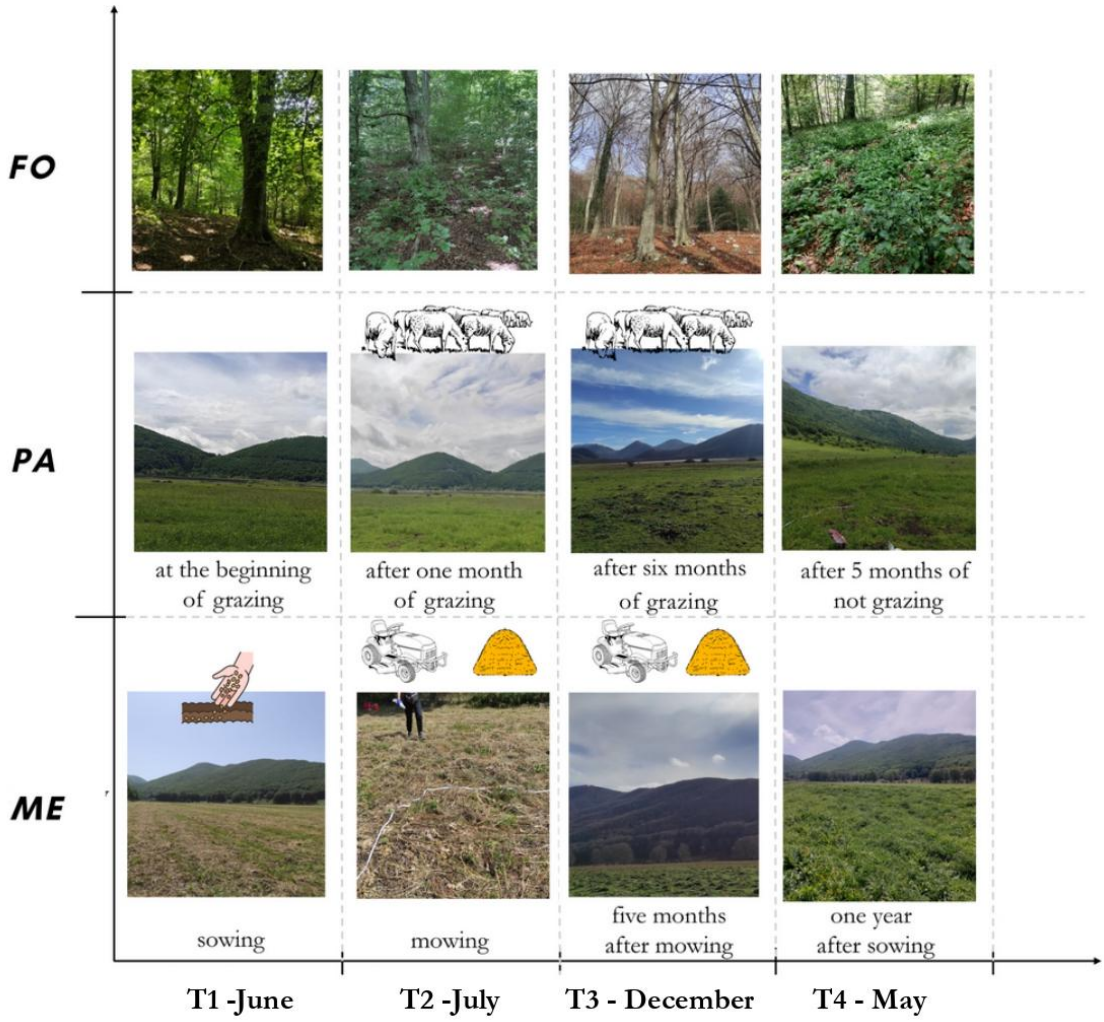

FIGURE 1 Sampling times (T1, T2, T3, T4) from June 2023 to May 2024 in Forest (FO), related to specific management practice in adjacent land use: in Pasture (PA) - at the beginning of grazing (T1), after one month (T2), after six months (T3), and after five months of not grazing (T4); and in Meadow (ME) - at sowing (T1), at mowing (T2), five months after mowing (T3), and one year after sowing (T4).

Soil chemical analysis

According to the methods of soil chemical analyses (Colombo and Teodoro, 2015), on fresh sieved soil samples, pH by a potentiometric method (SenseION+PH3, Hach) in a soil: distilled water (1:2.5 = v: v) suspension, and soil water content (WC) by oven-drying method (at 105 °C until constant weight), were measured. On oven-dried sieved soil samples (105 °C for 48 h; MMM Group Ecocell), soil grain size distribution was determined by a

combination of sieving of the >63-μm fraction and the sedimentation method based on Stokes' Law, for silt and clay fractions (2 to 63 μm) (Gee and Dani, 2002); soil organic matter (SOM) was measured by calcination in muffle (550 ◦C for 4 h, Nabertherm GmbH, Controller B 170) and soil organic carbon (SOC) calculated considering that the average carbon content in the SOM is ~ 58% (Pribyl, 2010). Phosphorus (P) and sulphur (S) total contents were determined by optical emission spectrometry with inductively coupled plasma (Thermo Scientific TM iCAP PRO XP Duo ICP-OES) after microwave-assisted acid digestion (USEPA, 1996). The soil physico-chemical properties of each land use were shown in the supplementary material (Table S1).

Soil biological analysis

Soil enzyme activities

Twelve enzymatic activities related to the main biogeochemical cycles were measured: α- and β-glucosidases (alfaG and betaG), cellobiohydrolase (cell), xylosidase (xilo), and glucuronidase (uroni), involved in the C cycle; chitinase (chit), leucine-aminopeptidase (leu), involved in N cycle; acid phosphomonoesterases (acP), phosphodiesterase (bisP), pyrophosphate-phosphodiesterase (piroP), involved in P cycle; arylsulphatase (aryS) involved in S cycle, and fluorescein diacetate (FDA) hydrolase, a proxy of total heterotrophic microbial activity (Gan et al., 2022).

Enzyme activities were determined on fresh sieved soil samples (0.25 g fresh weight, f.w.) by applying a heteromolecular exchange procedure (Cowie et al., 2013; Bardelli et al., 2017; Gómez-Brandón et al., 2022) using a lysozyme solution (3%) and bead beating step by using a Retsch 400 beating mill at 30 strokes s-1 for 3 min. The supernatant containing the desorbed enzymes was then centrifuged at 20,000 g for 3 min. Afterwards, 20 μL of diluted extracts were pipetted in duplicate on 384-well microplates with 50 μL of appropriate buffer in order to quantify enzyme activities using a

Synergy HT microplate reader (BIO-TEK). The activities were expressed as nanomoles of 4-methyl- umbelliferyl (MUF) h^{-1} g^{-1} soil (dry weight, d.w.), except for leucine-aminopeptidase and fluorescein diacetate hydrolase, whose activities were given as nanomoles of 7-amino-4-methyl coumarine (AMC) h^{-1} g^{-1} soil (d.w.) and nanomoles of carboxyfluorescein h^{-1} g^{-1} soil (d.w.), respectively.

Soil microbial biomass index (dsDNA)

Soil total microbial biomass (SMB) was measured from the double-stranded DNA (dsDNA) concentration using 0.25 g of fresh sieved in 0.12 M sodium phosphate solution at pH 7.8 as an extraction buffer (Fornasier et al., 2014). PicoGreen reagent (Life Technologies, Carlsbad, CA, USA) was then used to quantify dsDNA, expressed as nanograms of dsDNA g^{-1} soil d.w. after being corrected for soil moisture content.

Data treatment

Soil functions and mutifunctionality

The measured soil variables were used to calculate five soil functions: C (alfaG, betaG, cell, xilo and uroni), N (chit, leu), P (acP, piroP, bisP and P total) and S (aryS and S total) cycles and decomposition (SMB and FDA). Enzyme activities and microbial biomass were normalized per gram of organic carbon in each land use, given the positive correlation observed among these variables and SOC, to ensure consistent assessment of soil functions independently of SOC-driven variation (Figure S1a-c).

To ensure comparability and prevent a single function from having an overly strong weight in the calculation of multifunctionality, a variable selection was performed only for functions with four or more measured variables. For the functions C and P cycles, where four or more soil variables were measured, the selection was performed by principal component analyses (PCA), using the method described by Silva-Olaya et al. (2024), selecting

principal components with eigenvalue 1 (Kaiser criterion) and retaining the variables with weight loading ≥ 50% of the maximum value in each component. Subsequently, a correlation analysis was carried out among the variables maintained to identify possible redundancies. Consequently, two variables (betaG and uroni) and three variables (acP, piroP, and total P) were selected for the function C and P cycles, respectively. The results of this analysis are shown in Supplementary material (Table S2a, b). Subsequently, all variables related to the five functions were normalized on an ordinal scale from 0 to 1, and scores were given by applying the "more is better" or "less is better" function. The "more is better" function has been assigned to all variables (Cherubin et al., 2016; Silva-Olaya et al., 2024), using the equation S = X/Xmax, where S is the normalized value, X the measured value and Xmax is the maximum value observed in the study area. Soil functions were calculated by averaging the normalized variables (Byrnes et al., 2014).

Regarding the multifunctionality, two independent approaches, for each land use and sampling time, were used: (i) the averaging approach; (ii) the threshold approach (Byrnes et al., 2014). In the first approach, we calculated the soil multifunctionality index (SMF index) by averaging all the above-mentioned functions. In the second approach, we considered the same soil variables included in the above-mentioned soil functions. Three thresholds (25%, 50% and 75% of the maximum value, calculated as the average of the top 10% highest of values for each soil variable), were selected according to other studies (Cui et al., 2020; Zhang et al., 2023; Xu et al., 2024;) to evaluate how many functions exceed progressively more stringent levels. Multifunctionality was then estimated by calculating the number of functions that exceeded each of the three thresholds.

Statistical analysis

Correlation matrices among SOC and biological variables were calculated in FO, PA and ME. For this, a two-tailed Spearman correlation analysis was

performed. The permutation analysis of variance (PERMANOVA) was applied to test the overall response of the C, P, N and S cycles and decomposition functions against land use and time in each land use. P-values were calculated using the Monte Carlo permutation test (999 permutations) together with the permutation of residuals under an unrestricted permutation model. We used omega-squared (ω2) to incorporate the effect size in PERMANOVA tests. To visualize the variation of C, P, N and S cycles and decomposition functions among the different land uses and sampling times, we performed the PCA analysis. Significant differences in the investigated soil variables and their calculated functions were evaluated by two-way repeated measurements ANOVAs for the factors land use and time. Tukey HSD test, with Bonferroni corrected p-values ($p = 0.05$), was used for pairwise comparisons among land uses and sampling times. The "corrplot", "FactoMineR", "vegan", " effectsize", "factoextra", "tydiverse", "nlme", "multcomp", "eammeans", "ggplot2", "ggpubr" and "GGally" packages in the R 4.1.2 programming environment (R Core Team, 2021) were used.

Results

Soil functions

The PERMANOVA performed on soil functions calculated at FO, PA and ME during the year, highlighted overall significant differences among land uses ($\omega 2 = 0.56$; $F = 17.7$; $p < 0.001$) and among sampling times ($F = 5.91$; $p < 0.01$), as well as for their interaction ($F = 3.81$; $p < 0.01$). For the land use factor, the first component of PCA (Figure 2) explained 82% of the variance. The ellipses are partially overlapping, showing slight separation between PA and FO respect to ME, mainly determined by S, P and C cycles (Figure S2a).

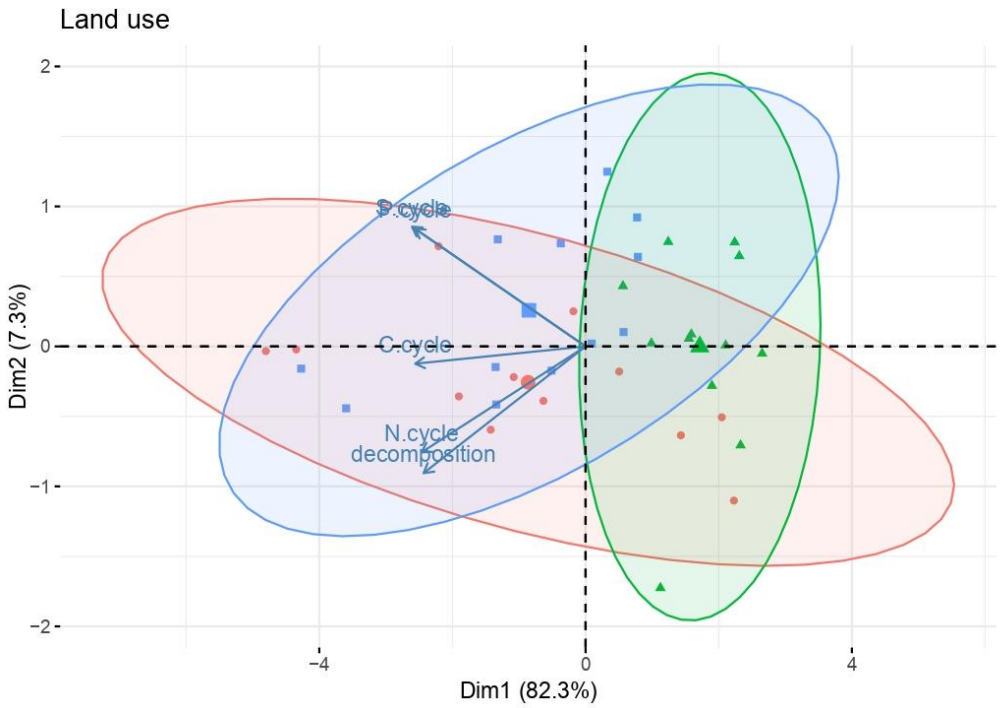

FIGURE 2 Biplot of the principal component analysis (PCA) with the superimposition of the confidence ellipses showing the differentiation among land uses. The graphs show the first two principal components (PC1 and PC2) and the variance explained by them. Forest (red), pasture (blue), and meadow (green)

Investigating the single function, the C cycle showed a value 1.9-fold higher ($p < 0.05$) in PA than in ME (Figure 3), as well as the variable uroni, linked to this function, was higher ($p < 0.001$) in PA than in FO and ME (Table S3). The functions P and S cycles showed a similar trend, with overall values for FO and PA 1.5-and 1.3-fold higher ($p < 0.01$) than ME (Figure 3), respectively. Regarding the variables associated with these functions (Table S3): for P cycle function, the variable acP showed higher value ($p < 0.001$) in FO respect to PA and ME, whereas the total P content showed higher value ($p < 0.001$) in PA respect to ME and FO; for S cycle function, variable Arys showed higher value ($p < 0.01$) in FO than ME, whereas the total S content showed higher ($p < 0.001$) value in PA than FO and ME (Table S3).

Decomposition function values (Figure 3) in FO were 1.2- and 1.6-fold higher ($p < 0.001$) than in PA and ME, respectively. In terms of variables

linked to this function (Table S3), SMB showed the same trend of the decomposition ($p < 0.001$), while FDA showed higher values ($p < 0.01$) in FO and PA than ME.

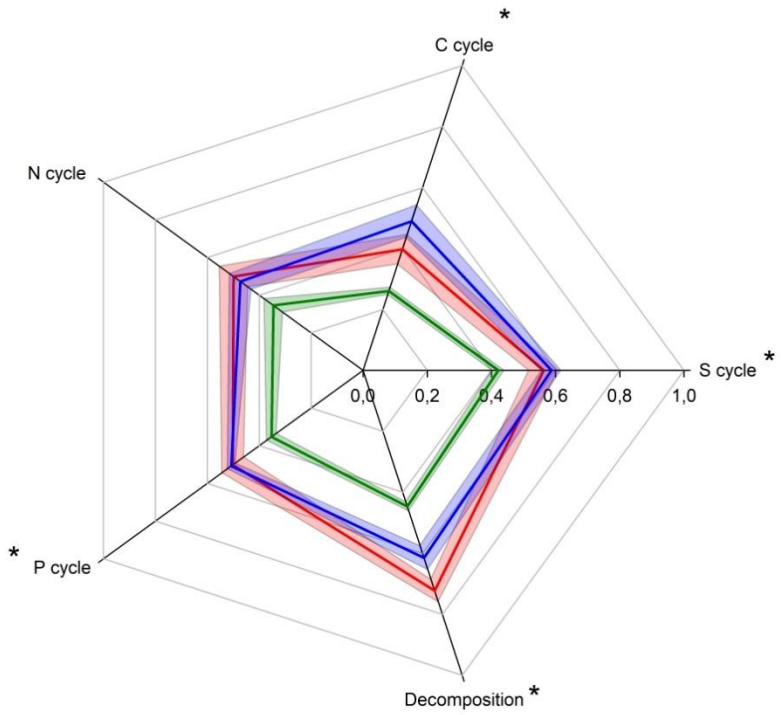

FIGURE 3 Radar plots of C, N, P and S cycle and decomposition functions. The plots show the mean values ± s.e. (filled area). Forest (red line), meadow (green line), pasture (blue line). When present, an asterisk indicates significant differences among land use

PERMANOVA was also applied for each land use to deepen the temporal differences in the measured soil functions: significant differences among sampling times resulted in PA ($\omega 2 = 0.86$; $F = 7.82$; $p < 0.01$) and ME ($\omega 2 = 0.64$; $F = 3.1$; $p < 0.01$).

In PA, the first component of PCA explained 82% of the variance (Figure 4a). The PCA plot showed a separation between T3 and the other times given by the functions N, S, and C cycles (Figure S2c). At T3, C and S cycle functions showed values from 1.5-to 2.0-fold ($p < 0.001$) and from 1.2-to 1.4-fold ($p < 0.001$) higher than the other sampling times, respectively (Table 1). Uroni variable ($p < 0.05$) and total S content (Figure S3a–b; $p < 0.001$) also followed a temporal pattern consistent with that of the associated functions.

Decomposition function values (Table 1) at T3 were from 1.2-to 1.5-fold higher ($p < 0.001$) than at the other sampling times. Also in this case, the associated SMB variable reflected a similar trend (Figure S3c), showing higher values at T3 ($p < 0.01$) than T1.

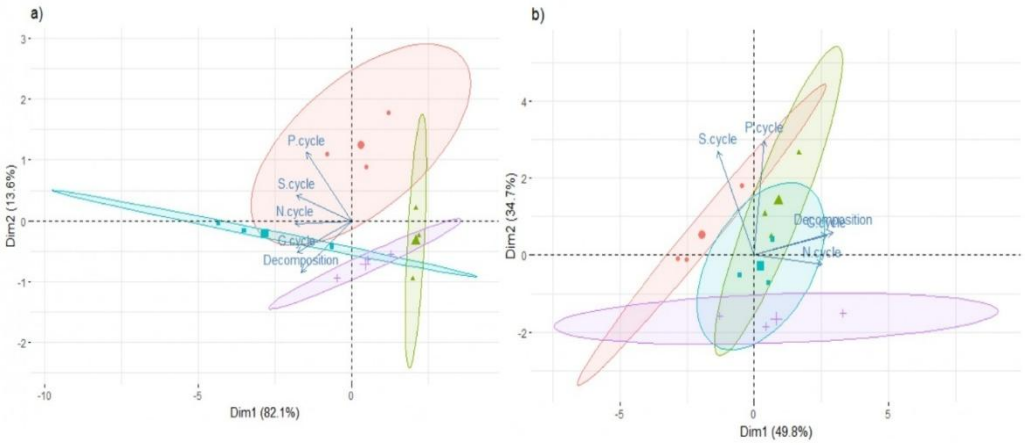

FIGURE 4 Biplot of the principal component analysis (PCA) with the superimposition of the confidence ellipses showing the differentiation among sampling times in pasture (a) and meadow (b). The graphs show the first two principal components (PC1 and PC2) and the variance explained by them. T1 (red), T2 (yellow), T3 (blue) and T4 (purple)

In ME, the first two components of PCA (Figure 4b) explained 84% of the variance and showed a separation of T4 from the T1 and T2 sampling times given by the functions S and P cycles (Figure S2f). At T4, the S ($p < 0.001$) and P ($p < 0.001$) cycle functions exhibited the lowest values (Table 1). Similarly, the associated variable, total S ($p < 0.001$) and P ($p < 0.001$) contents, showed differences among sampling times. These differences among sampling times were comparable in the S and P contents, reflecting the trends observed in their respective functions (Figure S3b, d).

In FO, although PERMANOVA did not show significant differences among sampling times ($\omega 2 = 0.70$; $F = 3.54$; $p = 0.062$), C, P and S cycle functions (Table 1; $p < 0.001$) showed an overall lower value at T4, whereas decomposition showed the higher values at T3 ($p < 0.001$).

TABLE 1 C, P, S and N cycles and decomposition functions and SMF index (mean ± s.e.) in forest (FO), pasture (PA) and meadow (ME) across the year. Significant differences (Tukey HSD test, with Bonferroni corrected *p*-values) among sampling times for each land use are indicated by different letters; n = 3.

Land use	C cycle	N cycle	P cycle	S cycle	Decomposition	SMF index
FO						
T1	0.44 ± 0.13 a	0.56 ± 0.19	0.54 ± 0.096 a	0.62 ± 0.092 a	0.71 ± 0.060 b	0.58 ± 0.11 a
T2	0.39 ± 0.070 ab	0.56 ± 0.0056	0.56 ± 0.046 a	0.60 ± 0.048 a	0.71 ± 0.021 b	0.56 ± 0.029 a
T3	0.53 ± 0.068 a	0.57 ± 0.054	0.59± 0.064 a	0.67 ± 0.060 a	0.89 ± 0.049 a	0.65 ± 0.059 a
T4	0.23 ± 0.042 b	0.30 ± 0.033	0.32 ± 0.018 b	0.35 ± 0.023 b	0.59 ± 0.014 b	0.36 ± 0.016 b
PA						
T1	0.39 ± 0.052 b	0.46 ± 0.045	0.55 ± 0.013 ab	0.60 ± 0.037 b	0.51 ± 0.038 c	0.50 ± 0.034 b
T2	0.37 ± 0.031 b	0.31 ± 0.015	0.45 ± 0.0022 b	0.50 ± 0.029 b	0.50 ± 0.045 c	0.43 ± 0.0066 b
T3	0.72 ± 0.13 a	0.65 ± 0.073	0.55 ± 0.024 a	0.73 ± 0.055 a	0.80 ± 0.028 a	0.69 ± 0.061 a
T4	0.48 ± 0.049 b	0.47 ± 0.043	0.48 ± 0.0041 ab	0.53 ± 0.025 b	0.65 ± 0.028 b	0.52 ± 0.029 b
ME						
T1	0.23 ± 0.023	0.26 ± 0.025	0.37 ± 0.030 ab	0.48 ± 0.012 a	0.39 ± 0.029	0.34 ± 0.022
T2	0.28 ± 0.017	0.40 ± 0.046	0.43 ± 0.025 a	0.46 ± 0.029 a	0.48 ± 0.021	0.41 ± 0.018
T3	0.29 ± 0.017	0.31 ± 0.022	0.32 ± 0.019 b	0.43 ± 0.011 ab	0.45 ± 0.0083	0.36 ± 0.0096
T4	0.26 ± 0.021	0.41 ± 0.13	0.29 ± 0.016 b	0.33 ± 0.011 b	0.46 ± 0.031	0.35 ± 0.035

Abbreviations: soil multifunctionality index (SMF index)

Soil multifunctionality

SMF index showed in FO and PA values 1.5-fold ($p < 0.01$) higher than ME (Figure 5). In FO, SMF decreased at T4 from 1.5-to 1.8-fold respect to the other sampling times ($p < 0.01$); in PA it increased at T3 from 1.3-to 1.6-fold respect to the other sampling times (Table 1; $p < 0.05$).

In the threshold approach, as for SMF index, the multifunctionality was higher in PA and FO respect to ME, at each threshold level (Figure 6a-c). Soil sampling time affected the multifunctionality and, as for SMF index, also the threshold approach showed: in FO a multifunctionality lower at T4, at each threshold level (Figure 6d-f) and in PA a multifunctionality higher at T1 and T3, providing 10 and 5 soil variables at 50 and 75% threshold, respectively (Figure 6e-f). In PA, among the examined variables, uroni activity reached the 75% threshold exclusively at T3, contributing to the

increase of multifunctionality in this sampling time (Table S4). In contrast to the SMF index, threshold approach highlighted differences in the multifunctionality among sampling times also in ME, where it resulted higher at T2, with 7 and 1 variables that reached the 50 and 75% threshold level, respectively (Figure 6e,f). Leu variable reached the 75% threshold exclusively at T2, compared to other sampling times, contributing to the increase of multifunctionality in this sampling time (Table S4).

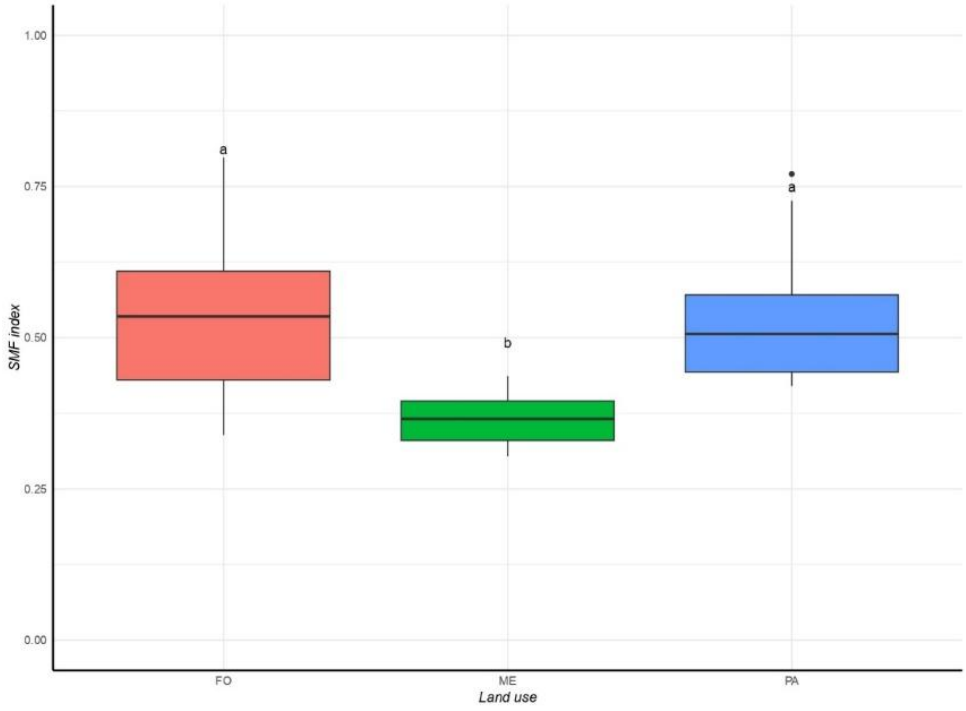

FIGURE 5 Box plots of soil multifunctionality index (SMF index) reported as the mean across sampling times. The box indicates the 25th and 75th percentiles, the continuous lines indicate the median values. Different letters indicate significant differences among land use. FO = forest (red), ME = (green) and PA = pasture (blue).

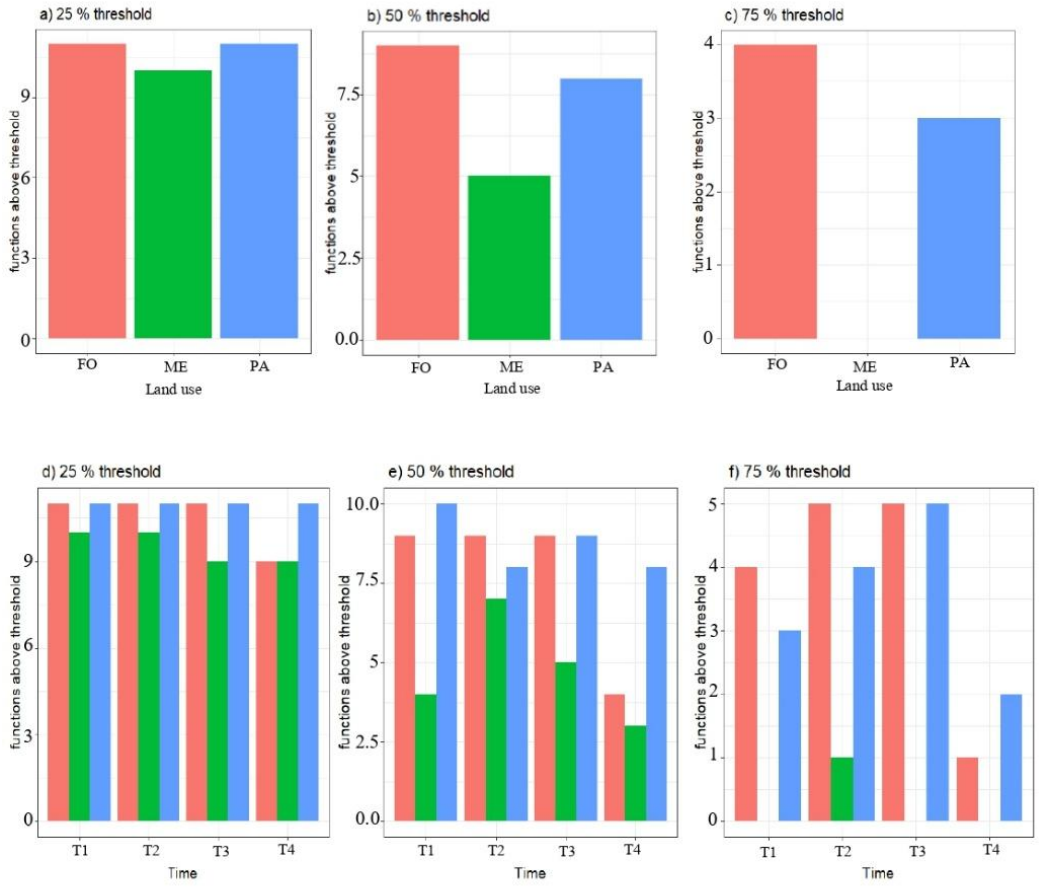

FIGURE 6 Distribution of the number of soil functions reaching a threshold (25%, 50%, and 75% of the maximum) across land uses (a–c), and in each land uses at four sampling times (d-f). FO = forest (red), ME = meadow (green) and PA = pasture (blue).

Discussion

Contrary to our hypotheses, the results demonstrate that soil multifunctionality varies across land use types, but not in a detrimental manner. Its variation was linked to periods of active management, such as grazing and mowing practices. The study of soil multifunctionality in grasslands has attracted increasing attention in recent years (Soliveres et al., 2016; Ma et al., 2022; Ding et al., 2023), also in relation to the role of belowground microbiome in maintaining soil ecosystem functions and stability (Li et al., 2024; Picariello and De Nicola, 2024; Long et al., 2025). In particular, enzymatic activities and microbial biomass, being sensitive

indicators to changes in soil properties (Bastida et al., 2016; Xue et al., 2020), proved to be determinants for assessing the soil functions and multifunctionality; in particular, microbial biomass regulates organic matter decomposition and nutrient mineralization through the production of extracellular enzymes. Given the crucial role of enzymatic activities and microbial biomass in sustaining soil multifunctionality, it is noteworthy that, contrary to our initial hypothesis, pasture soils did not show a reduction in multifunctionality. In particular, in forest and pasture (respect to meadow), the higher multifunctionality (regardless the approach considered for its evaluation) can be explained by the higher amount of organic matter due to the high input of litter (in forest) and livestock excrement (in pasture) to the soil during the year. This constant supply of carbon stimulates microbial activity (Bayranvand et al., 2021; Mencel et al., 2022; Baiano et al., 2024), mainly linked to the C, P and S nutrient cycles. Grazing appears to enhance several functions related to nutrient cycling, especially after six months of grazing, when an increase in C and S cycle and decomposition functions, as well as the soil multifunctionality was observed. This increase can be attributed to the role of trampling of livestock in pasture, which causes greater physical degradation of the litter, accelerates its decomposition, and consequently influences nutrient cycling (Wang et al., 2016). Although, on the one hand, the loss of plant biomass consumed by livestock reduces the quantity of litter on the soil and therefore the C and N input, worsening multifunctionality, on the other hand, grazing reduces the biomass of dominant plant species, enabling the less competitive species to thrive; this increases plant diversity and creates a nutritionally heterogeneous environment that leads to improved nutrient cycling (Wang et al., 2020). Indeed, several studies have shown that grazing stimulates nutrient cycling, thereby improving ecosystem multifunctionality (Wang et al., 2020; Zhang et al., 2021).

The multifunctionality evaluated using the threshold approach showed a stimulation of glucuronidase activity; since the enzyme involved in this activity is produced in large quantities by enteric bacteria present in excrement (Teriosina et al., 2025), this stimulation could be attributed to livestock excrement. Therefore, in addition to trampling, livestock excrement also played a direct role in improving soil multifunctionality by stimulating specific enzymatic activities.

Overall, the high values of the C and S cycles likely reflect well-functioning and tightly coupled biogeochemical cycles in this pasture system. As noted by Cao et al. (2024), biogeochemical cycles such as those of C, N, and S are highly interdependent, with key microbial processes - like denitrification and carbon mineralisation - occurring in parallel. When nutrient availability is high and balanced, microbial communities become more active, thereby enhancing these coupled processes and ensuring a continuous release of nutrients for plants and microorganisms. In contrast to systems affected by nutrient loss or intensive land management, where cycles become decoupled and microbial functioning declines (Dungait et al., 2012; Rumpel et al., 2015), these results suggest that the pasture studied site is characterized by stable conditions that promote microbial-mediated nutrient cycling. This stability contributes to enhanced soil multifunctionality.

In particular, our results showed that the pressure of grazing occurring in the investigated pasture land stimulated a high number of soil functions, which is likely also attributable to the spring-grazing rest. This practice is, in fact, used as a conservation measure to mitigate the damage caused by strong livestock trampling, which can have negative impacts, especially during periods when the soil is subjected to cycles of frost-thaw (Jing et al., 2024).

Our results showed that mowing did not negatively affect soil multifunctionality, contrary to our initial expectations. Although it is well known that grassland management practices, such as mowing and sowing, can affect soil properties and related functions (Mayel et al., 2021) by

reducing plant cover (consequently, organic matter inputs) and impairing nutrient cycle (Gilmullina et al., 2020; Mayel et al., 2021), in the studied meadow, the addition of manure as an organic amendment facilitated maintaining soil quality. Indeed, manure provides a continuous source of nutrients and organic matter, supporting microbial activity and promoting the closure of nutrient cycles. This input may therefore have compensated for any potential negative effects of mowing, thus preserving - or even enhancing - soil functions, as for S and P cycles, and multifunctionality.

In particular, mowing appears to have specifically stimulated the activity of enzymes, notably that of leucine-aminopeptidase, an enzyme involved in N cycle. In response to defoliation, plant produces root exudates, a readily available carbon source, promoting soil microbial biomass and activity. This raises the nitrogen demand and promotes the activation of N-leucine-aminopeptidase, leading to increased nitrogen availability in the soil (Gavrichkova et al., 2010; Khomutova et al., 2021).

Regarding forest, the differences in soil functions and multifunctionality among sampling times are attributable to the effect of climatic conditions. In particular, during winter, the low temperatures (average temperature of about 11° C; data reported in http://www.agricoltura.regione.campania.it/meteo/meteo_2022.html) reflect the inhibitory impact on soil functions, which then recover in late spring, as also found by Papa et al. (2014).

Conclusions

The high carbon inputs from livestock excrement deposition contributed to enhancing functions and multifunctionality in pasture, mainly after six months of grazing. In addition, the spring-rest grazing preserved the soil quality. Maintaining low grazing pressure seems fundamental to preserve soil functions and related services provided by the mountain ecosystems of the Mediterranean area.

Moreover, the mowing practice did not negatively affect the multifunctionality; on the contrary, it led to an increase in the N cycle, as highlighted by the threshold approach.

Our findings have important implications for implementing sustainable management strategies and to counteract ecosystem degradation caused by intensive soil exploitation through grazing and mowing, which is further amplified by climate change. In this context, organic inputs played a dual role in supporting both soil fertility and carbon storage, contributing positively to overall multifunctionality. Although our study of soil multifunctionality is based on one year of monitoring and a robust set of soil parameters, we acknowledge that expanding the analyses - for example, by including microbial biodiversity and multi-year monitoring would provide a deeper understanding of the factors that drive multifunctionality across different land uses.

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Supplementary material

TABLE S1 Soil physico-chemical properties (mean ± s.e.) of each land use (PA: Pasture; ME: Meadow and FO: Forest) across four sampling times (T1, T2, T3, T4). Soil texture is also reported, classified according to Shepard classification system*.

Land use	pH	WC %d.w.	SOC % d.w.	S g kg $^{-1}$ d.w.	P g kg $^{-1}$ d.w.	Texture
PA						
T1	6.3 ± 0.070	70 ± 3.8	13 ± 0.83	1.4 ± 0.096	4.9 ± 0.25	
T2	6.2 ± 0.044	15 ± 0.77	11 ± 1.1	1.4 ± 0.15	4.7 ± 0.28	Silty sand
T3	6.6 ± 0.15	92 ± 2.8	18 ± 0.62	1.7 ± 0.076	4.8 ± 0.033	
T4	6.3 ± 0.041	72 ± 1.9	15 ± 0.67	1.2 ± 0.045	4.0 ± 0.026	
ME						
T1	6.6 ± 0.21	73 ± 1.4	14 ± 0.76	1.3 ± 0.033	3.2 ± 0.33	
T2	6.4 ± 0.35	29 ± 4.6	14 ± 0.49	1.1 ± 0.053	3.1 ± 0.32	Silty sand
T3	6.1 ± 0.081	74 ± 0.29	15 ± 0.17	1.0 ± 0.030	2.6 ± 0.26	
T4	6.5 ± 0.13	70 ± 0.67	15 ± 0.098	0.78 ± 0.060	2.0 ± 0.15	
FO						
T1	6.5 ± 0.22	81 ± 2.8	15 ± 0.21	1.1 ± 0.029	2.2 ± 0.050	
T2	6.1 ± 0.081	48 ± 2.2	14 ± 0.11	1.1 ± 0.029	2.2 ± 0.051	Silty sand
T3	6.4 ± 0.15	82 ± 0.70	16 ± 0.19	1.0 ± 0.044	1.9 ± 0.069	
T4	5.9 ± 0.098	74 ± 0.59	14 ± 0.48	0.6 ± 0.083	0.99 ± 0.21	

Abbreviations: water content (WC); soil organic carbon (SOC); soil total sulphur content (S); soil total phosphorus content (P)

*Shepard FP (1954) Nomenclature based on sand-silt-clay ratios. J Sediment Res 24:151–158. https:// doi. org/ 10. 1097/ 00006 534- 19540 9000- 00011.

TABLE S2a Results of the principal component analysis (PCA) and correlation matrix for selection of the most representative soil variables (among alfaG, betaG, cell, xilo and uroni) related to the C cycle. The correlation matrix reports the Pearson correlation coefficients — commonly used for reducing the dimensionality of multivariate datasets — among the analysed variables, and displays the variables considered for the analysis. Variable selection is based on pairwise comparison: variables with correlation coefficients< 0.60 are retained; for coefficients > 0.60, the variable with the highest absolute loading is selected.

Principal components	**PC1**	**PC2**	**PC3**
Eigen value[a]	**3.9**	0.65	0.31
Percentage of variance	77	13	6.2
Cumulative percentage of variance	77	90	96
Loadings[b]			
alfaG	**0.88**	-0.077	0.43
betaG	**0.96**	-0.19	-0.0071
Cell	**0.91**	-0.094	-0.35
Xilo	**0.95**	-0.17	-0.056
uroni	**0.65**	0.76	-0.014

Correlation matrix[c]					
	alfaG	**betaG**	cell	xilo	**uroni**
alfaG	1.0	0.84	0.69	0.81	0.51
betaG	0.84	1.0	0.86	0.94	0.49
Cell	0.69	0.86	1.0	0.86	0.52
Xilo	0.81	0.94	0.86	1.0	0.50
uroni	0.51	0.49	0.52	0.50	1.0

[a]Boldface eigenvalues correspond to the PCs examined for selection; [b]Boldface loadings are considered highly weighted. [c] In the correlation matrix, soil variables in bold were included in the analysis.

Abbreviations: α- and β-glucosidases (alfaG and betaG); cellobiohydrolase (cell); xylosidase (xilo); glucuronidase (uroni).

TABLE S2b Results of the principal component analysis (PCA) and correlation matrix for selection of the most representative soil variables (among acP, bisP, piroP and total P content) related to the P cycle. The correlation matrix reports the Pearson correlation coefficients — commonly used for reducing the dimensionality of multivariate datasets — among the analysed variables, and displays the variables considered for the analysis. Variable selection is based on pairwise comparison: variables with correlation coefficients < 0.60 , all variables are retained; for coefficients > 0.60, the variable with the highest absolute loading is selected.

Principal components	**PC1**	**PC2**	**PC3**
Eigen value[a]	**1.8**	**1.7**	0.40
Percentage of variance	45	43	10
Cumulative percentage of variance	45	88	98
Loadings[b]			
acP	**0.58**	**-0.69**	0.43
bisP	**0.71**	**0.66**	-0.14
piroP	**0.97**	-0.076	-0.15
P	0.0015	**0.90**	0.42
Correlation matrix[c]			
	acP	bisP	**piroP**	**P**
acP	1.0	-0.080	0.53	-0.45
bisP	-0.080	1.0	0.63	0.52
piroP	0.53	0.63	1.0	-0.11
P	-0.45	0.52	-0.11	1.0

[a]Boldface eigenvalues correspond to the PCs examined for selection; [b]Boldface loadings are considered highly weighted. [c] In the correlation matrix, soil variables in bold were included in the analysis.

Abbreviations: acid phosphomonoesterases (acP); phosphodiesterase (bisP); pyrophosphate-phosphodiesterase (piroP); soil total phosphorus content (P).

TABLE S3 Soil uroni, acP, arys, FDA activities, SMB and soil total S and P content (mean ± s.e.) in forest (FO), pasture (PA) and meadow (ME). Significant differences (Tukey HSD test, with Bonferroni corrected *p*-values, $p = 0.05$) among land use are indicated by different letters; n = 12

	FO	**PA**	**ME**
Uroni **nmol MUF h^{-1} g^{-1} SOC**	392.9 ± 69.25 b	922.2 ± 102.6 a	263.7 ± 14.04 b
acP **nmol MUF h^{-1} g^{-1} SOC**	3103 ± 179.7 a	1610 ± 103.8 b	1400 ± 95.26 b
aryS **nmol MUF h^{-1} g^{-1} SOC**	1797 ± 169.1 a	1236 ± 76.51 ab	850.4 ± 46.19 b
FDA **nmol carboxyfluorescein h^{-1} g^{-1} SOC**	9729 ± 590.1 a	9055 ± 572.7 a	6817 ± 339.9 b
SMB **ng dsDNA g^{-1} SOC**	1439 ± 57.52 a	1218 ± 55.07 b	832.5 ± 46.16 c
S **g kg^{-1} d.w.**	0.9522 ± 0.07228 b	1.444 ± 0.07098 a	1.060 ± 0.05718 b
P **g kg^{-1} d.w.**	1.844 ± 0.1597 c	4.585 ± 0.1331 a	2.696 ± 0.1821 b

Abbreviations: glucuronidase (uroni); acid phosphomonoesterases (acP); arylsulphatase (aryS); fluorescein diacetate hydrolase (FDA); microbial biomass (SMB); soil total sulphur content (S); soil total phosphorus content (P).

TABLE S4 Threshold levels (25%, 50%, or 75% of the maximum value) and reaching of the threshold (***=> 75% of max; **= >50% of max; * => 25% of max and -= < 25% of max) in each land use (pasture-PA, meadow-ME and forest-FO) at each sampling times (T1, T2, T3 and T4).

Soil functions	Soil variables	Treshold (%)			PA				ME				FO			
		25	50	75	T1	T2	T3	T4	T1	T2	T3	T4	T1	T2	T3	T4
S cycle	S	0.4309	0.8619	1.292	***	***	***	**	**	**	**	*	**	**	**	*
	aryS	621.1	1242	1863	**	**	**	**	**	**	**	**	**	**	**	**
C cycle	betaG	274.4	548.8	823.1	**	**	**	**	*	**	**	*	***	**	***	*
	uroni	326.3	652.7	979.0	**	**	***	**	-	-	-	-	*	*	*	-
N cycle	chit	153.3	306.6	459.9	**	*	**	*	*	*	*	**	**	***	**	*
	leu	729.1	1458	2187	***	***	***	***	*	***	*	*	***	***	***	*
P cycle	acP	935.7	1871	2807	*	*	*	*	*	*	*	*	***	***	***	**
	piroP	177.6	355.2	532.8	**	*	*	*	*	*	-	-	**	**	**	*
	P	1.265	2.530	3.795	***	***	***	***	**	**	**	*	*	*	*	-
Decomposition	SMB	409.2	818.5	1227	**	***	***	**	*	**	**	**	**	***	***	***
	FDA	3180	6361	9542	**	**	**	**	**	**	*	*	***	***	***	**

Abbreviations: soil total sulphur content (S); arylsulphatase (aryS); α- and β-glucosidases (alfaG and betaG); cellobiohydrolase (cell); xylosidase (xilo); glucuronidase (uroni); chitinase (chit), leucine-aminopeptidase (leu); acid phosphomonoesterases (acP); phosphodiesterase (bisP); pyrophosphate-phosphodiesterase (piroP); soil total phosphorus content (P); soil microbial biomass (SMB); fluorescein diacetate hydrolase (FDA)

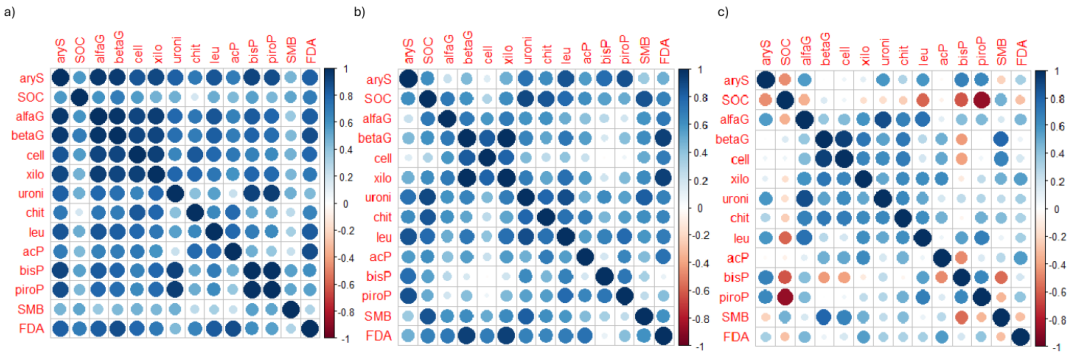

FIGURE S1 Correlation matrices among soil organic carbon (SOC), twelve soil enzyme activities (alfaG, betaG, cell, xilo, uroni, chit, leu, acP, bisP, piroP, aryS, FDA) and microbial biomass (SMB) measured in different land use: forest (a), pasture (b) and meadow (c) (Spearman critical value = 0.570).

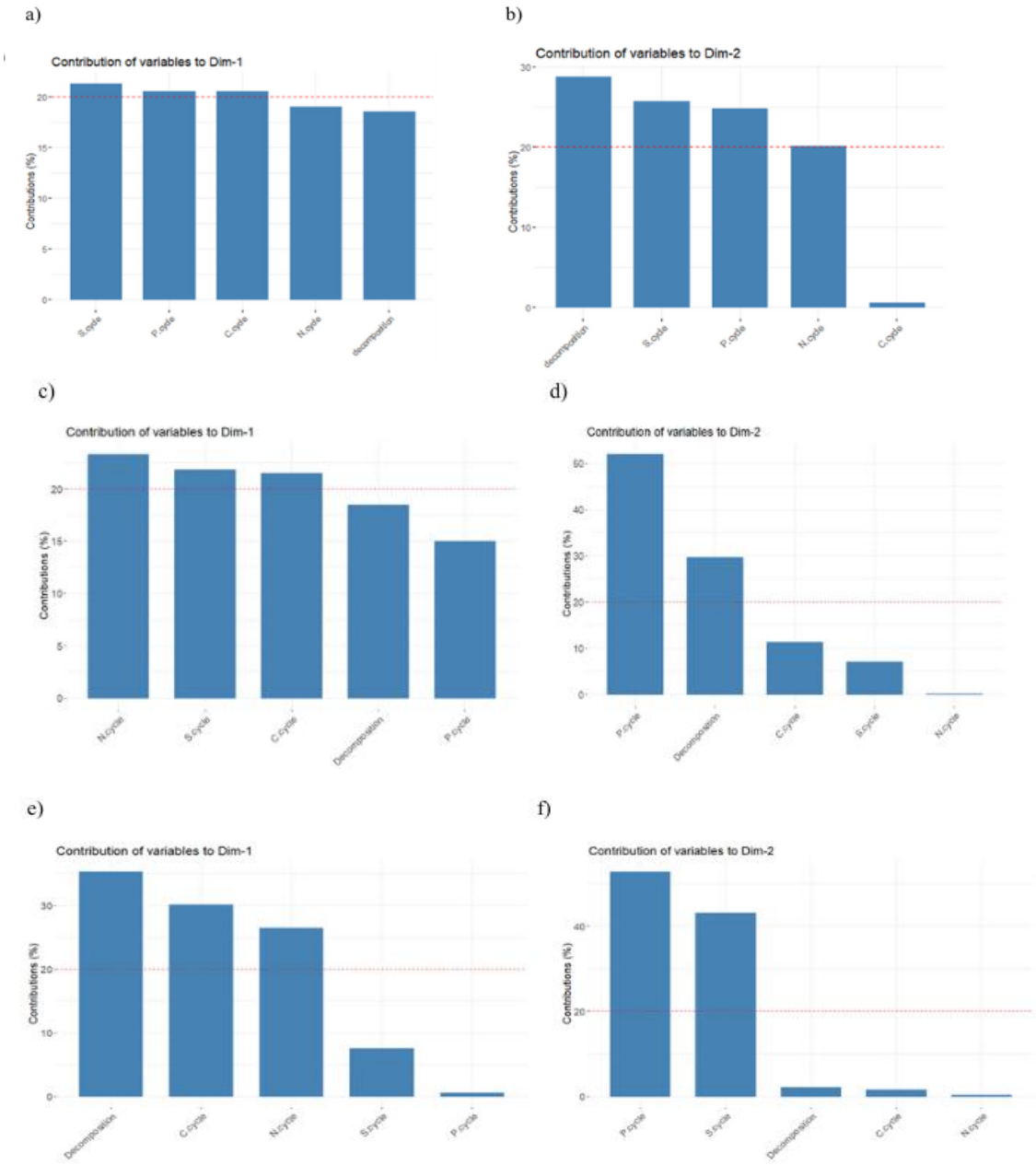

FIGURE S2 Variance explained by Dim1 and Dim2 of the C, N, P and S cycle and decomposition functions processed by means of two separated principal component analyses (PCA) among the different land uses (a-b) and sampling times in pasture (c-d) and meadow (e-f).

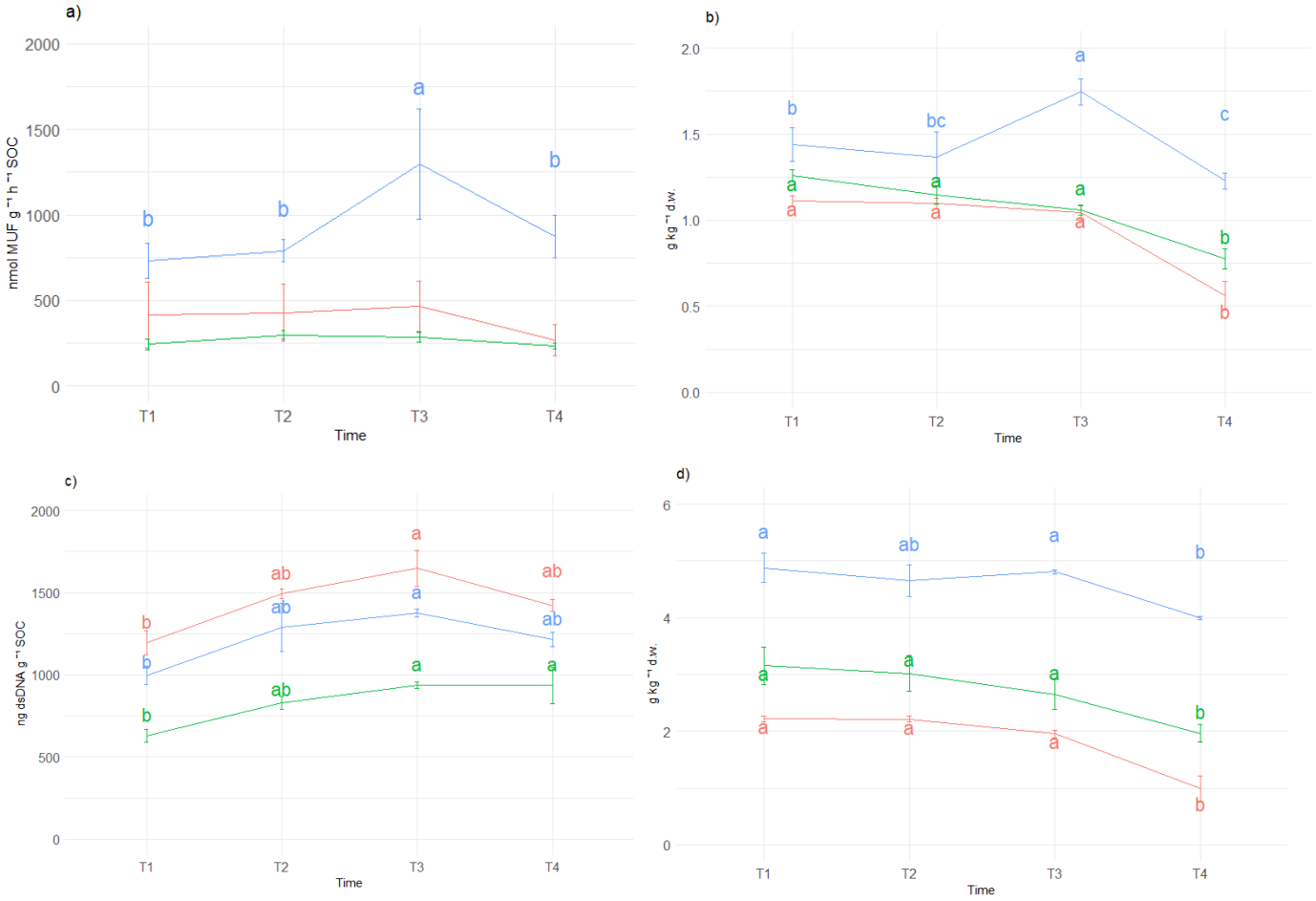

FIGURE S3 Mean values ± s.e. of (a) uroni activity, (b) total S content, (c) SMB, (d) total P content in soil of the different land use: forest (red line), pasture (blue line) and meadow (green line) at four sampling times (T1, T2, T3, T4). Significant differences (Tukey HSD test, with Bonferroni corrected p-values, p = 0.05) among sampling times are indicated by different letters; n = 3

CHAPTER 7

GENERAL CONCLUSIONS

This doctoral research provides a comprehensive assessment of soil multifunctionality and microbial community dynamics across four land-use systems in southern Italy, including forest, pasture, meadow, and cropland. Disturbances associated with land use and related management practices emerge as central factors affecting the capacity of soils to sustain multiple functions. The results highlight how different management practices shape microbial community composition, activity, and interactions, ultimately influencing the regulation of key ecosystem functions. By integrating chemical and biological indicators within a multifunctionality framework and employing advanced analytical approaches, the study elucidates the mechanisms linking soil microbial biodiversity to ecosystem functioning across distinct land-use systems in the Mediterranean area, where such integrative studies remain relatively scarce.

Although the findings are based on a limited number of sites and a single year of observation, they nonetheless offer robust and detailed insights into soil ecosystem dynamics. Moreover, the work underscores the effectiveness of integrated and novel methodological approaches for assessing multifunctionality, contributing valuable perspectives for future research and the sustainable management of soils.

Across all sites, microbial communities were consistently associated with soil multifunctionality, highlighting their key role as mediators of nutrient cycling, organic matter decomposition, and carbon stabilization. Forest soils, minimally disturbed and enriched with litter inputs, exhibited high microbial biomass, enzyme activities, and relatively complex microbial co-occurrence networks. These networks, characterized by numerous interactions among microbial taxa, supported a stable and highly multifunctional ecosystem. In contrast, pasture and meadow soils, subject to moderate disturbance through

grazing and mowing, showed fewer microbial species interactions, reflecting structurally simpler networks. Despite this reduced connectivity, soil multifunctionality was maintained, illustrating that a lower number of microbial interactions does not equate to functional impairment. Rather, soil microbial communities demonstrated adaptive resources allocation, maintained high functional diversity, and responded dynamically to environmental conditions, ensuring the persistence of key ecosystem functions, such as nutrient cycling, carbon stabilization, and organic matter decomposition. This observation provides an important refinement to traditional assumptions that directly link network complexity to ecosystem functioning.

Grassland systems further illustrated the role of management intensity and timing. Mountain pastures, grazed at moderate stocking rates of approximately 1.9 livestock units per hectare and subjected to spring rest-grazing, demonstrated enhanced nutrient cycling and improved soil multifunctionality several months after grazing. Similarly, meadows subjected to annual mowing maintained high microbial diversity and functional performance despite lower network connectivity. The addition of organic matter from livestock excrement further contributed to maintaining nutrient availability, aligning microbial nutrient demand with soil supply. Meadows, managed through annual mowing, maintained high microbial diversity, although microbial networks show minor connections respect to forest soils. These results are consistent with the intermediate disturbance hypothesis, suggesting that moderate disturbances can promote species coexistence by limiting competitive exclusion. These managed soils sustain ecosystem processes even with fewer microbial interactions. Such findings underscore the importance of timing, intensity, and type of disturbance in shaping microbial community structure and associated soil functions.

In cropland soils, the use of biodegradable plastic mulch films enhanced microbial activity, nutrient cycling, and overall soil multifunctionality while

minimizing environmental impacts relative to conventional polyethylene films. This demonstrates that targeted and sustainable management interventions can preserve or improve soil functions under intensive management, highlighting the applied relevance of the research in demonstrating the feasibility of environmentally compatible practices to reconcile productivity with the maintenance of multifunctionality in agricultural systems.

A key strength of this thesis lies in the integration of complementary approaches to unravel the ecological mechanisms underlying soil multifunctionality. Random Forest modeling identified the most influential predictors of multifunctionality among interacting chemical and biological factors, enabling robust interpretation despite the limited number of sites. Vector-based stoichiometric analysis quantified microbial nutrient acquisition strategies, linking enzyme activity patterns to ecosystem-level processes. Co-occurrence network analysis captured both direct and indirect microbial interactions, linking network structure to functional outcomes. The combined application of these approaches, applied in a Mediterranean context and across multiple land-use types, represents a significant methodological advance in the study of soil multifunctionality. Together, these methods provide a holistic framework that goes beyond traditional diversity metrics, linking microbial traits, interactions, and resource allocation strategies to ecosystem functioning.

The research also underscores the relevance of local conditions and management context. Pastures and meadows, located adjacent to forested areas, likely benefited from landscape connectivity, supporting microbial functional diversity. Furthermore, although these grasslands are situated within the Mediterranean region, their location in Matese mountain exposes them to less stressful climatic conditions than those typically of Mediterranean environments. These local environmental conditions likely contributed to the maintenance of microbial activity and soil functionality.

These findings highlight the need to consider both local conditions and management practices when evaluating soil ecosystem performance.

Although the focus of this research was on microbial communities, it is acknowledged that other biotic and abiotic factors—such as vegetation, soil fauna, and climate—also contribute to ecosystem processes. Nonetheless, the results provide clear, case-based evidence of the mechanisms through which microbial communities regulate multifunctionality, emphasizing the utility of integrative methodological approaches in capturing these dynamics.

Overall, the findings show that soil multifunctionality can be sustained across diverse land use types under moderate and context-specific management. Grasslands benefited from controlled grazing and mowing regimes, croplands from sustainable technological interventions, and forests from minimal disturbance and continuous organic inputs. Across all systems, multifunctionality was resilient to management disturbances, even in systems with simpler microbial network structures, emphasizing the role of microbial functional diversity and adaptive resource allocation over network complexity alone.

In conclusion, this thesis adopts a case-study approach that enables a detailed understanding of mechanisms linking microbial communities dynamics to ecosystem functions, revealing that multifunctionality can be preserved under moderate management even in different ecosystems. By connecting microbial dynamics, resource allocation, and community interactions to ecosystem processes, the research provides a robust scientific foundation for developing sustainable management strategies aimed at preserving soil integrity, enhancing ecosystem services, and promoting long-term resilience in managed landscapes.

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