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Changes in Microbial Necromass Carbon in Soil Profiles of Grasslands with Different Stages of Restoration in a Karst Region

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Abstract: Ecological restoration has increasingly been employed to reverse land degradation and increase carbon (C) sink, especially in ecologically fragile karst areas. Microbial necromass carbon (MNC) constitutes a critical pool within soil organic carbon (SOC), contributing substantially to long-term C sequestration through mineral stabilization. However, its distribution patterns across soil profiles and grassland restoration stages in karst areas remain unclear. To address this knowledge gap, the contents of bacterial necromass C (BNC), fungal necromass C (FNC), and their contributions to SOC were estimated based on glucosamine and muramic acid contents across the soil profile (0–20 cm, 20–40 cm, 40–60 cm, 60–80 cm, and 80–100 cm) for four subalpine restoration stages (grazing enclosure for 5, 11, 17, and 25 years) in the karst region. Our findings demonstrated that both soil depth and grassland restoration stages effectively influenced the BNC and FNC contents. On average, the soil BNC, FNC, and total MNC at the depth of 80–100 cm reduced by 70.50%, 59.70%, and 62.18% compared with in topsoil (0–20 cm), respectively. However, the FNC/BNC ratio gradually increased with the increase in soil depth, which was 43.15% higher at 80–100 cm soil depth than in topsoil, suggesting that the accumulation efficiency of FNC was higher compared to BNC in the deep soil. The BNC, FNC, and MNC were positively correlated with the grassland restoration stage, while FNC/BNC ratio had a negative relationship with the restoration stage (R^2 = 0.45, $p < 0.001$). FNC contributed significantly more to SOC (28.6–36.4%) compared to BNC (7.7–9.9%) at all soil depths, indicating that soil fungal necromass has an essential effect on SOC sequestration. The results of the random forest model and distance-based redundancy analysis identified that pH, soil water content, and dissolved organic carbon were the three most essential predictors for the contribution of MNC to SOC. Our study highlights the importance of microbial necromass to SOC accumulation, providing significant scientific implications for the C pool management during the restoration of degraded grasslands in karst regions.

Keywords: microbial necromass; soil organic carbon; grasslands; restoration stage; karst area

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1. Introduction

Grasslands are a vital component of terrestrial ecosystems, covering 40% of the Earth's land surface and accounting for 69% of global agricultural land area [1]. They not only provide

essential resources for human survival and development but also have an essential effect in protecting biodiversity and maintaining ecosystem stability. However, due to the combined impacts of human activities (such as overgrazing and excessive cultivation) and global climate change, approximately 49% of grasslands worldwide have experienced varying degrees of degradation [2], which has become an increasingly serious global environmental issue. Grassland degradation not only leads to a reduction in vegetation productivity, but also makes the soil carbon pool more vulnerable and unstable. Globally, the soil organic carbon (SOC) pool is around 1550 Pg, making it twice the size of the atmospheric C pool and three times that of the plant C pool [3]. Consequently, even minor fluctuations in the SOC pool can have a substantial impact on atmospheric carbon levels [4]. Therefore, gaining insight into the dynamics of the SOC pool is crucial for evaluating the carbon sequestration potential in ecosystems.

SOC accumulation involves a dynamic balance between inputs and decomposition. Although plant carbon serves as a direct source of carbon input into the soil, microbes act as bridges between plants and soil and play crucial roles in the process of SOC accumulation [5]. Microorganisms are essential not only for breaking down SOC but also for incorporating available carbon into their cellular components. After their life cycle ends, these microbial byproducts remain in the soil as microbial necromass [6]. This necromass can account for as much as 50% of the SOC pool and is known for its greater stability in soil compared to plant residues, underscoring its critical function in the long-term storage and stabilization of SOC [7]. Previous studies have been carried out to examine the accumulation of soil microbial necromass and the contributions to SOC in grasslands. For instance, the previous study found that microbial necromass carbon (MNC) at a soil depth of 0–40 cm in meadow grasslands contributed 72.51% on average to SOC, which was mainly affected by habitat and soil moisture [8]. A field study conducted in the Qinghai–Tibet Plateau revealed that MNC of the alpine grassland contributed up to 45.4% to SOC [9]. Their findings also indicated that the key factors influencing MNC varied with soil depth. At a soil depth of 0–10 cm, MNC was primarily regulated by plant C inputs and mineral protection. On the other hand, in the subsoil (30–50 cm), its preservation was mainly driven by physico-chemical stabilization mechanisms, including the protective effects of soil aggregate, iron–aluminum oxides, and exchangeable calcium [9]. In addition, a meta-analysis revealed that bacterial necromass carbon (BNC) consistently contributed less to SOC than fungal necromass C (FNC) across cropland, forest, and grassland soils [10]. This was attributed to the greater biomass of living fungi compared to bacteria, and the slower decomposition of fungal cell compounds, allowing them to persist longer in the soil [10]. Therefore, the sequestration of MNC and its contribution to SOC vary among soil depths and grassland types. Currently, research on restored subalpine grasslands is relatively limited.

Karst landscapes cover about 15% of the Earth's land surface and serve as a vital source of drinking water for a quarter of the world's population [11]. China has the most extensive karst distribution, comprising nearly one-third of its land area [12]. The karst regions in southern China, particularly in Yunnan, Guizhou, and Guangxi, experience slow soil formation, thin soil layers, rapid drainage of rainwater, and vegetation highly susceptible to human disturbances, ultimately leading to severe degradation and SOC loss [13]. Subalpine grasslands serve as keystone primary producers in subtropical montane ecosystems, sustaining regional biodiversity, carbon sequestration, and other critical ecosystem functions [14]. Given the ecological vulnerability of karst regions, the carbon sink function of subalpine grasslands is highly susceptible to both climate change impacts and anthropogenic disturbances. Therefore, the Chinese government has implemented various measures to restore degraded subalpine grasslands and increase soil carbon sink, such as enclosure. Soil MNC has a crucial effect on SOC accumulation and is an important component of the carbon pool. Therefore, underpinning the characteristics of MNC and its driving factors is of great significance for understanding SOC sequestration and the restoration of karst ecosystems. The objects of this study were to (1) determine the BNC,

FNC, and their contributions to SOC among soil depths in grasslands with different stages of restoration, and (2) reveal the key factors driving MNC and their contributions to SOC in restored subalpine grasslands in the karst region of southwest China.

2. Materials and Methods

2.1. Study Site and Sampling

We conducted the study in a transitional zone between the eastern Yunnan Plateau and the central Guizhou mountainous hills (Figure S1), located within 104°05′54″–104°47′21″ E and 26°45′06″–27°09′21″ N, with an average elevation around 1500 m. The main soil type is calcareous soil, which is classified as a Calcisol according to the FAO taxonomy. The soil texture is loam, with soil particle size distributions of 39.47% sand (0.05–1.00 mm), 42.84% silt (0.001–0.05 mm), and 17.69% clay (<0.001 mm). The region exhibits a subtropical humid monsoon climate, with an annual mean temperature of 12.8 °C, 950 mm precipitation, and approximately 1377 sunshine hours. The northwestern region of Guizhou Province is a major distribution area for natural grassland resources in the province, predominantly characterized by subalpine grasslands. Dominant and subdominant plant species include *Mazus longipes*, *Trifolium repens*, *Cirsium monocephalum*, *Potentilla fulgens*, *Festuca ovina*, *Anaphalis margaritacea*, *Swertia bimaculate, Gentiana lineolata*, and *Eragrostis pilosa*. Before the 1990s, frequent human practices (i.e., extreme livestock overgrazing) and climate change caused severe grassland degradation in the region, which exacerbated soil erosion, biodiversity decline, and ecosystem function decrease. China has carried out a series of grassland ecological protection and restoration programs to restore degraded grassland ecosystems since the 1990s, such as rotation grazing, enclosure, prohibiting grazing, and supplementary sowing. Among them, enclosure has been proven to be a prominent nature-based restoration strategy. By prioritizing ecological self-regulation over artificial management, grazing exclusion minimizes financial costs while delivering synergistic environmental, economic, and social benefits.

Through the utilization of governmental records, literature reviews, and field investigations, a selection of subalpine meadows with four stages of restoration, namely 5 years (RS5, enclosed since 2018), 11 years (RS11, enclosed since 2012), 17 years (RS17, enclosed since 2006), and 25 years (RS25, enclosed since 1998), was conducted. The selected grasslands with different stages of restoration were systematically enclosed, forming a 25-year restoration chronosequence free from grazing and anthropogenic pressures. Detailed information about the dominant plant species, subdominant plant species, and plant richness of the study sites is shown in Table S1. Each restoration stage comprised five replicated plots, totaling twenty sampling sites in this study. These sites were chosen based on their similar vegetation types, site conditions, and soil textures, notwithstanding the variations in restoration duration. Within each site, five randomly placed 20 m × 20 m plots were established to facilitate the collection of soil samples. Vegetation surveys and soil sampling were completed across field plots during peak growing season in July 2023. Within each plot, five quadrats (50 × 50 cm) were randomly selected, and the litter was removed from each quadrat. Two soil profiles were chosen in each quadrat and we collected soil samples using a soil core (10 cm diameter, 20 cm depth) with 2 replicates per quadrat at 20 cm intervals from 0 to 100 cm (5 depths), excluding plant and litter. Soil samples were split into two subsamples: one air-dried for physicochemical analysis, and the remaining stored in the refrigerator for the determinations of microbial necromass and soil enzyme activity.

2.2. Laboratory Analysis

The pH of the soil was assessed through the potentiometric method, using a soil-to-water ratio of 1:5. Soil water content (SWC) was measured using the gravimetric

method. SOC and total nitrogen (TN) were assessed using the Walkley–Black method and automated Kjeldahl distillation, respectively. Soil NH_4^+-N and NO_3^--N were extracted with 2 M KCl and then determined using a continuous flow analyzer (Futura, Alliance, Frépillon, France). Dissolved organic carbon (DOC) was quantified via high-temperature catalytic oxidation (680 °C) with a total organic carbon analyzer. Soil total phosphorus (TP) was determined using the molybdenum–antimony anti-spectrophotometric method. The activities of two hydrolytic enzymes (glucosidase, BG; cellobiohydrolase, CB) and one oxidative enzyme (polyphenol oxidase, PPO) in soils were assessed according to previous studies [14,15]. In brief, 10 g fresh soil was disrupted in cold (≤4 °C) sodium acetate buffer (pH 5.5) for two minutes. Soil slurry of 200 µL was then added into each well in 96-well microplates along with 50 µL of fluorometric substrate. For all enzymes, six analytical replicates for each soil sample and control were used in this study. The micro-plates were incubated at 20 °C in a constant-temperature incubator and continuously shaken before the measurement. We determined BG activity and CB activity after 1 h incubation and 4 h incubation using pNP–β–glucopyranoside and pNP–cellobioside as substrates, respectively. In addition, we determined PPO activity after 2 h incubation using L–3, 4–dihydroxyphenylala–nine (DOPA) as the substrate. The absorbance of the products was determined using a fluorescence spectrometer set to 365 nm for excitation and 450 nm for emission.

Soil amino sugars were quantified via gas chromatography according to the previous study [16]. Briefly, air-dried soils (ground to <0.25 mm) were hydrolyzed in 6 M HCl at 105 °C for 8 h. After adding myo-inositol (internal standard), hydrolysates were filtered and adjusted to pH 6.6–6.8. Supernatants were lyophilized, and residues dissolved in methanol (5 mL) and dried under N_2 (45 °C). Derivatization employed hydroxylamine hydrochloride (32 mg mL^{-1}) and 4–(dimethylamino)–pyridine (40 mg mL^{-1}) in pyridine:methanol (4:1 v/v) at 75–80 °C for 30 min to form aldononitrile derivatives prior to GC analysis. The concentrations of BNC and FNC were estimated from the amino sugar content using the conversion factors using Equations (1)–(3) [17]:

$$\text{BNC} = \text{MurA} \times 45 \quad (1)$$

$$\text{FNC} = (\text{GlcN}/179.2 - 2 \times \text{MurA}/251.2) \times 179.2 \times 9 \quad (2)$$

$$\text{MNC} = \text{BNC} + \text{FNC} \quad (3)$$

where 45 serves as the conversion factor for transforming bacterial MurA into bacterial necromass C, while 9 is used for converting fungal GlcN into fungal necromass C. Additionally, the molecular weights of GlcN and MurA are 179.2 and 251.2, respectively.

2.3. Statistical Analysis

Data normality and variance homogeneity were first assessed using the Shapiro–Wilk and Levene tests, respectively. One-way ANOVA was conducted to assess the grassland restoration- or soil depth-induced differences in soil properties, microbial necromass C, and enzyme activities. Significant variations ($\alpha = 0.05$) were further evaluated with Duncan's post hoc test using IBM SPSS statistics 21. Since data from different soil layers were not completely independent, we employed Repeated Measures Analysis of Variance (RMANOVA) to analyze the effects of restoration stage and soil depth on soil properties, MNC, and enzyme activities based on Pillai's Trace index using SPSS software (version 21). Redundancy analysis (RDA) was conducted to elucidate relationships between the environmental factors and MNC and their contributions to SOC with the package "vegan" in the R software (version 4.4.1). To explore the key factors regulating BNC, FNC, and MNC, and their contributions to SOC, a "random forest" algorithm was used with the "randomForest"

package (1000 trees, $p < 0.05$) [18]. The relationships between concentrations of MNC and soil properties were evaluated according to Spearman's correlation coefficient using "psych" packages in R (version 4.4.1).

3. Results

3.1. Soil Properties Among Different Soil Depths and Grassland Restoration Stages

The soil properties varied significantly among different grassland restoration stages and soil depths ($p < 0.05$), showing a decrease with rising soil depth along all restoration stages (Figure 1). The DOC, SOC, and NH_4^+-N decreased from the highest values at the topsoil (0–20 cm) to the lowest values at the bottom soil (80–100 cm). Similarly, the TN decreased from 1.03, 1.05, 1.01, and 1.02 mg g^{-1} at the topsoil for RS5, RS11, RS17, and RS25 grasslands to 0.50, 0.61, 0.63, and 0.75 mg g^{-1} at the bottom soil (Figure 1). On the other hand, the pH at the bottom soil was higher than those at topsoil along all restoration stages, ranging from 6.47 to 6.67, 6.42 to 7.15, 6.99 to 7.36, and 7.31 to 7.49 for RS5, RS11, RS17, and RS25 grasslands, respectively. On the other hand, SWC increased first and then decreased with soil depth, and the largest value was found at a soil depth of 40–60 cm. Compared with farmland, vegetation restoration increased pH and SOC in all soil depths (Figure S2). SWC, DOC, TN, TP, NH_4^+-N, and NO_3^--N also gradually increased from RS5 to RS25 in 80–100 cm soil depth ($p < 0.05$). However, there was no significant influence of grassland restoration on SWC and DOC in 40–60 cm soil depth ($p > 0.05$). The soil properties were impacted by the interactive effects between restoration stage (RS) and soil depth (D), except for pH and NO_3^--N content (Figure 1). The SOC, TP, and NH_4^+-N were positively associated with grassland restoration stage ($p < 0.01$), while they had a negative relationship with soil depth ($p < 0.01$). By contrast, pH and SWC had a significantly positive correlation with both soil depth and grassland restoration stage ($p < 0.05$).

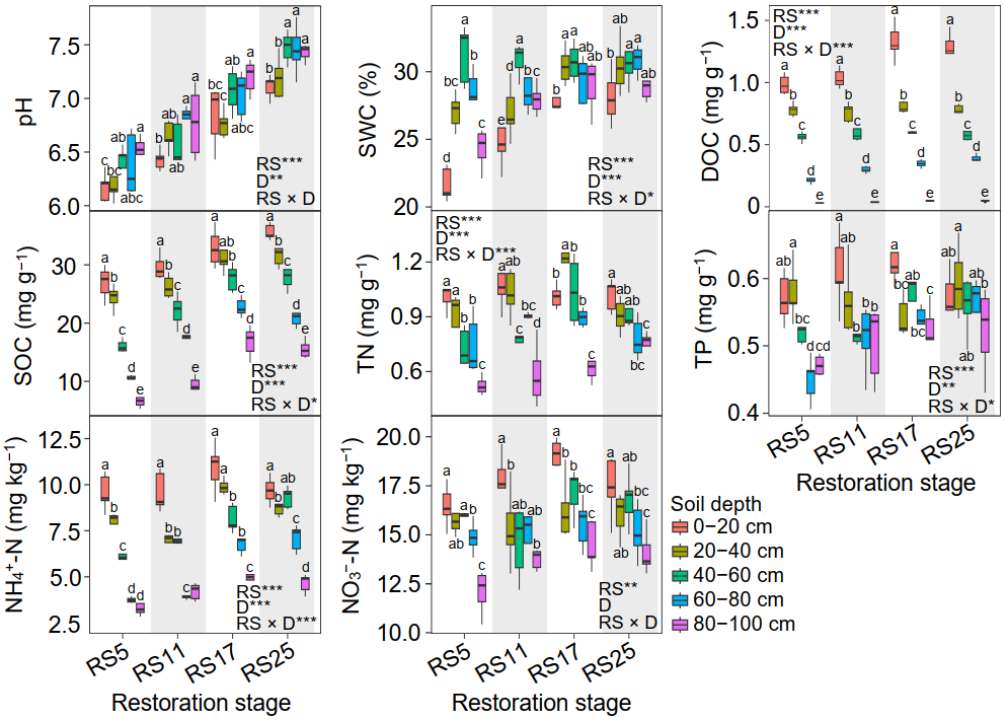

Figure 1. Variation in soil properties among soil depths and grasslands with different stages of restoration. RS5, 5 years of grassland restoration; RS11, 11 years of grassland restoration; RS17, 17 years of grassland restoration; RS25, 25 years of grassland restoration. Different letters represent significant differences among soil depths at $p < 0.05$. * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$, respectively.

3.2. Microbial Necromass Carbon C Among Different Soil Depths and Grassland Restoration Stages

The BNC, BNC, and MNC differed among soil depths considerably ($p < 0.05$), indicating a decreasing trend with rising soil depth (Figure 2). FNC decreased from 9.53 to 10.64 mg g^{-1} in 0–20 cm soil to 2.91–6.12 mg g^{-1} in 80–100 cm soil, while BNC ranged from an average of 3.10, 2.80, 1.85, 1.38, and 0.96 mg g^{-1} in 0–20, 20–40, 20–60, 60–80 and 80–100 cm soils, respectively (Figure 2). Overall, FNC was greater than BNC in all soils (7.09 vs. 2.02 mg g^{-1} of the total MNC). By contrast, the ratio of FNC/BNC greatly increased from 3.96 to 7.30 at bottom soil, to 3.05 to 4.17 at topsoil. The BNC, FNC, MNC, and FNC/BNC ratio were significantly influenced by soil depth, grassland restoration stage, and their interactive effects (Figure 2). The BNC, FNC, and MNC increased significantly along the restoration gradient, while the ration of FNC/BNC decreased following the grassland restoration stages (Figure S3). There was no significant difference in BNC, FNC, MNC, and FNC/BNC ratio between RS17 and RS25 in 0–80 cm soil depth ($p > 0.05$), while the BNC, FNC, and MNC were significantly higher in RS17 than RS25 in 80–100 cm soil depth ($p < 0.05$). The BNC, FNC, and MNC were positively correlated with grassland restoration stage, while they had negative relationship with soil depth ($p < 0.01$) (Figure S4).

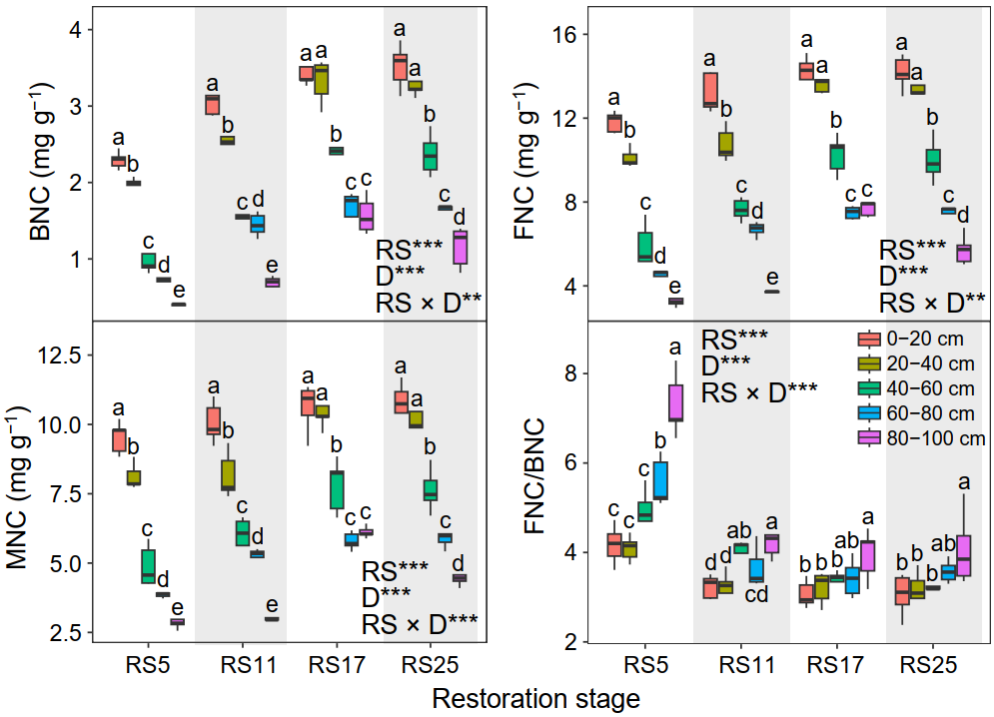

Figure 2. Variation in bacterial necromass C (BNC), fungal necromass C (FNC), total microbial necromass C (MNC), and the ratio of fungal/bacterial necromass C (FNC/BNC) among soil depths and grasslands with different stages of restoration. RS5, 5 years of grassland restoration; RS11, 11 years of grassland restoration; RS17, 17 years of grassland restoration; RS25, 25 years of grassland restoration. Different letters represent significant differences among soil depths at $p < 0.05$. ** $p < 0.01$; and *** $p < 0.001$, respectively.

3.3. Soil Enzyme Activities Among Different Soil Depths and Grassland Restoration Stages

The BG and PPO activities were higher in 0–40 cm soils and lower in 80–100 cm soils, and varied considerably among grassland restoration stages ($p < 0.05$) (Figure 3). In 0–20 cm soils, BG activity was highest in RS5 grassland, whereas in 80–100 cm soils, there was no difference in grasslands with different restoration stages. The CB activity varied from 0.29 to 0.32 nmol g^{-1} h^{-1} among all grasslands in 0–20 cm soils and it was lower in the RS25 grassland at the 80–100 cm depth. The PPO activity increased first and then declined with

grassland restoration and attained the maximum in RS17 (Figure S5). Compared to RS5, grassland restoration decreased the activities of BG by 14.27% and 15.71% compared with RS25 in 0–20 cm and 60–80 cm soil depths ($p < 0.05$). Nevertheless, there were negligible changes in CB activity between RS5 and RS11 in 40–100 cm soil depth ($p > 0.05$). BG, CB, and PPO activities were significantly affected by both soil depth and grassland restoration stage, while only CB was impacted by interactive effects between soil depth and grassland restoration stage ($p < 0.05$).

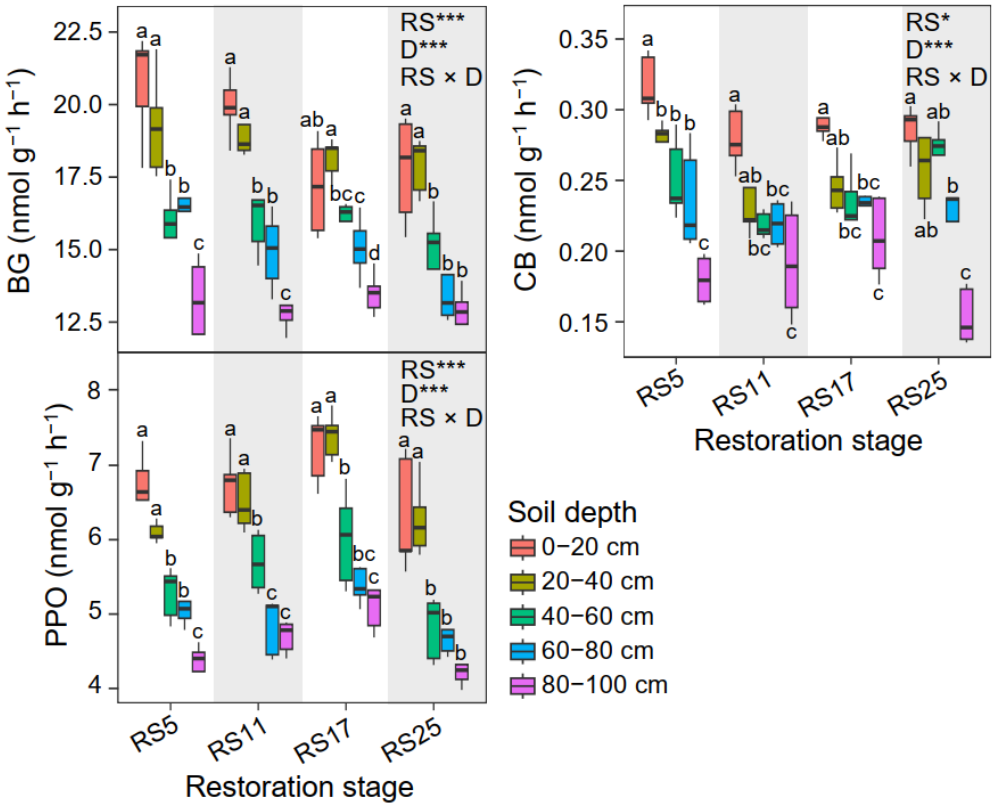

Figure 3. Variation in soil enzyme activities among soil depths and grasslands with different stages of restoration. BG, glucosidase; CB, cellobiohydrolase; PPO, polyphenol oxidase. RS5, 5 years of grassland restoration; RS11, 11 years of grassland restoration; RS17, 17 years of grassland restoration; RS25, 25 years of grassland restoration. Different letters represent significant differences among soil depths at $p < 0.05$. * $p < 0.05$; and *** $p < 0.001$, respectively.

3.4. Effects of Soil Properties and Enzyme Activities on MNC and the Contribution to SOC

In the current study, we found that BNC, FNC, and MNC were positively correlated with DOC, TN, TP, NH_4^+-N, NO_3^--N, BG, and PPO activities, while the FNC/BNC ratio had a negative correlation with these variables (Figure 4). Based on the random forest model, pH, DOC, NH_4^+-N, and PPO activity showed significant effects on BNC, FNC, and MNC, while pH, DOC, TN, and TP strongly impacted the FNC/BNC ratio. Moreover, the BNC/SOC ratio was significantly affected by DOC and PPO activity, while the FNC/SOC ratio and MNC/SOC ratio were strongly impacted by pH, SWC, and DOC. The results indicated that soil microbial necromass was a critical component of SOC, and the nutrient-rich soils are beneficial for the formation of microbial residues. Moreover, grassland restoration increased bacterial necromass higher than fungal, leading to a decreased FNC/BNC ratio.

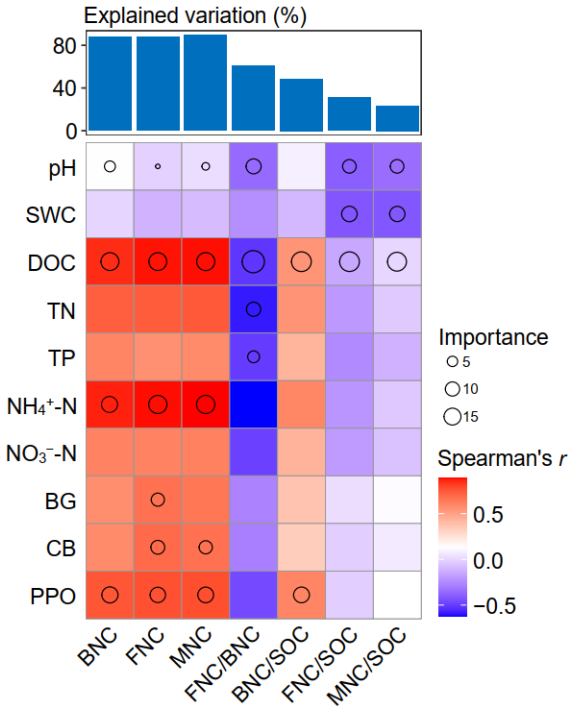

Figure 4. Effects of soil properties and enzyme activities on the bacterial necromass C (BNC), fungal necromass C (FNC), MNC, total microbial necromass C (MNC), FNC/BNC, the contribution of BNC to SOC (BNC/SOC), the contribution of FNC to SOC (FNC/SOC), and the contribution of MNC to SOC (MNC/SOC) based on random forest model. BG, glucosidase; CB, cellobiohydrolase; PPO, polyphenol oxidase. SWC, soil water content; DOC, dissolved organic carbon; TN, total nitrogen; TP, total phosphorus; NH_4^+-N, ammonium nitrogen; NO_3^--N, nitric nitrogen; BG, β-1,4-glucosidase; CB, β-D-cellobiohydrolase; PPO, polyphenol oxidase.

4. Discussion

4.1. Variations in Soil Properties Among Soil Depths and Grassland Restoration Stages

In the present study, the SOC content in grasslands with different restoration stages ranged from 6.06 to 26.90 mg g^{-1}, and the TN content ranged from 0.81 to 1.94 mg g^{-1}, with a highly significant correlation between them ($r = 0.789$, $p < 0.001$). As the grassland restoration stage increased, the accumulation of SOC and TN in the topsoil (0–20 cm) became more pronounced. The surface accumulation characteristic of SOC and TN in soil has been confirmed by numerous studies [19]. Climate and vegetation are the main factors affecting the vertical distribution of soil nutrients [20]. The primary sources of SOC and TN in soil are surface litter, root exudates, animal remains, and feces. Plant and animal residues first enter the topsoil, and approximately 90% of the underground biomass is concentrated in the 0–20 cm soil layer. Therefore, the content of SOC and TN is relatively higher in the surface soil layer [21]. Additionally, with increasing soil depth, the amount of roots decreases, and the influence of vegetation on soil nutrient content gradually weakens, resulting in low content of SOC and TN in the deeper soil layers [22]. Moreover, soil organic matter functions as a primary P reservoir, where changes in its quantity directly modulate soil P content. Soil organic matter serves as a crucial reservoir of P, and alterations in its content can lead to variations in soil P content. The correlation analysis revealed a significant positive correlation between TP and SOC ($r = 0.607$, $p < 0.001$). This suggests that the distribution of TP in soil profiles is strongly affected by the soil organic matter [23].

SOC compounds can be decomposed by soil microorganisms into different forms of N that plants can absorb and utilize, such as nitrate N, ammonium N, and various amino acids [24]. In the study area, the contents of ammonium N (NH_4^+-N) and nitrate N (NO_3^--N) increased with

grassland restoration stage, with NO_3^--N being the primary form of N accumulation. This is mainly due to the increased abundance of legumes during grassland restoration, which have N-fixing nodules that could increase the N content in the soil [25].

During the restoration of grassland, a large amount of litter returns to the ecosystem annually, providing ample material for nutrient supply and soil improvement [26]. These materials decompose into humus through microbial activity, increasing SOC content and fixing atmospheric N into the soil, continuously improving soil properties. Improved soil, in turn, provides more nutrients for vegetation recovery, creating a mutually reinforcing cycle of soil and vegetation production. After 17 years of grassland restoration, the NH_4^+-N and NO_3^--N contents in the topsoil (0–20 cm) increased from 9.58 to 10.93 mg kg^{-1} and 16.46 to 19.17 mg kg^{-1}, respectively, significantly enhancing soil fertility. Compared with SOC, soil TP did not significantly change with restoration stage (Figure 1). Soil P is mainly affected by parent material, which is a sedimentary mineral with poor migration in soil, vegetation type, and climatic condition [27]. As the climate and parent material is similar across all restoration stages, the variation in soil TP is not as obvious as that of SOC.

Generally, climate condition (i.e., precipitation and temperature) was the key factor influencing SWC; for instance, precipitation was the only source of water for soil water supplementation. However, climate variables were insignificant for significant SWC changes in the current study, because due to the close distance between sample sites and their identical sampling time, the climatic conditions were essentially consistent across restoration stage. On the other hand, with the increase in restoration ages, due to the long-term lack of anthropogenic disturbance, the vegetation cover became larger. The increased vegetation cover was beneficial to soil water retention and the dense distribution of plant roots in the grassland, which can effectively intercept water and increase SWC [28]. Additionally, vegetation cover can protect surface soil from wind erosion, intercept nutrient-rich dust and organic detritus, and help retain water by alleviating the speed of wind [29].

4.2. Variations in Soil Enzyme Activities Among Soil Depths and Grassland Restoration Stages

Soil enzymes have a crucial effect on the material cycling and energy flow within grassland ecosystems [30]. Monitoring soil enzyme activity in restored grassland is essential for assessing soil quality, which has significant implications for the management and maintenance of the grassland ecosystem. In this study, soil depth significantly affected enzyme activity (Figure 3). The cycling of soil nutrients is closely related to soil profile structure, and different soil enzymes respond differently to soil depth. This variability is likely due to the higher abundance of plant and animal residues, microorganisms, and root distribution in the soil layer. Since the primary sources of soil enzymes were microorganisms, plant and animal residues, and root exudates, the enzyme secretion and activity were higher in the surface soil layer. As soil depth increases, enzyme activity diminishes due to the decrease in humus content and soil nutrients. Consequently, enzyme activity and nutrient content at a soil depth of 0–20 cm were larger than those at deeper soil depths (Figure 3).

The restoration stage significantly affected soil physicochemical properties, hydrothermal conditions, and biotic communities, which in turn influenced soil enzyme activity. Comparing grasslands with different restoration stages revealed that two hydrolytic enzymes related to the carbon cycle (β-glucosidase (BG) and cellobiohydrolase (CB)) exhibited higher activity in the short-term restored grassland, indicating a rapid decomposition of organic matter. However, with the increase in restoration stage, the activities of these enzymes were unchanged or slightly declined (Figure 3). In the initial stage of restoration, the aboveground biomass and underground roots continued to grow and develop with increasing restoration stage [31], resulting in an increase in the soil nutrient contents, mi-

crobial growth, and enzyme activity. On the other hand, in the late stage of restoration, the increased plant height and canopy cover reduced photosynthetically active radiation (PAR) at ground level, thereby intensifying light competition among plants and decreasing plant diversity [32]. These changes would result in either a decrease or no change in microbial populations and enzyme activity.

4.3. Variations in Microbial Necromass C Among Soil Depths and Grassland Restoration Stages

Due to the strong influence of vegetation and soil properties on microbes, MNC varies considerably across different soil depths and grassland restoration stages. The previous study found that FNC and BNC were 12 and 4.8 mg g^{-1} in the global grassland ecosystem, respectively [10]. Our study indicates that the average FNC and BNC in grasslands with different restoration stages were 7.09 and 2.02 mg·g^{-1}, respectively, which was lower than that of the global grassland ecosystem. These findings suggest that soil depth and restoration stage probably affect the amino sugar content in soils.

The contribution of microbes to soil carbon pools is directly related to the dynamics of microbial communities and the balance between the production and decomposition of microbial products [33]. Generally, the microbial community structure remains consistent. Hence, the contribution of microbial residues can effectively indicate the stability and status of SOC pools in ecosystems. Soil fungi play a dominant role in decomposing recalcitrant substrates [34], whereas bacterial communities were more competitive than fungi in utilizing available substrates [35]. Our study indicated that FNC contributed between 25.7% and 46.8% to SOC, while BNC contributed between 6.4% and 10.8% (Figure S6), suggesting that fungi have a more significant impact on SOC accumulation in karst grasslands.

4.4. Driving Factors of Microbial Necromass C

The variations in MNC are closely related to soil properties. The previous study indicated that particulate organic carbon (POC) and easily oxidizable carbon (EOC) exhibited a positive effect on fungal-derived glucosamine, with EOC or POC being the appropriate indicators for SOC sequestration potential [36]. Our study indicated that the changes in soil MNC in karst grasslands were closely linked to soil properties. A redundancy analysis showed that the marginal effects of pH, SWC, DOC, and CB were significant for BNC, FNC, and MNC accumulation ($p < 0.05$), making it the primary factor influencing microbial necromass (Figure 5).

This study demonstrated that the accumulation of MNC was predominantly regulated by DOC, which is a type of plant C input to soils [37,38]. Therefore, DOC could stimulate microbial residue formation by serving as the growth substrate. In addition, the high level of DOC (C availability) could increase the likelihood of metabolic processing and eventual transformation into necromass-derived products, including extracellular polymeric substances and osmolytes [39]. This aligned with the observed positive correlation between DOC and BNC, FNC, and MNC across grasslands (Figure 4).

In addition to the detected importance of DOC, the influence of soil pH in regulating MNC should be considered. The results suggested that soil pH exerted a significant negative influence on both microbial necromass C and the contributions to SOC (Figure 4). Acidic conditions enhanced microbial necromass C accumulation (BNC, FNC, and MNC), likely through three mechanisms: (i) fungi tend to thrive in slightly acidic environments; (ii) therefore, the low pH promoted the growth of fungi and the accumulation of microbial necromass C (FNC constitutes the majority of the MNC) [40]; and (iii) relatively high pH accelerated the decomposition process of microbial necromass, resulting in the reduction in MNC [41]. Therefore, the future efforts to increase the sequestration of soil microbial necromass in karst grasslands should focus more on regulating pH and C availability (DOC) in soils.

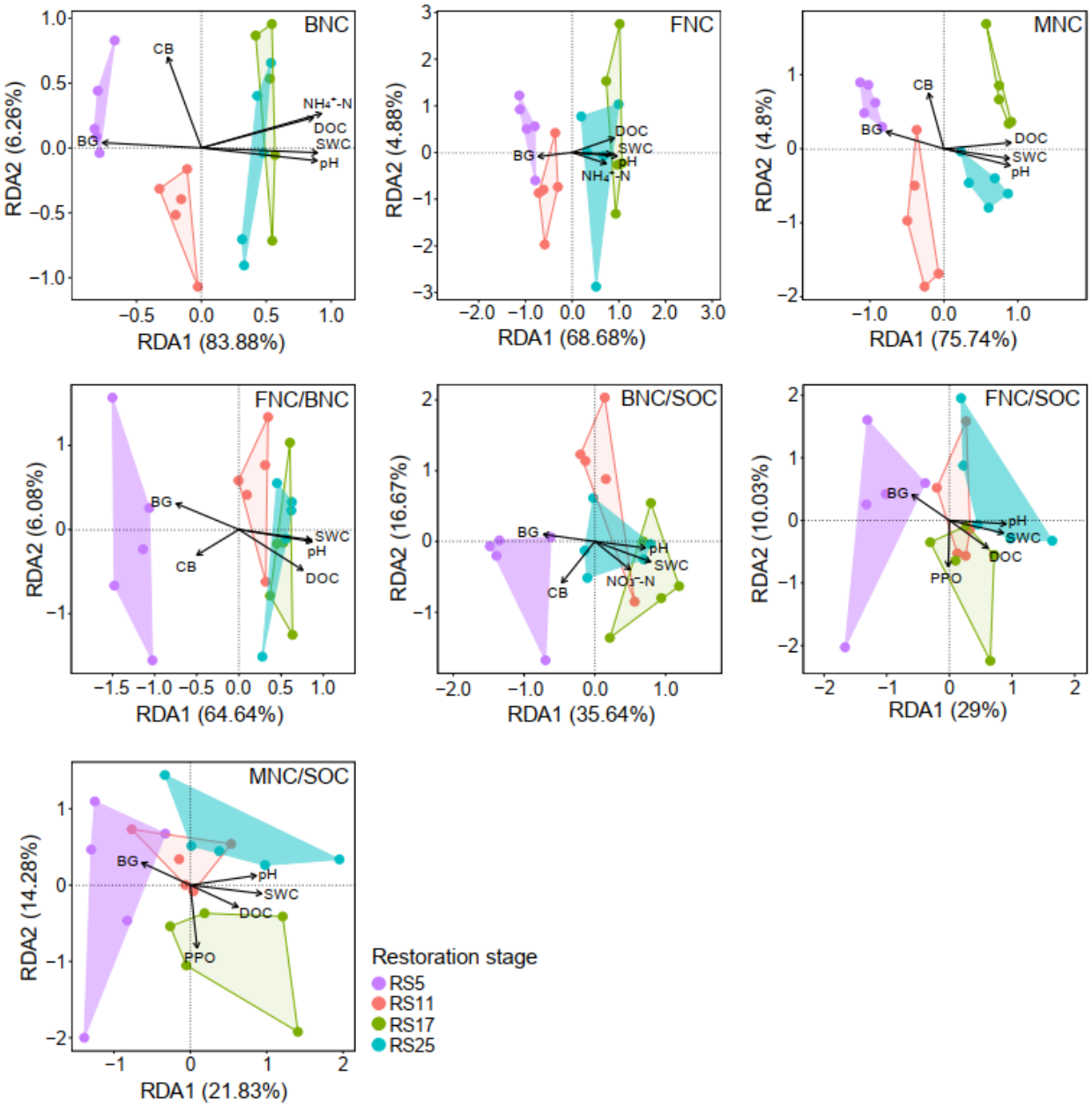

Figure 5. Effects of soil properties and enzyme activities on the bacterial necromass C (BNC), fungal necromass C (FNC), MNC, total microbial necromass C (MNC), FNC/BNC, the contribution of BNC to SOC (BNC/SOC), the contribution of FNC to SOC (FNC/SOC), and the contribution of MNC to SOC (MNC/SOC) based on distance-based redundancy analysis (RDA). The black arrows represent explanatory variables. The circular symbols in different colors in the figure show the distribution of data points for different grassland restoration stages. The variables that appear in the figure indicate significant influences on the response variables. SWC, soil water content; DOC, dissolved organic carbon; BG, glucosidase; CB, cellobiohydrolase; PPO, polyphenol oxidase.

These findings demonstrate that grazing exclusion (enclosure) can be recognized as an effective strategy for grassland restoration to enhance soil carbon sequestration in karst ecosystems by regulating diverse biotic and abiotic factors. These results are consistent with previous studies [42], indicating the importance of restoration in carbon sinks within fragile ecosystems. The mechanism of carbon sequestration and accumulation is a complex process, involving multiple biotic and abiotic factors. Our study demonstrated that pH, SWC, and DOC were the three most essential factors influencing microbial necromass

and their contributions to SOC. These findings deepen our comprehension of how carbon accumulation within the karst ecosystem responds to diverse biotic and abiotic factors across grassland restoration stages in soil profiles. Moreover, the regulation of soil pH, water, and nutrient availability for incorporation into the restoration of fragile ecological areas carries significant implications for future carbon sequestration and subalpine grassland restoration.

5. Conclusions

This study provides a comprehensive analysis of the accumulation of MNC, the contribution of MNC to SOC, and their driving factors across grasslands with different restoration stages in soil profiles. The BNC, FNC, and MNC were gradually decreased with an increase in the soil depth, with FNC content generally higher than BNC. The pronounced decline in root biomass and associated exudates with soil depth possibly limited the contribution of plant-derived C to microbial necromass C accumulation in the deep soil layer. MNC was dominated by FNC, which contributed more to SOC accumulation than BNC. Based on the random forest model and distance-based redundancy analysis (RDA), the most influential factors on MNC accumulation were soil pH, DOC, NH_4^+-N, and PPO, while pH, SWC, and DOC were the three most important predictors for the contribution of MNC to SOC. In near-neutral pH soils with high SWC and rich in DOC, microbes efficiently utilized substrates to synthesize biomass, resulting in greater carbon storage potential and microbial necromass accumulation. These findings highlight valuable implications for C pool management during the restoration of degraded grasslands in the karst region. A better understanding of the links between the restoration process and MNC accumulation will aid in the development of effective practices for C sequestration in the karst region. Further studies are still required to underpin the relationships between microbial community characteristics and microbial necromass, which can help gain more profound insights into the dynamics and driving factors of MNC and microbial necromass contribution to SOC in grassland soils.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agronomy15061436/s1, Table S1: Information on the dominant plant species, subdominant plant species, and plant richness of the study sites; Figure S1: Location of the study area and sampling sites in the karst region of Guizhou Province; Figure S2: Variation in soil properties among grasslands with different stages of restoration and soil depths; Figure S3: Variation in bacterial necromass C (BNC), fungal necromass C (FNC), total microbial necromass C (MNC), and the ratio of fungal/bacterial necromass C (FNC/BNC) among grasslands with different stages of restoration and soil depths; Figure S4: The correlations between grassland restoration stage and bacterial necromass C (BNC), fungal necromass C (FNC), total microbial necromass C (MNC), and FNC/BNC ratio; Figure S5: Variation in soil enzyme activities among grasslans with different stages of restoration and soil depths; Figure S6: Variation in the contribution of bacterial necromass C (BNC/SOC), fungal necromass C (FNC/SOC), and total microbial necromass C (MNC/SOC) to SOC among soil depths and grasslands with different stages of restoration.

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Abbreviations

The following abbreviations are used in this manuscript:

C	Carbon
SOC	Soil organic carbon
BNC	Bacterial necromass carbon
FNC	Fungal necromass carbon
MNC	Microbial necromass carbon
RS	Restoration stage
TN	Total nitrogen
TP	Total phosphorus
MurA	Muramic acid
GlcN	Glucosamine
GalN	Galactosamine
RDA	Redundancy analysis
DOC	Dissolved organic carbon
NH_4^+-N	Ammonium nitrogen
NO_3^--N	Nitrate nitrogen
SWC	Soil water content
BG	Glucosidase
CB	Cellobiohydrolase
PPO	Polyphenol oxidase

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