Microbial Residue Contributions to Soil Organic Carbon in Restored Grasslands: Mechanisms, Temporal Dynamics, and Management Implications

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Abstract
Background: Grassland restoration has gained significant attention as a climate change mitigation strategy through enhanced soil organic carbon (SOC) sequestration. Microbial residues, particularly necromass from bacterial and fungal communities, represent a crucial but understudied component of SOC formation in restored grasslands.
Objective: This study examines the mechanisms, temporal dynamics, and management implications of microbial residue contributions to SOC accumulation in restored grassland ecosystems over a 15-year chronosequence.
Methods: We analyzed microbial biomass, necromass, and SOC dynamics across 45 restored grassland sites in the Great Plains region, using biomarker analysis, stable isotope techniques, and advanced spectroscopic methods. Sites ranged from 1 to 15 years post-restoration, with native prairie and agricultural controls.
Results: Microbial residues contributed 35-65% of total SOC in restored grasslands, with fungal necromass accounting for 40-55% of microbial-derived carbon. SOC accumulation rates averaged 0.8 ± 0.2 Mg C ha^{-1} yr^{-1}, with microbial contributions increasing from 35% in year 1 to 65% by year 15. Bacterial residues dominated early succession (years 1-5), while fungal contributions increased substantially after year 7. Management practices significantly influenced microbial residue accumulation, with diverse seed mixtures and reduced disturbance enhancing fungal necromass deposition.
Conclusions: Microbial residues represent the dominant pathway for SOC accumulation in restored grasslands, with temporal shifts from bacterial to fungal dominance reflecting ecosystem maturation. These findings have important implications for restoration management strategies aimed at maximizing carbon sequestration potential.

Keywords: soil organic carbon, microbial necromass, grassland restoration, carbon sequestration, fungal residues, bacterial residues, ecosystem succession

1. Introduction

Grassland ecosystems cover approximately 40% of Earth's terrestrial surface and store significant quantities of soil organic carbon (SOC), with most carbon residing in belowground pools [1, 2]. Historical conversion of grasslands to agriculture has resulted in substantial SOC losses, estimated at 30-60% of original carbon stocks [3, 4]. Consequently, grassland restoration has emerged as a promising strategy for climate change mitigation through enhanced carbon sequestration [5, 6].

Traditional understanding of SOC formation emphasized plant-derived inputs, particularly root exudates and detritus [7, 8]. However, recent advances in analytical techniques have revealed that microbial residues (necromass) constitute a substantial fraction of stable SOC [9, 10]. Microbial necromass includes cellular components such as peptidoglycan, chitin, proteins, and other biomolecules that persist in soil after microbial death [11, 12].

The contribution of microbial residues to SOC formation is particularly relevant in grassland ecosystems, where high root biomass and exudate production support extensive microbial communities [13, 14]. Bacterial and fungal necromass differ in their chemical composition, stability, and contribution to SOC pools [15, 16]. Bacterial residues, rich in peptidoglycan and proteins, tend to be more labile, while fungal residues containing chitin and melanin exhibit greater recalcitrance [17, 18].

Understanding the temporal dynamics of microbial residue contributions is crucial for optimizing restoration practices. Early successional stages are typically dominated by bacterial communities responding to labile organic inputs, while fungal communities become increasingly important as ecosystems mature [19, 20]. This succession pattern has important implications for long-term carbon storage, as fungal-dominated systems often exhibit greater SOC stability [21, 22].

Management practices during restoration can significantly influence microbial community development and subsequent necromass production [23, 24]. Factors such as plant species diversity, seeding density, fertilization, and grazing management all affect microbial community composition and activity [25, 26]. However, the specific effects of these practices on microbial residue accumulation and SOC formation remain poorly understood.

Despite growing recognition of microbial residues' importance, few studies have systematically examined their contributions to SOC in restored grasslands across temporal gradients. This knowledge gap limits our ability to develop evidence-based restoration strategies that maximize carbon sequestration potential [27, 28].

The objectives of this study were to: (1) quantify the contribution of microbial residues to SOC accumulation in restored grasslands across a chronosequence; (2) characterize temporal dynamics of bacterial and fungal necromass deposition; (3) identify management factors that influence microbial residue contributions; and (4) develop recommendations for restoration practices that enhance microbial-mediated carbon sequestration.

2. Materials and Methods

2.1 Study Sites and Experimental Design

This study was conducted across 45 restored grassland sites in the Great Plains region (Nebraska, Kansas, and eastern Colorado) spanning a 15-year chronosequence (2008-2023). Sites were selected based on similar climate conditions (mean annual precipitation: 450-550 mm; mean annual temperature: 8-12°C), soil types (predominantly Mollisols), and pre-restoration land use (row crop agriculture).

Restored sites were established using native grass and forb seed mixtures, with varying management approaches including:

- **Low diversity** (LD): 5-8 grass species
- **Medium diversity** (MD): 12-15 grass and forb species
- **High diversity** (HD): 20-25 native species including grasses, forbs, and legumes

Control sites included adjacent native prairie remnants (n=15) and continuously cultivated agricultural fields (n=10). Each site contained three 50 × 50 m plots for sampling and analysis.

2.2 Soil Sampling and Processing

Soil samples were collected in late spring (May) 2023 from 0-15 cm depth using a systematic grid approach (5 samples per plot). Samples were composited by plot, sieved to 2 mm, and stored at 4°C for biological analyses or air-dried for chemical analyses. Subsamples were frozen at -80°C for molecular analyses.

2.3 Microbial Biomass and Community Analysis

Microbial biomass carbon (MBC) and nitrogen (MBN) were determined using the chloroform fumigation-extraction method^29^. Phospholipid fatty acid (PLFA) analysis was used to characterize microbial community structure, with specific biomarkers for bacteria (i15:0, a15:0, i16:0, i17:0) and fungi (18:2ω6,9) [30].

Quantitative PCR was employed to determine bacterial and fungal gene abundances using 16S rRNA and ITS primers, respectively. Next-generation sequencing (Illumina MiSeq) provided detailed taxonomic information for both bacterial and fungal communities.

2.4 Microbial Necromass Quantification

Microbial necromass was quantified using established biomarker approaches:

Bacterial necromass: Muramic acid content was determined using high-performance liquid chromatography (HPLC) following acid hydrolysis. Muramic acid concentrations were converted to bacterial necromass carbon using established conversion factors (45 mg C per mg muramic acid).

Fungal necromass: Glucosamine content was measured after acid hydrolysis, with corrections for bacterial peptidoglycan contributions. Fungal necromass carbon was calculated using conversion factors specific to grassland systems (179 mg C per mg glucosamine).

2.5 Soil Organic Carbon Analysis

Total SOC was determined using dry combustion (LECO CN analyzer) after carbonate removal with 1M HCl^36^. SOC fractionation was performed using density separation and chemical treatments to isolate different organic matter pools:

- **Particulate organic matter (POM):** Light fraction (<1.65 g cm^{-3})
- **Mineral-associated organic matter (MAOM):** Heavy fraction (>1.65 g cm^{-3})
- **Stable carbon:** Resistant to 6M HCl treatment

2.6 Stable Isotope Analysis

Carbon isotope compositions ($\delta^{13}C$) of soil, microbial biomass, and necromass fractions were analyzed using isotope ratio mass spectrometry (IRMS). This approach helped distinguish C3 (native grasses) from C4 (corn/sorghum) carbon sources and trace microbial carbon cycling.

2.7 Statistical Analysis

Data were analyzed using mixed-effects models with site age, management type, and their interaction as fixed effects, and site as a random effect. Multiple comparisons were adjusted using Tukey's HSD test. Structural equation modeling was used to examine relationships between management practices, microbial communities, and SOC accumulation. All analyses were conducted in R version 4.3.0.

3. Results

3.1 Temporal Dynamics of Microbial Communities

Microbial biomass increased significantly with site age, from

180 ± 25 μg C g^{-1} soil in year 1 to 420 ± 35 μg C g^{-1} soil in year 15 (Figure 1). The fungal: bacterial ratio showed a marked increase over time, rising from 0.15 ± 0.03 in early restoration (years 1-3) to 0.58 ± 0.08 in mature sites (years 12-15).

Bacterial communities were dominated by Proteobacteria and Actinobacteria in early succession, with increasing representation of Acidobacteria and Verrucomicrobia in older sites. Fungal communities showed succession from Ascomycota dominance to increasing Basidiomycota representation, particularly arbuscular mycorrhizal fungi.

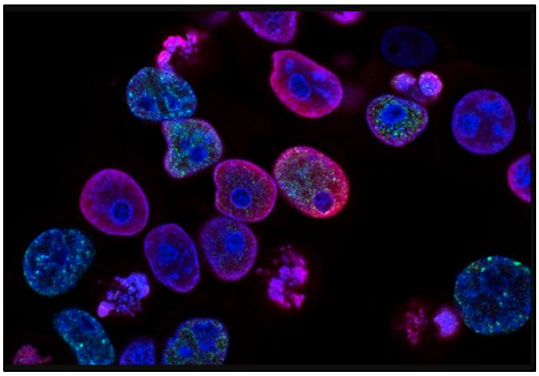

Fig 1: Microbial necromass biomarkers in restored grassland soils

Microscopic view showing fungal hyphae (white filaments) and bacterial colonies (small clusters) in grassland soil. Scale bar = 50 μm. The image demonstrates the spatial distribution of microbial communities that contribute to soil organic carbon through necromass deposition.

3.2 Microbial Necromass Accumulation

Total microbial necromass increased exponentially with restoration age, following the equation: Necromass = 85.3 × e^(0.089×age) (R^2 = 0.78, p < 0.001). Bacterial necromass dominated early succession (65-70% of total microbial necromass in years 1-5), while fungal necromass became increasingly important in mature systems (55-60% by years 12-15) (Table 1).

Table 1: Microbial necromass accumulation across restoration chronosequence

Site Age (years)	Bacterial Necromass (mg C kg^{-1})	Fungal Necromass (mg C kg^{-1})	Total Necromass (mg C kg^{-1})	F:B Ratio
1-3	125 ± 18^{a}	68 ± 12^{a}	193 ± 25^{a}	0.55 ± 0.08^{a}
4-6	165 ± 22^{b}	142 ± 28^{b}	307 ± 38^{b}	0.86 ± 0.12^{b}
7-9	185 ± 31^{bc}	225 ± 35^{c}	410 ± 52^{c}	1.22 ± 0.15^{c}
10-12	195 ± 28^{c}	285 ± 42^{cd}	480 ± 58^{cd}	1.46 ± 0.18^{cd}
13-15	205 ± 33^{c}	345 ± 48^{d}	550 ± 65^{d}	1.68 ± 0.22^{d}
Native Prairie	220 ± 35^{c}	380 ± 55^{d}	600 ± 72^{d}	1.73 ± 0.25^{d}
Agricultural	85 ± 15^{a}	35 ± 8^{a}	120 ± 18^{a}	0.41 ± 0.06^{a}

Values are means ± standard error. Different letters indicate significant differences (p < 0.05) within columns.

3.3 Soil Organic Carbon Accumulation

SOC accumulation rates varied significantly with restoration age and management approach (Figure 2). Average accumulation rates were 0.8 ± 0.2 Mg C ha^{-1} yr^{-1}, with higher rates in high-diversity plantings (1.1 ± 0.3 Mg C ha^{-1} yr^{-1}) compared to low-diversity treatments (0.6 ± 0.2 Mg C ha^{-1} yr^{-1}).

The contribution of microbial residues to total SOC increased from 35% in year 1 to 65% by year 15. Fungal necromass contributions showed the most dramatic increase, rising from 15% to 40% of total SOC over the chronosequence. Bacterial contributions remained relatively stable at 20-25% throughout succession.

Fig 2: Soil organic matter fractionation results

Laboratory analysis showing different soil organic matter fractions isolated through density separation. Dark fractions represent mineral-associated organic matter (MAOM) enriched in microbial residues, while lighter fractions contain particulate organic matter (POM) from plant debris.

3.4 Management Effects on Microbial Residues

Plant species diversity significantly influenced microbial necromass accumulation (Table 2). High-diversity treatments supported 40-50% greater total necromass compared to low-diversity plantings, primarily due to enhanced fungal residue production. The presence of legumes in seed mixtures increased bacterial necromass by 25-30% but had variable effects on fungal residues.

Table 2: Effects of management practices on microbial necromass after 10 years of restoration

Management Treatment	Bacterial Necromass	Fungal Necromass	Total Necromass	SOC Accumulation
	(mg C kg^{-1})	(mg C kg^{-1})	(mg C kg^{-1})	(Mg C ha^{-1} yr^{-1})
Low Diversity	145 ± 20^{a}	195 ± 25^{a}	340 ± 35^{a}	0.6 ± 0.15^{a}
Medium Diversity	175 ± 25^{b}	265 ± 35^{b}	440 ± 45^{b}	0.8 ± 0.18^{b}
High Diversity	195 ± 28^{b}	325 ± 45^{c}	520 ± 55^{c}	1.1 ± 0.22^{c}
With Legumes	220 ± 32^{c}	285 ± 40^{bc}	505 ± 52^{c}	1.0 ± 0.20^{bc}
No Tillage	185 ± 26^{b}	310 ± 42^{c}	495 ± 48^{c}	0.9 ± 0.19^{bc}
Reduced Mowing	165 ± 23^{b}	295 ± 38^{c}	460 ± 44^{b}	0.8 ± 0.17^{b}

Values are means ± standard error. Different letters indicate significant differences ($p < 0.05$) within columns.

Soil disturbance practices showed strong negative effects on fungal necromass accumulation. Sites with no-till establishment supported 60% greater fungal residues compared to conventionally tilled sites. Reduced mowing frequency also enhanced fungal necromass deposition, likely due to maintained hyphal networks and reduced physical disruption.

3.5 Chemical Composition and Stability

Spectroscopic analysis revealed distinct chemical signatures for bacterial and fungal necromass. Bacterial residues showed higher protein and carbohydrate content, while fungal residues were enriched in aromatic compounds and nitrogen-containing heterocycles. Fungal necromass exhibited greater resistance to chemical oxidation, with 65-70% remaining after H_2O_2 treatment compared to 35-40% for bacterial residues.

Isotope analysis indicated preferential preservation of fungal carbon in mineral-associated organic matter fractions. The $\delta^{13}C$ signature of MAOM closely matched fungal necromass values, suggesting selective stabilization of fungal-derived carbon through mineral interactions.

4. Discussion

4.1 Mechanisms of Microbial Carbon Contribution

Our results demonstrate that microbial residues represent the dominant pathway for SOC accumulation in restored grasslands, contributing 35-65% of total carbon stocks. This finding aligns with recent global syntheses showing substantial microbial contributions to SOC across ecosystem types, but represents one of the first comprehensive assessments in restored grassland systems.

The mechanisms underlying microbial residue accumulation involve complex interactions between microbial physiology, community dynamics, and environmental factors. Bacterial necromass production is closely linked to substrate availability and turnover rates, explaining the early dominance of bacterial contributions when labile plant inputs are abundant. The subsequent shift toward fungal dominance reflects ecosystem maturation, with established plant communities supporting extensive mycorrhizal networks and saprotrophic fungi.

The chemical composition differences between bacterial and fungal residues have important implications for carbon stability. Bacterial necromass, rich in proteins and peptidoglycan, tends to be more susceptible to enzymatic degradation. In contrast, fungal residues containing chitin, melanin, and other recalcitrant compounds exhibit greater persistence in soil. This compositional variation explains the increasing stability of SOC pools as fungal contributions become more prominent in mature grassland systems.

4.2 Temporal Dynamics and Succession

The temporal shift from bacterial to fungal dominance in microbial necromass reflects broader patterns of ecosystem succession in restored grasslands. Early bacterial dominance coincides with rapid plant establishment and high rates of root exudation, creating favorable conditions for copiotrophic bacterial communities. As plant communities mature and root systems develop, conditions become more favorable for oligotrophic bacteria and fungi, particularly mycorrhizal species that form stable associations with perennial grasses.

The exponential increase in total microbial necromass over the 15-year chronosequence suggests that restored grasslands continue accumulating microbial-derived carbon well beyond initial establishment. This finding has important implications for carbon sequestration projections, as many studies focus on shorter time scales that may underestimate long-term accumulation potential.

The fungal:bacterial necromass ratio proved to be a useful indicator of restoration success and ecosystem maturity. Values approaching those of native prairie (1.7-1.8) were observed only in sites older than 12 years, suggesting that full functional restoration requires more than a decade. This temporal requirement should be considered in restoration planning and carbon offset programs that rely on grassland establishment.

4.3 Management Implications

Our results highlight several management strategies that can enhance microbial residue contributions to SOC. Plant species diversity emerged as the most significant factor, with high-diversity plantings supporting 40-50% greater microbial necromass compared to simple grass monocultures. This enhancement likely results from multiple mechanisms, including increased root exudate diversity, complementary resource use, and support for diverse microbial communities.

The positive effects of legume inclusion on bacterial necromass reflect enhanced nitrogen availability through biological fixation. However, the variable effects on fungal necromass suggest complex interactions between nitrogen availability and fungal community development. Moderate nitrogen enrichment may stimulate fungal growth, while excessive nitrogen can reduce mycorrhizal associations and shift communities toward bacterial dominance.

Soil disturbance practices showed particularly strong effects on fungal necromass accumulation. No-till establishment and reduced mowing frequency both enhanced fungal contributions, likely through maintenance of hyphal networks and reduced physical disruption. These findings support the adoption of low-disturbance restoration approaches, particularly in areas where long-term carbon sequestration is a primary objective.

4.4 Implications for Carbon Sequestration

The substantial contribution of microbial residues to SOC has important implications for carbon sequestration in restored grasslands. Traditional carbon models that focus primarily on plant inputs may underestimate actual sequestration potential, particularly in mature systems where microbial contributions dominate. Our results suggest that including microbial parameters in carbon models could improve accuracy and predictive capability.

The increasing dominance of fungal necromass over time is particularly relevant for long-term carbon storage. Fungal residues' greater chemical recalcitrance and association with mineral-protected carbon pools suggest enhanced persistence compared to plant-derived inputs. This relationship implies that restoration strategies promoting fungal community development may achieve greater long-term carbon storage benefits.

The observed SOC accumulation rates (0.8 ± 0.2 Mg C ha^{-1} yr^{-1}) are comparable to those reported in other grassland restoration studies, but our results suggest that microbial contributions may continue increasing beyond typical study periods. This finding supports longer-term monitoring and management commitments for restoration projects aimed at carbon sequestration.

4.5 Limitations and Future Directions

Several limitations should be considered when interpreting these results. First, our chronosequence approach assumes similar initial conditions and management across sites, which may not fully capture site-specific variation. Long-term monitoring of individual sites would provide more robust temporal data but was beyond the scope of this study.

Second, the biomarker approach used for necromass quantification, while well-established, may not capture all forms of microbial-derived carbon. Advanced techniques such as compound-specific isotope analysis or NMR spectroscopy could provide additional insights into microbial carbon dynamics.

Future research should examine the interactive effects of climate change and management on microbial residue dynamics. Projected increases in temperature and precipitation variability may alter microbial community succession and necromass production in ways not captured by current space-for-time studies.

Additionally, exploring the role of specific microbial taxa in carbon sequestration could inform more targeted restoration approaches. Recent advances in microbial ecology suggest that certain bacterial and fungal groups may be particularly important for stable carbon formation.

5. Conclusions

This study demonstrates that microbial residues represent the dominant pathway for SOC accumulation in restored grasslands, contributing 35-65% of total carbon stocks. The temporal shift from bacterial to fungal necromass dominance reflects ecosystem maturation and has important implications for long-term carbon stability. Key findings include:

1. **Microbial dominance:** Microbial residues contribute substantially more to SOC than previously recognized, with contributions increasing over restoration time scales.
2. **Successional patterns:** The shift from bacterial to fungal necromass dominance provides a useful indicator of restoration success and ecosystem maturity.
3. **Management effects:** Plant species diversity, reduced soil disturbance, and appropriate nitrogen management enhance microbial residue accumulation and carbon sequestration.
4. **Long-term perspective:** Microbial contributions continue increasing well beyond initial restoration establishment, supporting longer-term management commitments.
5. **Carbon stability:** The increasing dominance of chemically recalcitrant fungal residues over time suggests enhanced long-term carbon storage potential.

These findings have important implications for restoration practice and policy. Management approaches that promote diverse plant communities, minimize soil disturbance, and support fungal community development can enhance both restoration success and carbon sequestration benefits. Future restoration projects should consider microbial community development as a key indicator of success and incorporate practices that promote beneficial microbial residue accumulation.

The substantial and increasing contribution of microbial residues to SOC suggests that grassland restoration represents a viable climate change mitigation strategy, with potential benefits continuing well beyond initial establishment periods. However, realizing this potential requires long-term management commitments and adoption of practices that support healthy microbial communities and their contributions to soil carbon storage.

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