Perennial Forage Polycultures and Organic Amendments Drive Soil Carbon Sequestration and Organic Matter Stabilization: Results After 90 Years of Management at the Breton Plots

by

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Abstract

Increasing agricultural intensification threatens to strain already degrading global soil resources while simultaneously escalating anthropogenic greenhouse gas emissions. Loss of soil organic matter (SOM) from agroecosystems is a major cause of soil degradation and a source of considerable carbon (C) emissions. By corollary, agricultural practices that increase SOM can improve soil productivity and sequester atmospheric C. However, SOM responses to land management can take decades to become apparent, necessitating long-term experiments to accurately identify them. This project aimed to quantify how diverse agricultural management practices impact soil health and agroecosystem sustainability and, more specifically, SOM accumulation and persistence and, in turn, soil C storage. To address these questions, soils from various long-term agricultural treatments from the Breton Plots (est. 1929) were evaluated, including manure amendment, conventional fertilizer application, alternating fallow, continuous and rotational cropping with and without perennials and legumes, no-till, and green manuring. This project explored the impacts of these practices on soil C stock and microbial necromass, along with various physicochemical properties important to soil health and agroecosystem sustainability. Soil C stocks down to 90 cm below the soil surface were evaluated and contrasted between different management practices. Further, the monetary values of C stock differences were quantified using the Government of Canada’s current minimum price on C pollution ($80 CAD per tonne carbon dioxide equivalent). Following up on this, soil microbial necromass in the top 7.5 cm of soil was estimated, and relationships between necromass and soil C and nitrogen (N) pools, along with various soil physicochemical properties in three soil particle size and density fractions, were examined. Fallow was found to cause severe soil degradation in both the topsoil (i.e., 0-15 cm depth) and subsoil (> 15 cm depth) through C and N loss in all measured soil fractions, as well as SOM destabilization from a reduction in the proportion of fungal necromass and generally limited microbial necromass accumulation. Conventional fertilizer application consistently increased soil acidification and bacterial necromass C accumulation. Fertilization also increased topsoil C and N when paired with continuous cropping but not when paired with fallow. In contrast, manure application consistently raised soil pH and N levels while increasing C stored in mineral-associated organic matter fractions and all necromass pools. Similarly, perennial grass-legume forages increased C and N stored in all soil fractions and necromass pools in direct proportion to how long forages were consecutively grown within a given cropping system, with soil C enrichments reaching well into the subsoil. No-till generated an extremely C-rich uppermost soil layer enriched in fresh plant C residues, with a high proportion of SOC stabilized in fungal necromass. On the other hand, legume green manuring appears to have contributed to C accrual in both the topsoil and subsoil while favouring bacterial necromass accumulation in the surface soil layer. In total, compared to the annual cash crop system lacking fallow, the continuous forage system and the green-manure-inclusive eight-year rotation led to more than \$5000 CAD ha^{-1} of additional C storage, while fallow reduced C storage by more than \$11,000 CAD ha^{-1}. Finally, microbial necromass was found to be a strong predictor of overall soil organic C, with fungal necromass contributing more to predictive power than bacterial necromass. These findings demonstrate that soil health, fertility, and SOM accumulation and stability benefit from increased organic matter inputs, whether from increased crop biomass inputs or organic amendments like manure. Further, these benefits translated into considerable monetary sums under current C valuation practices.

Preface

This thesis is an original work by Jamin N. Achtymichuk, and no part of it has been previously published.

"The nation that destroys its soil destroys itself."
- Franklin Delano Roosevelt

Acknowledgements

No man is an island, and indeed, this research was far from an individual, isolated effort. In that spirit, I would like to thank the many colleagues, friends, and family members who helped support me through this chapter of my life.

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Table of Contents

List of Tables

List of Figures

List of Acronyms

5Y	5-year rotation
8Y	8-year rotation
BD	bulk density
BNC	bacterial necromass carbon
C	carbon
CF	continuous forage cropping system
CG	continuous grain cropping system
CMAOM	coarse mineral-associated organic matter
CO_2	carbon dioxide
CO_2-e	carbon dioxide equivalent
ESM	equivalent soil mass
FAO	Food and Agriculture Association
FMAOM	fine mineral-associated organic matter
FNC	fungal necromass carbon
GC	gas chromatography
GHG	greenhouse gas
GLM	generalized linear model
GLMM	generalized linear mixed-effects model
Glu	soil total glucosamine content
Glu_{fun}	soil fungal glucosamine content
HCl	hydrochloric acid
K_2SO_4	potassium sulfate
LF or LFOM	light fraction organic matter
LM	linear regression model
LMW	low-molecular-weight
LTAE	long-term agroecosystem experiment
MAOM	mineral-associated organic matter
MNC	microbial necromass carbon
MOA	mineral-organic association

MurA	soil muramic acid content
N	nitrogen
N_2	nitrogen gas
N_2O	nitrous oxide
NaI	sodium iodide
OM	organic matter
P	phosphorus
PLFA	phospholipid fatty acid
POM	particulate organic matter
R_{sample}	sample ^{13}C:^{12}C ratio
R_{VPDB}	VPDB reference ^{13}C:^{12}C ratio
S	sulphur
SOC	soil organic carbon
SOM	soil organic matter
TC	total soil carbon content
TN	total soil nitrogen content
VPDB	Vienna Peedee Belemnite
WF	wheat-fallow rotation
ΔTC	change in total soil carbon content

List of Appendices

Chapter 1 – General Introduction to Soil Organic Matter and Long-Term Agroecosystem Experiments on the Canadian Prairies

1.1. Agricultural Management for Climate Change Mitigation and Food Security

Over the past several decades, growing concerns about the detrimental effects of global change have prompted the international community to commit to ambitious goals to limit future climate change. Amongst the most pivotal of these goals is reaching net-zero global greenhouse gas (GHG) emissions before the end of the century. As a result, nations are looking at various industrial sectors—such as agriculture—as potential targets for emission reduction. Globally, agriculture contributes one-fifth to one-third of total annual GHG emissions (FAO, 2021a; Poore & Nemecek, 2018; Rosenzweig et al., 2020). Locally, the Canadian agriculture industry contributed an estimated 8% of national GHG emissions in 2019 (Environment and Climate Change Canada, 2021). Due to the significant emission footprint associated with agricultural activity, there is mounting pressure from both governments and consumers to shift towards enhanced agricultural sustainability (i.e., by limiting environmental impacts). At the same time, a global population increasing in both size and prosperity is swelling demand for agricultural products, with a notable increase in the demand for animal-derived protein. For example, a recent meta-analysis found that by 2050, the demand for food could increase as much as 56% compared to the demand in 2010 (van Dijk et al., 2021). Similarly, global meat consumption by 2050 is projected to increase by up to 60% compared to 2010 levels, bolstered primarily by increasing prosperity in developing nations (Revell, 2015). Therefore, there is a concomitant need for agricultural producers to increase productivity and reduce net GHG emissions.

To promote GHG emission reductions in various industrial sectors, various governments are implementing carbon (C) valuation mechanisms to incentivize C storage or reduce C pollution. However, these developing C economies must be based on a robust knowledge of GHG source and sink dynamics in the relevant industrial systems to effectively reduce GHG pollution. In the case of agricultural production systems, the development of effective C economies is hampered by an incomplete understanding of how soil C sequestration responds to agricultural management practices (Amelung et al., 2020). In Canada, as of 2024, the Government of Canada's *Greenhouse Gas Pollution Pricing Act* sets a minimum price on C pollution of $80 CAD per tonne of C dioxide (CO_2) equivalent (CO_2-e), which will progressively increase to $175 CAD per tonne CO_2-e by 2030 (Greenhouse Gas Pollution Pricing Act, 2018). These C prices could represent significant increases in costs or profits for agricultural producers if used to tax or credit producers for C emitted or stored, respectively. Given that C valuation policies are increasingly being realized, taxation and compensation levels will rely on policymakers' understanding of C dynamics in agroecosystems. Thus, ongoing C dynamics in relation to specific agricultural practices must be well understood so that any such taxation and compensation is accurate and appropriate in scale.

Agricultural management systems focusing on increasing soil organic matter (SOM) levels can simultaneously combat agricultural GHG emissions and improve agricultural productivity. Soil organic matter is the most significant terrestrial C reservoir, with a global total of about 1500 petagrams (Pg) of organic C stored within the first metre of soil, dwarfing the estimated 560 Pg of C stored globally as biomass (Batjes, 1996; Falkowski et al., 2000; Jobbágy & Jackson, 2000; Lal, 2008). This high C storage capacity makes soils an attractive target for C sequestration efforts. Globally, agricultural and managed soils are also estimated to have lost 40 Pg to 230 Pg of soil organic C (SOC) since the advent of agriculture, 50% to 75% of which could be restored, representing a sizeable potential C sink (Lal, 2001; Lal et al., 2007; Paustian et al., 1997). Moreover, SOM is a vital component of soil systems that improves multiple facets of soil health. For example, SOM improves soil aggregation and structure, adds critical plant nutrients such as nitrogen (N), sulphur (S), and phosphorus (P) to the soil, and benefits important soil chemical features, such as cation exchange capacity (Johnston et al., 2009). Thus, by increasing SOM content, agricultural management strategies can promote significant C fixation while benefitting long-term productivity.

Amongst the litany of different management practices that can help increase SOM levels, the use of soil amendments to improve crop productivity and soil biomass inputs remains widespread. Inorganic (i.e., conventional fertilizers) and organic (e.g., manure, straw) soil amendments are extensively documented to increase crop production and, consequently, SOM, SOC, and soil N levels (Baumhardt et al., 2015; Li et al., 2014; Nielsen & Calderón, 2011; Plaza-Bonilla et al., 2015; Ramesh et al., 2019; Rodgers et al., 2021; Tiefenbacher et al., 2021). Fertilizer and manure are among the most important cropland soil amendments. While both these amendments improve soil productivity, they affect soils and their surrounding environments in unique ways. Nitrogen fertilizer and manure application can both lead to N leaching and eutrophication of nearby waterbodies (Bijay-Singh & Craswell, 2021; Jwaideh et al., 2022; Miao et al., 2011), as well as increased nitrous oxide (N_2O) emissions (Bouwman et al., 2002; Snyder et al., 2009; Tian et al., 2020). However, manuring generally contributes less to these N pollution issues than conventional fertilization, though effluents rich in mineral N (e.g., pig slurry) can be an exception to this trend (Abbasi & Sepaskhah, 2023; Bijay-Singh & Craswell, 2021; Gu et al., 2023; He et al., 2019). Long-term conventional N fertilization also generally leads to soil acidification (Tian & Niu, 2015). In contrast, manure application tends to increase soil buffering capacity, causing the soil to approach a neutral pH (Liu et al., 2020; Ning et al., 2020). However, manure amendments often lead to excess soil P levels and increased P leaching, which also presents waterbody eutrophication risks (Hu et al., 2024; Ma et al., 2020). Further, the production, procurement, transport, and application of soil amendments (e.g., manure, conventional fertilizer) are associated with considerable lifecycle costs and GHG emissions in agricultural systems (Aguirre-Villegas & Larson, 2017; Gareau, 2012; Hasler et al., 2015; Menegat et al., 2022). As a result, attempts to improve agricultural profitability and sustainability using soil amendments require judicious application rates, timings, amendment selection, and placement (i.e., 4R nutrient stewardship) to minimize the deleterious side effects of soil amendments.

Importantly, N fertilizer and manure application are the primary sources of agricultural N_2O emissions (Shcherbak et al., 2014). These emissions are particularly concerning, as N_2O is a potent GHG with a global warming potential 273 times greater than CO_2 over a 50- to 100-year timescale (IPCC, 2021b). Furthermore, N_2O is currently the most significant stratospheric ozone-depleting gas and is projected to remain the dominant such gas throughout this century (Ravishankara et al., 2009). Notably, agriculture is the predominant anthropogenic source of N_2O globally (IPCC, 2021a). Therefore, limiting the amounts of applied N fertilizer and manure could significantly reduce the total environmental footprint of agricultural activity, but alternative sources of N (e.g., legume cultivation) would be required to avoid crop N-limitation-induced losses to productivity. Given the high costs associated with soil-amendment-based nutrient management, often reaching or exceeding 24% to 30% of the total variable cost of conventional agricultural production (Gareau, 2012), limiting or judiciously applying soil amendments to optimize agricultural productivity could also improve farm profitability.

Increasing the inclusion of perennials (e.g., forages, bioenergy crops, perennial grains) into cropping systems is another SOM-enhancing management practice that could decrease the environmental impacts of agricultural activity. Due to their continuous growth over multiple years, perennial crops produce extensive underground biomass and generally input greater amounts of organic matter (OM) into the soil than annual crops (King & Blesh, 2018; Ledo et al., 2020; Sprunger et al., 2020). Further, these belowground biomass inputs increase in scale over multiple growing seasons as perennial crops continue to expand their root systems (Bolinder et al., 2012; Houde et al., 2020; Sakiroglu et al., 2020; F. Zhang et al., 2023). Additionally, perennial crops inherently provide extended periods of soil cover and reduce tillage frequency (i.e., within tillage-inclusive systems), thereby helping to preserve soil structure and moisture while reducing erosion (Baumhardt et al., 2015; Desta et al., 2021; Durán Zuazo & Rodríguez-Pleguezuelo, 2008; Schlautman et al., 2021). Extended periods of soil cover mean that perennials maintain greater soil OM inputs, constrain microbial nutrient limitations and allow more efficient conversion of plant inputs into microbial residues (Blagodatskaya et al., 2014; Kästner et al., 2021; Ruf & Emmerling, 2020; Sinsabaugh et al., 2013). These factors make perennial crops effective tools for increasing SOM levels and soil C storage.

Similarly, increasing the inclusion of leguminous crops, such as alfalfa (*Medicago sativa* L.) or faba beans (*Vicia faba* L.), which biologically fix and assimilate atmospheric N into their tissues, can improve SOM generation and retention. Legumes improve soil fertility as N-enriched plant residues cycle into the soil, improve future crop productivity, and reduce the need for nitrogenous fertilizer and manure applications (Stagnari et al., 2017). Additionally, the high quality (i.e., low C:N ratio) of legume residues enhances microbial substrate use efficiency and allows a higher proportion of plant biomass to be converted into microbial biomass rather than lost to respiration (Cotrufo et al., 2013; Cotrufo & Lavallee, 2022; Frasier et al., 2016; Kallenbach et al., 2015). This process encourages the formation of stabilized mineral-associated OM (MAOM), as these soil microbes eventually die and become entombed on mineral surfaces as necromass (Buckeridge et al., 2020; Lavallee et al., 2020; Miltner et al., 2012). Consequently, the increased inclusion of legumes in crop rotations can potentially increase SOM levels, as well as elevate overall agricultural productivity and profitability while bolstering environmental sustainability on multiple fronts.

Many animal forage crops are both perennial and leguminous (e.g., alfalfa), while non-leguminous forages (e.g., grasses) are often grown as part of legume-inclusive polyculture hays. As such, forage cropping systems generally experience the combined benefits of perennials, legumes, and enhanced crop diversity. Increasing crop diversity is noteworthy as a SOM-increasing management strategy, as it promotes microbial diversity, nutrient cycling, root biomass generation, and general soil health (Frasier et al., 2016; Plaza-Bonilla et al., 2015; Sprunger et al., 2020). On top of the ensuing potential provided by diverse cropping systems for enriching soils and decreasing agriculture's environmental footprint, forage crops are the basis of several livestock feed supply chains (i.e., for large herbivores).

The impact of increased forage production on feed supply is a critical consideration for the animal agriculture industry, as supplying feed is one of the costliest activities associated with livestock production. For instance, in Canada, between 2012 and 2016, supplying cattle with feed was responsible for 64% of the total cost of cow-calf production (CANFAX, 2017). As such, increasing forage crop acreage would help stabilize feed supply chains and drive down feed-associated costs. In turn, this feed cost reduction would help the agricultural industry economically meet the growing consumer demand for meat and animal-derived protein. Furthermore, a robust understanding of the impacts of forage growth on the lifecycle emissions of livestock production systems will be necessary to effectively integrate them into developing C economies. As a result, a comprehensive understanding of how common forage systems affect soil C storage and stabilization, and how these systems modulate the need for soil N amendments and associated N pollution, is required.

To complicate matters, many soil processes occur on relatively slow timescales (i.e., decadal or greater), including the accumulation and turnover of recalcitrant SOM pools (Gerzabek, 1998; Kleber et al., 2015). Consequently, the effects of different agricultural management systems on SOM accrual can be obscured due to their tendency to be too minimal to be reliably observed on shorter timescales. To overcome this barrier to evaluation, it is critical to have long-term data that can effectively capture the soil's ongoing response to different management strategies. Long-term agroecosystem experiments (LTAEs) are an invaluable source of such long-term data and are the focus of the current investigation.

Numerous LTAEs across the Canadian Prairies include either permanent forage components or forages as parts of crop rotations (Carkner et al., 2020; Dyck et al., 2012; Lafond et al., 2012; Lemke et al., 2012; Smith et al., 2012). Many of these LTAEs have indicated improved soil health and productivity metrics in response to forage crop cultivation (Dyck et al., 2012; Lafond et al., 2012; Lemke et al., 2012; Smith et al., 2012). Observed agronomic benefits include improved SOM (and SOC) content, soil macronutrient levels, soil structure, and crop yields, as well as reductions in N fertilizer inputs needed to maintain crop productivity (Dyck et al., 2012; Lafond et al., 2012; Lemke et al., 2012; Smith et al., 2012). However, significant uncertainty remains surrounding the stability of these benefits over time, the extent to which forage crop cultivation can be credited for these improvements, and the specific modes of action through which forage crops may promote long-term soil health and productivity. Of special interest are the long-term impacts of forage crop inclusion on the overall storage and turnover rate of SOC, including the impact on microbially derived SOM dynamics.

1.2. Changing Conceptions of SOM

Traditionally, SOM was thought to be primarily composed of stable (i.e., slow to turnover) “humic substances” that were characterized by their reactivity (or lack thereof) to highly alkaline solutions (e.g., aqueous sodium hydroxide) and subsequent solubility in water after reacidification (International Humic Substances Society, 2007; Lehmann & Kleber, 2015). Humic substances, collectively known as humus, were commonly separated into three pools: humic acid, the material that precipitates upon reacidification; fulvic acid, which remains soluble following reacidification; and humin, the fraction of SOM that did not react with the alkaline extractant (Lehmann & Kleber, 2015). As such, humic substances represented an operational proxy, comprising an amalgamation of distinct SOM types reduced to inextricable chemical slurries that obscured the mechanisms behind stable SOM formation (known as ‘humification’ in the humic substances model) and its decay (Lehmann & Kleber, 2015). This view of stable SOM generation traditionally conceptualized chemically recalcitrant plant compounds as the major building blocks of humic substances (Flaig et al., 1975; Martin et al., 1980). Despite generally falling out of favour, this paradigm has continued to inform aspects of modern soil science.

Perhaps inspired by the chemistry- and plant-focused view of stable SOM formation, the ‘selective preservation’ model has also been influential. Selective preservation assumes that chemically recalcitrant plant compounds (e.g., lignin, cellulose) remain in the soil as other more labile compounds are preferentially broken down, leading to increasing concentrations of inherently recalcitrant materials in the soil (Marschner et al., 2008; Sollins et al., 1996; von Lützow et al., 2008). By corollary, this viewpoint implies that stable SOM must be primarily composed of recalcitrant plant compounds (von Lützow et al., 2006). This concept is indeed old, as Flaig et al. (1975) suggested that lignin and phenolic plant constituents must be major originators of humic substances due to their (assumed) slow decay rates. Providing further support for this view, living microbial biomass has been observed to typically only make up 1% to 5% of total SOM at any given time (Anderson & Domsch, 1989; Dalal, 1998), ostensibly confirming the supremacy of plant-derived compounds in stable SOM formation.

However, the selective preservation model has been largely invalidated due to research indicating that stable SOM is not characterized by a narrow range of biochemically stable compounds, and that many of the supposedly recalcitrant plant compounds often break down quickly in the soil (Dungait et al., 2012; Marschner et al., 2008). For example, in an experiment conducted by Rasse et al. (2006), stable isotope analysis was used to show that most lignin decomposed within a year of addition to soil. Additionally, more recent studies have demonstrated that senesced microbial residues (i.e., necromass) can be stabilized against decay in the soil via associations with mineral surfaces and other necromass (Buckeridge et al., 2020; Kleber et al., 2015; Miltner et al., 2012). As an example, Miltner et al. (2012) combined ^{13}C-labelling and scanning electron microscope imaging to track the decay of bacterial cells added to soil, concluding that, after a 224-day incubation, 50% of the added bacterial C remained in the soil, primarily as mineral-associated fragments. Similarly, Buckeridge et al. (2020) used stable-isotope-labelling and laboratory soil incubation to demonstrate that necromass adsorbs to both mineral surfaces and to other necromass, and roughly 60% to 100% of labelled necromass C and N were retained after 3 days of incubation. These results allude to other stable SOM formation pathways, depending more on microbial modification and generation of OM than on the selective preservation of recalcitrant plant compounds.

Further cementing the importance of microbial SOM formation, contemporary research has indicated that the majority (or at least a considerable minority) of SOM comprises microbial necromass generated from soil fungi and bacteria. Recent estimates of microbial contributions to SOM show necromass C typically ranges between 30% to 70%—and averages 50%—of total SOC (Li et al., 2023; Liang et al., 2019; Wang et al., 2021; Zhu et al., 2020). While these findings are broadly valid for a range of ecosystems, forests tend to have the lowest mean microbial C contribution (~29% to 35% of SOC), whereas grasslands (~47% to 65.9% of SOC) and croplands (~51% to 58.4% of SOC) are comparable (Liang et al., 2019; Wang et al., 2021). The characterization of amino sugar biomarkers using tools such as gas chromatography (GC) (Liang et al., 2012) and high-performance liquid chromatography (Salas et al., 2023), followed by back conversion to dry-weight microbial necromass is currently the favoured technique for estimating long-term microbial contributions to SOM. However, various techniques, such as molecular fingerprinting, have been used to complete these estimations. For example, Simpson et al. (2007) used nuclear magnetic resonance spectroscopy to examine the fingerprint of key plant and microbial biomass indicator compounds, estimating that more than 50% of alkaline-extractable SOM, approximately 45% of humin SOM, and more than 80% of soil N were of microbial origin. Irrespective of the technique used to quantify microbial contributions to SOM, the importance of the microbial SOM formation pathways appears undeniable and the primacy of plant-derived SOM, questionable.

Regardless of the sizable contribution of soil microbes to SOM generation outlined above, it should be noted that around half of total SOM is generally plant-derived (i.e., composed of preserved plant biomolecules that have not been recycled into microbial biomass), with forests being notable regions where the majority of SOM is typically plant-derived (Angst et al., 2021). Therefore, it is critical to understand the mechanisms through which plant-derived OM is converted to stable SOM. Similar to microbial residues, it appears that plant-derived OM is also primarily stabilized through interactions with soil mineral particles, as well as by occlusion within soil aggregates (Angst et al., 2021). In particular, molecular fingerprinting studies indicate that short-chain root lipids, root exudates (e.g., galactose), and the products of extracellular decomposition of plant lipids and hemicelluloses may be important contributors to the MAOM pool (Whalen et al., 2022).

In any case, recent work on SOM formation appears to favour a soil continuum model sensu Lehmann and Kleber (2015), in which SOM comprises a continuum of organic compounds being iteratively decomposed into smaller molecules with increasing capacity for mineral-organic association (MOA). In this view, the most important stabilizer of SOM is the generation of MOAs, which appear to have turnover times 4 to 100 times slower than either free or aggregate-occluded SOM (Kleber et al., 2015). In turn, these MOAs are primarily formed when plant-derived OM is converted to microbial necromass or modified via extracellular decomposition (Kleber et al., 2015; Whalen et al., 2022). An apparently smaller proportion of MOAs form from low-molecular-weight (LMW) plant compounds (e.g., root exudates) directly interacting with soil mineral surfaces (Kleber et al., 2015; Whalen et al., 2022). Given the importance of MOAs to SOM stabilization—and the dominant role of microbes in facilitating these associations—understanding how agricultural practices impact soil microbes and MOA formation will be critical to efforts aimed at enhancing soil health and C storage.

1.3. Quantifying SOM

1.3.1. SOM Fractions

Given the much-enhanced stability of MOAs (Kleber et al., 2015) and the sensitivity of physically uncomplexed SOM to land management practices (Gregorich et al., 2006); analyzing how each of these pools responds to management is a critical step in developing practices that increase SOM. This necessitates fractioning soil into functional pools prior to analyzing soil properties (e.g., C and N) so that each pool can be evaluated independently. Perhaps the most common fractionation technique involves separating soil into particulate OM (POM) and MAOM based on soil particle size (following aggregate dispersion), with POM consisting of all particles larger than 20-63 µm (cut-offs vary between fractionation protocols) in diameter, and MAOM consisting of all particles smaller than 20-63 µm in diameter (Lavallee et al., 2020). This fractionation technique is appealing because it is parsimonious and relatively non-labour-intensive while effectively separating SOM into pools with distinctly different turnover times, chemical makeups, and soil functions (Lavallee et al., 2020; Poeplau et al., 2018). For example, as reviewed by Lavallee et al. (2020), POM and MAOM have turnover times spanning from years to decades, and decades to centuries, respectively. However, the fraction of POM that exceeds a density of 1.6-1.85 g cm^{-3} (again varying based on the protocol), variously called heavy POM, sand-sized SOM, or coarse-MAOM (CMAOM), includes MOAs and displays unique formation, turnover, and soil nutrient provisioning dynamics (Gregorich et al., 2006; Lavallee et al., 2020; Maillard et al., 2015; Poeplau et al., 2018; Samson et al., 2020). Meanwhile, the POM fraction with a density of less than 1.6-1.85 g cm^{-3}, also known as light fraction OM (LF or LFOM) or light POM, is principally composed of physically uncomplexed SOM closely associated with fresh (i.e., minimally microbially processed) plant and faunal detritus (Gregorich et al., 2006; Lavallee et al., 2020; Maillard et al., 2015; Poeplau et al., 2018; Samson et al., 2020). The lack of MOAs in the LFOM fraction leaves it vulnerable to rapid turnover (at rates 2 to 11 times that of the bulk soil), particularly in response to disturbances to the soil environment (Gregorich et al., 2006).

As a result, there are strong motivations to eschew the POM-MAOM dichotomy and examine an expanded number of fractions. This can be done by including additional particle size or density intervals, examining various soil aggregate size classes, or including chemically determined fractions (Poeplau et al., 2018). However, there are diminishing returns in analytical clarity as the cost and labour-intensiveness of the overall fractionation process increases (Poeplau et al., 2018). In response to this issue, Poeplau et al. (2018) concluded that examining three to five soil fractions resulted in optimal analytical returns relative to labour and cost inputs. This study also found that particle size fractionation was superior to aggregate size fractionation for identifying soil fractions with unique behaviour and turnover rates (Poeplau et al., 2018). Samson et al. (2020) provide an example of a physical fractionation procedure utilizing particle size and density fractionation to examine three fractions, namely LFOM ($\rho < 1.7$ g cm^{-3}), CMAOM ($\rho > 1.7$ g cm^{-3}, d > 53 μm), and fine-MAOM (FMAOM; $\rho > 1.7$ g cm^{-3}, d < 53 μm). Ultimately, the authors concluded that the CMAOM fraction (which would normally not be distinguished from LFOM) was the primary locus for new SOM formation and stabilization because it acted as an intermediate phase in the long-term generation of FMAOM (Samson et al., 2020). Further, while LFOM was often considered the primary source of available soil nutrients (due to being readily decomposable), it appears that the heavy fraction (i.e., CMAOM and FMAOM) supplies the majority of mineralizable N within both forest and cropped soils (Gregorich et al., 2006). As a result, the commonly applied POM-MAOM dichotomy obscures the unique dynamics of the CMAOM fraction, and more research should be conducted to assess the influence of this critical SOM pool.

1.3.2. Microbial Necromass

Much recent work has focused on identifying microbial residues (i.e., necromass) to better understand the contributions of soil microbes to SOM formation and stabilization. These efforts have primarily fallen into two categories: molecular fingerprinting studies using techniques such as nuclear magnetic resonance spectroscopy (e.g., Simpson et al., 2007); and amino sugar extraction and analysis using chromatographic techniques (e.g., Liang et al. 2012; Salas et al., 2023). The current study will focus on the latter, as amino sugar analysis currently dominates necromass quantification efforts.

Amino sugar analysis is based on the quantification of microbe-specific amino sugars (e.g., glucosamine, muramic acid) that are not constituents of plant or faunal tissues and occur in known, relatively stable stoichiometries within microbial cells, specifically those of fungi and bacteria (Liang et al., 2019). Once the quantities of these amino sugars as bioindicators in soil have been determined, they can then be back converted to total necromass C or N using average ratios of the amino sugars to total microbial dry weight (Liang et al., 2019). Specifically, as reviewed by Liang et al. (2019), bacterial necromass C (BNC) is calculated using soil muramic acid content (MurA), and average values for bacterial biomass C (0.46 g C per g bacteria) and muramic acid (0.0103 g C muramic acid per g bacteria) content:

$$BNC = MurA\left(\frac{0.46\ \frac{g\ C}{g\ bacterial\ biomass}}{0.0103\ \frac{g\ muramic\ acid}{g\ bacterial\ biomass}}\right) \cong \left(45\frac{g\ C}{g\ muramic\ acid}\right)MurA,$$

while fungal necromass C (FNC) is calculated in two steps. First, fungal glucosamine content (Glu_{fun}) is calculated using total soil glucosamine content (Glu), the average bacterial ratio of glucosamine to muramic acid (2:1), and the molar masses of muramic acid (251.23378 g/mol) and glucosamine (179.17 g/mol) (Liang et al., 2019):

$$Glu_{fun} = MurA\left(2\frac{mol\ glucosamine}{mol\ muramic\ acid}\right)\left(\frac{179.172\frac{g\ glucosamine}{mol\ glucosamine}}{251.23378\frac{g\ muramic\ acid}{mol\ muramic\ acid}}\right)$$

$$\cong \left(1.43\frac{g\ glucosamine}{g\ muramic\ acid}\right)MurA$$

Second, FNC is calculated using average values for fungal biomass C (assumed to be the same as for bacteria; 0.46 g C per g fungi) and glucosamine contents (0.049 g glucosamine per g fungi) (Liang et al., 2019):

$$FNC = Glu_{fun}\left(\frac{0.46\ \frac{g\ C}{g\ fungal\ biomass}}{0.049\ \frac{g\ glucosamine}{g\ fungal\ biomass}}\right) \cong \left(9\frac{g\ C}{g\ muramic\ acid}\right)Glu_{fun}.$$

Fungal and bacterial necromass N can then be calculated by dividing FNC and BNC by an averaged C:N ratio for microbial biomass of 6.7, and total microbial necromass N and C by summing the complementary fungal and bacterial necromass pools (Liang et al., 2019).

Using the above relationships to estimate total necromass C and N from amino sugar contents has recently enjoyed broad acceptance (Liang et al., 2020; Wang et al., 2021). Nonetheless, the many assumptions required to convert amino sugar contents to necromass suggest caution is warranted, as the potential for error is considerable (Joergensen, 2018; Whalen et al., 2022). In particular, as summarized by Whalen et al. (2022), estimates of BNC from muramic acid are highly uncertain, largely due to the assumed ratio of Gram-positive to Gram-negative bacteria (65%:35%)—based on a single experimental site—that is conventionally used to estimate bacterial muramic acid content. However, this issue could be ameliorated by pairing amino sugar measurements with measurements of the Gram-positive to Gram-negative bacteria ratio (Joergensen, 2018; Whalen et al., 2022). Furthermore, amino sugar analysis does not capture extracellular microbial products that may be preserved in the soil, which could represent a sizeable portion of microbial contributions to SOM (Whalen et al., 2022). Despite these shortcomings, the estimation of necromass via amino sugars appears to be a relatively robust method for quantifying the microbial SOM contribution and is likely the best tool for the job while more precise methods are being developed (Liang et al., 2020; Wang et al., 2021).

1.3.3. C Isotope Natural Abundance Analysis

Stable C isotope abundances can provide valuable insights into soil C fluxes and serve as valuable tracers of SOM formation, turnover, and transfer between biological pools (Paterson et al., 2009; Staddon, 2004; Werth & Kuzyakov, 2010). Isotope abundances are commonly conveyed using ‘delta notation’ that expresses, in parts per thousand, the abundance of a particular isotope in a sample relative to that of a reference standard (Hayes, 2004). For ^{13}C, this reference standard is Vienna Peedee Belemnite (VPDB), and the relative isotopic abundance of a sample is denoted as $\delta^{13}C$ such that:

$$\delta^{13}\mathrm{C}\ (‰) = 1000\left(\frac{R_{sample} - R_{VPDB}}{R_{VPDB}}\right),$$

where R_{sample} and R_{VPDB} are the ^{13}C:^{12}C ratios in the sample and VPDB reference standard, respectively (Staddon, 2004). This means that $\delta^{13}C$ values are negative when the sample's ^{13}C:^{12}C ratio is less than that of VPDB, and positive when it is greater. More generally, the more positive the $\delta^{13}C$ value, the more ^{13}C-enriched a sample is, and vice versa.

The abundance of soil C isotopes is often used to quantify plant- or microbe-driven SOM dynamics (Staddon, 2004). This quantification is possible as plant and microbial metabolisms are discriminatory against the heavier ^{13}C atoms, preferentially using and releasing the more metabolically favourable ^{12}C atoms as $^{12}CO_2$, leading to ^{13}C enrichment in the remaining substrate (Blaser & Conrad, 2016; Conrad, 2007; Werth & Kuzyakov, 2010). Further, photosynthetic pathways vary between plants, with the C_3 and C_4 photosynthesis pathways being relatively less and more discriminatory against metabolic ^{13}C use, respectively (Werth & Kuzyakov, 2010). These differences allow for the tracking of processes such as SOM changes following changes in vegetation from one dominant photosynthetic pathway to another or preferential feeding of soil biota on a particular vegetation type (Staddon, 2004). In any case, microbial processing will cause further enrichment in ^{13}C as OM is passed from one trophic level to the next (Werth & Kuzyakov, 2010). Carbon isotope natural abundance can also be used to track the incorporation of organic amendments such as manure into SOM, as manure often has ^{13}C:^{12}C ratios that differ from the bulk soil and, therefore, will shift the soil’s isotope natural abundance (Gerzabek et al., 1997). As a result, soil isotope ratios can serve as important sources of information regarding the previous activity of soil microbial communities, and thereby help to clarify the impacts of land management practices on SOM.

1.3.4. Equivalent Soil Mass

Soil properties are often expressed in terms of spatial coordinates, such as the stock of an element per unit area in a particular fixed depth interval, typically calculated by multiplying the elemental content by the bulk density (BD) and by the thickness of the interval (Gifford & Roderick, 2003). This practice may seem intuitive considering the important influence of depth on many soil properties, but as has been noted since at least the 1990s, analyzing soil properties using fixed-depth intervals can be a source of sizeable analytical error (Ellert & Bettany, 1995; von Haden et al., 2020). Consider the example provided in the seminal work by Ellert and Bettany (1995), where the top 15 cm of soil at a hypothetical site was sampled for SOC before and after tillage, with no loss of SOC occurring between sampling events. Following tillage, the surface soil was reduced in BD, expanding to the depth of tillage, meaning what was originally the top 15 cm of soil has now expanded to be more than 15 cm in depth (Ellert & Bettany, 1995). Consequently, when the top 15 cm of soil was sampled following the tillage event, the SOC content, soil BD, and ultimately SOC stock will appear to have been reduced (despite no loss of SOC at the site) because the sampling is no longer capturing the same amount of mineral soil (Ellert & Bettany, 1995).

In a review conducted by von Haden et al. (2020), it was reported that soil surface BD commonly changes by 5% to 20% following land use or management changes, including those that cause (or alleviate) compaction or change plant residue inputs to the soil. Ultimately, if fixed depth intervals are used to estimate soil responses to changing land management, this can lead to the same type of issues enumerated by Ellert and Bettany (1995). To avoid such issues, soil properties can instead be evaluated on a cumulative mass basis, where equivalent soil masses per unit area (e.g., Tg ha^{-1}) are compared (Ellert & Bettany, 1995; Gifford & Roderick, 2003; von Haden et al., 2020). This approach ensures similar quantities of mineral soil are compared regardless of management-induced surface BD variations (Gifford & Roderick, 2003).

By using the equivalent soil mass (ESM) approach, errors in soil property measurements due to management-induced BD changes (which can easily be as large as 10% to 20%) can be avoided (Gifford & Roderick, 2003; von Haden et al., 2020). Consequently, it is now broadly recommended that the ESM basis be used in lieu of the fixed depth basis wherever land management changes may impact relevant soil property quantification (von Haden et al., 2020). Nonetheless, fixed-depth-based comparisons remain common and are likely obscuring soil responses to land management practices (von Haden et al., 2020). Thus, more researchers should utilize the ESM framework to ensure accurate quantification of the responses of SOC stocks, SOM dynamics, and other crucial soil properties to management practices.

1.4. Thesis Overview and Objectives

This project utilized data from two experiments (the Classical Plots and Hendrigan Plots) at the Breton Plots LTAEs to quantify changes in soil C storage, soil C stability, and microbe-mediated SOM cycling in response to various agricultural management practices. Specifically, the experiments examined here compared intermittently bare fallowed and continuously cropped systems; unamended, conventionally fertilized, and manure-amended treatments; and differing temporal intervals of perennial forage and legume inclusion within long-term crop rotations. Elucidating the impacts of fallow, soil amendment, legume presence, and perennial forage contributions to SOM sheds light on the processes leading to the formation and stabilization of this essential soil property. This enhanced understanding will allow for better estimations of lifetime GHG emissions from common agricultural systems and facilitate more informed policy decisions. Ultimately, it will help form a basis for future agricultural C budgets and valuation strategies, as well as inform practices meant to improve soil health and productivity. Furthermore, by highlighting the specific long-term benefits to soil health and productivity derived from perennial forage production, producers may be motivated to increase forage inclusion in their cropping systems, potentially leading to improved livestock feed supply and simultaneous reductions to their environmental footprint.

The first research chapter (Chapter 2) provides a snapshot of soil C stocks at the Breton Plots as of 2021, after 41 years (Hendrigan Plots) and 91 years (Classical Plots) of continuous management, allowing unprecedented clarity regarding how management practices have affected SOC at the site. The objectives of this chapter were to:

1. Document how alternate year bare fallow impacted soil C storage.
2. Contrast the effects of three nutrient management regimes (no amendment, vs. fertilization, vs. manuring) on SOC stocks.
3. Quantify the impacts of different intervals of perennial forage inclusion (none, vs. 2/5-year rotation, vs. 3/8-year rotation, vs. 40 years of continuous forage growth) on SOC.
4. Provide a dollar valuation of the SOC stocks for each treatment compared to the ‘business-as-usual’ continuous annual grain system that closely represents the dominant cropping systems in Alberta.

The second research chapter (Chapter 3) focused on microbial contributions to the SOC dynamics at the Breton Plots. These contributions were evaluated by examining microbial necromass C contents in each treatment and the associations between microbial necromass, various soil physicochemical properties, and soil C and N pools. For this chapter, the objectives were to:

1. Quantify the effect of alternate year bare fallowing on bacterial, fungal, and total necromass C.
2. Examine the specific impacts of fertilizer and manure amendments on necromass C pools.
3. Analyze how necromass C pools respond to increasing the consecutive years of perennial forage growth in a rotation (annual-only, vs. 2 years, vs. 3 years, vs. continuous).
4. Estimate microbial contributions to total SOC by evaluating the ratio of necromass C pools to SOC.

5. Explore relationships between necromass C pools and whole soil, LFOM, CMAOM, and FMAOM fraction C, N, and $\delta^{13}C$ pools.
6. Evaluate and contrast the ability of each necromass C pool to explain total SOC content.

Chapter 4 provides a synthesis of the two research chapters, discusses the significance of the observed results, and proposes directions for future research following the current study.

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Chapter 2 – Soil Carbon Stocks Respond Positively to Manuring and Perennial Forage Inclusion in a Gray Luvisol

2.1. Abstract

Agricultural practices that increase soil organic carbon (SOC) storage can improve soil health and reduce agricultural greenhouse gas emissions. However, SOC pools have long response times to changes in soil management. As such, long-term experimental data are required to reliably determine the effects of different management practices on SOC. Here, soil samples from two experiments within the Breton Plots research facility were used to test the effects of various long-term agricultural practices on SOC stocks. Samples were taken from the Classical Plots (est. 1930), which includes a wheat-fallow rotation and a 5-year rotation with three years of annual cash crops and two consecutive years of perennial grass-legume forage hay. Further, both of these cropping systems include non-amended, fertilized, and manured sub-treatments that were sampled. Samples were also taken from the three cropping systems comprising the Hendrigan Plots (est. 1980): a continuous forage system; a continuous grain system; and a complex 8-year agro-ecological rotation that includes annual cash crops, leguminous green manure, and three consecutive years of perennial grass-legume forage hay. Differences in SOC stocks down to 90 cm were assessed using linear contrasts. Additionally, the relative value of SOC stored under each treatment was assessed by comparing their SOC stocks to the continuous grain treatment, which was used as a proxy for business-as-usual practices. Total SOC stock was reduced by 51% under fallow but increased by 7%, 39%, and 9% following fertilization, manuring, and the inclusion of forages, respectively. Using the 2024 valuation of carbon ($80/tonne CO_2-equivalent) in Canada, the economic impacts associated with SOC changes were losses of approximately $11,000 CAD/ha of SOC due to fallow and gains of more than $5000 CAD/ha of SOC due to continuous forage production or the inclusion of forages and green manuring. This work informs producers and policymakers about the effects of agricultural practices on SOC storage and lays a foundation for understanding how agricultural practices can optimize this important ecosystem service.

2.2. Introduction

Globally, most nations have committed to climate change mitigation efforts, including striving to reach net-zero greenhouse gas (GHG) emission targets by 2050 (United Nations, n.d.). This goal will have to be achieved despite a growing global population projected to reach 9.8 billion by 2050 (United Nations, 2017) while simultaneously increasing food supply by 56% compared to 2010 levels (van Dijk et al., 2021). Furthermore, increases in the average global standard of living are projected to increase demand for meat and animal-derived protein by 60% between 2010 and 2050 (Revell, 2015). At the same time, human population growth will require more land for habitation and other non-agricultural uses, generating higher competition for the remaining land that could be converted to agriculture (Smith et al., 2010). Compounding this issue is that much of the land supporting global agricultural expansion is part of biodiverse tropical ecosystems, making it desirable to maintain these areas for conservation purposes rather than for agriculture (Zabel et al., 2019). As a result, global agricultural production must increase, likely through intensification (Kopittke et al., 2019), while reducing environmental impacts and facing greater land availability limitations.

Agriculture accounts for one-fifth to one-third of annual global GHG emissions (FAO, 2021a; Poore & Nemecek, 2018; Rosenzweig et al., 2020). In particular, livestock production contributes a large amount to anthropogenic emissions, accounting for 12% to 16% of annual global GHG emissions (FAO, 2023; Gerber & FAO, 2013). By 2050, if livestock production increases in step with projected changes in demand, under business-as-usual conditions, associated emissions are estimated to rise by 47% compared to 2015 (FAO, 2023). At the same time, ongoing intensification of agricultural practices further increases the risk of soil degradation (Kopittke et al., 2019). Currently, 33% of global soils are moderately to highly degraded, presenting a threat to food supply (FAO, 2015). Therefore, it is critical to develop agricultural practices that balance the need for increased production (both per unit area and in aggregate) while lowering environmental impacts and maintaining soil health.

Much of the environmental burden of contemporary agricultural production has been caused by soil degradation in the form of soil carbon (C) stock depletion. During these last ten millennia, an estimated 40 Pg to 537 Pg of soil organic C (SOC) has been lost from agricultural and managed soils, 50% to 75% of which may be restorable (Lal et al., 2007; Paustian et al., 1997). Soil C loss not only resulted in a proportional increase in the amount of C in the atmosphere (Amundson, 2001; Lal et al., 2007), but also reflects a decrease in soil organic matter (SOM). SOM is primarily composed of SOC and influences all metrics of soil health, including soil aggregation and structure, water holding capacity, nutrient levels and supply, and buffering capacity, making it of considerable importance to agriculture and the environment (Johnston et al., 2009). Therefore, agricultural soils represent an attractive potential C sink, with the refinement of land management strategies that maximize soil C sequestration a desirable goal. Such strategies could decrease net agricultural GHG emissions while maintaining or increasing soil health.

Going forward, the development and implementation of soil C storage strategies will hinge on a robust understanding of how various agricultural practices interact to influence soil C dynamics. One major limitation to understanding how land use change influences SOM is the slow pace of soil C processes and the necessity for long-term soil data to determine how SOC equilibriums will be affected (Beillouin et al., 2023). Certain SOM pools, such as mineral-associated organic matter (MAOM), can take decades to centuries to respond to changes in management (Kleber et al., 2015; Lavallee et al., 2020). Consequently, long-term experimental data are required to confidently identify causal relationships between management and SOC responses. Considering all these factors, data from long-term agroecological experiments (LTAEs) examining the impact of different agricultural practices on soil C are critical to developing agricultural C storage strategies.

To limit GHG emissions, governments are enacting policies to provide both disincentives for C pollution, and incentives for C sequestration (e.g., C taxes and credits, respectively), such as the Government of Canada’s Greenhouse Gas Pollution Pricing Act (Greenhouse Gas Pollution Pricing Act, 2018). Such policies must be supported by an accurate accounting of C flows if they are to be effective in limiting C pollution and withstanding scrutiny. Regarding soil C and the influences of agricultural practices, C valuation regimes are still faced with a paucity of evidence needed to understand soil C sequestration potential and establish sound pricing levels (Amelung et al., 2020). As of 2024, C pollution in Canada is taxed at \$80 CAD per tonne of C dioxide (CO_2) equivalent (CO_2-e), and this price will progressively increase to \$170 per tonne of CO_2-e by 2030 (Greenhouse Gas Pollution Pricing Act, 2018). If translated into payments for C sequestered, this could provide a considerable incentive for agricultural producers to shift their practices to enhance soil C sequestration. However, to ensure appropriate compensation, policymakers must understand how specific agricultural practices affect soil C stocks.

To address the growing interest in reducing the agricultural footprint on C emissions and support the need for data-driven policies on agricultural practices striving to increase SOC stocks, the potential long-term benefits of different agricultural practices, including diversified cropping systems, must be rigorously quantified. This research investigates the effects of different cropping practices, including cropping systems of varied complexity, on soil C stocks at the University of Alberta's Breton Plots LTAEs in west central Alberta after more than 40 years of consistent management. More specifically, the effects of fallowing, nutrient management, and perennial forage inclusion, in varying temporal intervals, on soil C stocks are evaluated in various intervals of the soil profile down to 90 cm. Specific objectives include 1) documenting the impact of alternate year fallowing on soil C levels, 2) comparing the impact of different nutrient management regimes (fertilization vs. manure vs. no amendment) on resulting SOC stocks, and 3) quantifying the effect of different temporal intervals of perennial forage inclusion (none, vs. 2/5-year rotation, vs. 3/8-year rotation, vs. continuously for 40 years) among long-term plots. An increased understanding of the potential benefits of SOC accrual may not only convince agricultural producers to shift their agricultural practices towards enhanced sustainability but will also provide tools for policymakers to develop more effective C crediting systems that help offset the cost to producers of adopting C-friendly management strategies.

2.3. Materials and Methods

2.3.1. Site Description

The University of Alberta’s Breton Plots are a set of LTAEs located in central Alberta, near the town of Breton (53°09’N, 114°44’W; Fig. S1). Soils at the site are classified as Orthic Gray Luvisols, Typic Cryoboralfs, and Luvisols according to the Canadian System of Soil Classification, United States Department of Agriculture, and World Reference Base for soil resources, respectively (Izaurralde et al., 2001; Soil Classification Working Group, 1998).. These soils were developed on glacial till parent material under a boreal forest ecosystem, which was cleared for agricultural production in 1919 and 1920 (Dyck et al., 2012). The treatments examined here are summarized in Tables S1 and S2 and are described below.

The first set of experimental plots at this site, established in 1930, remains today and is known as the Classical Plots experiment (Dyck et al., 2012). While the treatments within the Classical Plots have been broadly similar since their inception, some management changes have occurred. Initially, the Classical Plots consisted of a continuous wheat (*Triticum aestivum* L.) system and a four-year rotation comprised of three years of cereals and a single year of legumes (Dyck et al., 2012). In 1938 and 1941, respectively, the four-year rotation was converted into a five-year rotation (5Y) comprising three years of cereals—namely wheat, oats (*Avena sativa* L.), and barley (*Hordeum vulgare* L.)—and two years of perennial forage hay comprising downy brome (*Bromus tectorum* L.) and alfalfa (*Medicago sativa* L.), while the continuous wheat system was converted into a wheat-fallow rotation (WF) (Dyck et al., 2012). Additionally, both crop rotations are subjected to various fertility treatments. The current study specifically examines the complete conventional fertilization treatments (NPKS), manured treatments (Manure), and control treatments (Check) that receive no fertilizer or manure amendment (Dyck et al., 2012). These rotations and fertility treatments have persisted but undergone minor changes. In 1980, the fertilizer rates applied to the Classical Plots were modernized (increased), and starting in 2000, straw—which had previously been removed—was retained on the plots after harvest (Dyck et al., 2012).

These were the last substantive changes to the Classical Plots, and management has remained consistent since 1980.

Concurrent with the management update of the Classical Plots, another experiment known as the Hendrigan Plots was established in 1980 (Dyck et al., 2012). This experiment was established on formerly cropped land to compare three cropping systems: continuous grain (CG), continuous perennial forage (CF), and an eight-year agroecological rotation (8Y) (Dyck et al., 2012). In the CG system, only barley is grown, whereas the CF system combines creeping red fescue (*Festuca rubra* L.), tall fescue (*Lolium arundinaceum* (Schreb.) Darbysh.), and white Dutch clover (*Trifolium repens* L.). The 8Y rotation (see Table S1 for details on rotation phases) is the most complex, consisting of annual cash crops for five years, including faba beans (*Vicia faba* L.; historically as a green manure plow down, but recently harvested for seed), and finished with three years of perennial forage hay (Dyck et al., 2012; Ross et al., 2008). The CF and CG systems are designed to be prototypical of livestock-exclusive cropping systems where the crops are mechanically harvested and exported, with conventional fertilizer being the only external nitrogen (N) source available to the crops (Dyck et al., 2012). In contrast, the 8Y rotation is designed to reflect a mixed farming, rotational grazing style system with little to no external N supplementation. To this end, the 8Y system is designed to receive all N from fixation by legume crops and the presence of livestock is simulated by removing hay and recycling nutrients by applying manure at a rate equaling 70% of the N removed during the harvest of the perennial forage hay over three years (Dyck et al., 2012).

Soil amendment application rates vary among the cropping systems and are summarized in Table 2. However, management differences extend beyond fertility rates and crop rotations, with each system being subject to differing amounts of tillage. The Classical Plots undergo tillage each fall and spring, with the exception of the 5Y system during its perennial phase (Grant et al., 2001). Similarly, the CG and 8Y systems are tilled twice yearly, in spring and fall, except the 8Y treatment during its perennial phase (Ross et al., 2008). In contrast, the CF treatment is never tilled, but white clover seed is occasionally broadcasted into the stand at a rate of approximately 1 kg ha^{-1} (Ross et al., 2008). In all relevant perennial-inclusive treatments for both experiments, forage crops are cut twice per season (Dyck et al., 2012; Ross et al., 2008).

2.3.2. Soil Sampling

All soil sampling was completed on October 12th to 15th, 2021, 41 and 91 years after the initiation of the Hendrigan and Classical Plots experiments, respectively. Soil cores were extracted using a hydraulic Giddings soil coring unit mounted on a Dodge 3500 flat deck truck and affixed with a sampling tube 6 cm in diameter and 1.2 m long. At three random locations within each plot, the sampling tube was plunged to at least 90 cm below the soil surface and extracted. Cores were removed from the sampling tube and separated into 0-7.5 cm, 7.5-15 cm, 15-30 cm, 30-60 cm, and 60-90 cm layers. For each plot, soil from one core was bagged for bulk density (BD) analysis, while the soil from the remaining two cores was bulked within each depth layer and bagged for evaluation of soil C and N. An additional two cores were taken down to 30 cm within each plot at random locations, with the soil added to the bulked soil for the top three layers to increase the volume of soil available for analysis and reduce uncertainty in SOC caused by surface variation.

2.3.3. Sample Processing

Samples retained for BD were oven-dried at 65°C for at least 24 hours and the oven dried weight was divided by the initial bulk sample volume to determine BD:

$$BD\ \left(\frac{g\ soil}{cm^3\ soil}\right) = \frac{oven\ dried\ soil\ weight\ (g)}{bulk\ soil\ volume\ (cm^3)}.$$

All other samples were air-dried and finely ground using a Retsch MM 400 Mixer Mill (Retsch GmbH, Haan, Germany) at 30 Hz for 2 minutes per sample, then set aside for further analysis.

2.3.4. Total C and Organic C Measurement

Processed soil samples were submitted to the Natural Resources Analytic Laboratory in the University of Alberta's Department of Renewable Resources for total C (TC) and total organic C (TOC) analysis via flash combustion (AOAC International, 2000) using a Thermo FLASH 2000 Organic Elemental Analyzer (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.). In brief, subsamples were weighed into tin capsules and placed into a combustion tube filled with silvered cobaltous/cobaltic oxide catalysts and chromium (III) oxide. Oxygen was then introduced to the tube to create a flash combustion reaction at temperatures of 1800-2000°C, which converted sample C to CO_2, which was then measured by the analyzer. Samples submitted for TOC analysis were weighed into open silver capsules, then iteratively treated with hydrochloric acid until no further effervescence was observed, indicating exhaustion of the soil carbonate (inorganic C) pool. Once effervescence was no longer detectable, these samples were oven-dried at 70°C overnight, sealed, and analyzed for TOC via flash combustion as described above. Final measurements were reported as TC, or organic C percent by mass (i.e., elemental content).

2.3.5. Soil Mass and Elemental Stock Calculations

Soil mass in each soil layer was calculated by taking the product of the BD and the thickness of each soil layer:

$$soil\ mass\ \left(\frac{kg\ soil}{ha\ soil}\right) = \left[BD\ \left(\frac{g\ soil}{cm^3\ soil}\right)\right] * \left(\frac{1\ kg}{1000\ g}\right) * [layer\ thickness\ (cm)] * \left(\frac{10^8\ cm^2}{1\ ha}\right),$$

and thereafter, soil elemental stocks within each sampled depth layer were calculated as the product of elemental content in that layer multiplied by soil mass:

$$X\ Stock\ \left(\frac{kg\ X}{ha\ soil}\right) = \left(\frac{1}{100}\right) * \left[X\ content\ \left(\%\frac{kg\ X}{kg\ soil}\right)\right] * \left[soil\ mass\ \left(\frac{kg\ soil}{ha\ soil}\right)\right].$$

2.3.6. Equivalent Soil Mass Calculations

Various studies have shown that management-induced changes in soil BD can obscure concurrent changes in other soil properties when analyzed within fixed-depth intervals and have instead advocated for the use of equivalent soil mass (ESM) comparisons (Ellert & Bettany, 1995; von Haden et al., 2020). For example, tilling a field may reduce the BD of the soil to the depth of tillage, creating a thicker but less dense surface soil layer. Consequently, if the SOC stock of this surface layer were sampled in the same fixed depth interval before and after tillage, a reduction in SOC stock would be found irrespective of any actual loss of SOC from the profile (Ellert & Bettany, 1995). ESM comparisons avoid such issues by comparing equivalent masses of mineral soil rather than comparing equivalent depths and have been shown to lead to reduced error when estimating management-induced changes to soil properties (von Haden et al., 2020).

Due to the issues associated with fixed-depth sampling, ESM-based comparisons were utilized in the current study. To achieve this, cumulative soil mass and elemental stock at the endpoint of each measured soil depth interval (7.5 cm, 15 cm, 30 cm, 45 cm, 60 cm, 90 cm) were calculated for each sample plot as described above. Thereafter, cumulative soil mass at the endpoint of each interval was combined with the cumulative elemental stock at that depth, and these values were used to create a cumulative stock vs. cumulative mass dataset. Similar to Gifford & Roderick (2003), linear spline fits were applied individually to the cumulative elemental stock vs. cumulative soil mass data for each sample plot to facilitate the interpolation of soil C stocks along the soil profile. Between all plots, the minimum cumulative soil mass value at 90 cm was determined and used to define the endpoint soil mass value (10.6 Tg ha^{-1}) for all plots so that no extrapolation outside the observed data was necessary. The soil profile in each stock was then separated into six soil mass intervals (0-0.75 Tg ha^{-1}, 0.75-1.5 Tg ha^{-1}, 1.5-3 Tg ha^{-1}, 3-5 Tg ha^{-1}, 5-7.5 Tg ha^{-1}, and 7.5-10.6 Tg ha^{-1}) by using the splines to estimate the elemental stock at the endpoint of each interval and subtracting off the elemental stock present in overlaying depth intervals. Similarly, the cumulative elemental stocks for each plot were also determined for six different intervals (0-0.75 Tg ha^{-1}, 0-1.5 Tg ha^{-1}, 0-3 Tg ha^{-1}, 0-5 Tg ha^{-1}, 0-7.5 Tg ha^{-1}, and 0-10.6 Tg ha^{-1}) using the spline fits. All spline fits were completed in R and RStudio versions 4.3.1 (Posit team, 2023; R Core Team, 2023).

2.3.7. C Pricing Calculations

The value of C stored as SOC was calculated using the Canadian federal government's CO_2-e price, as laid out in the Minimum National Carbon Pollution Price Schedule in accordance with the Greenhouse Gas Pollution Pricing Act (Greenhouse Gas Pollution Pricing Act, 2018). As of 2024, this schedule set the price of C as $80 CAD per tonne of CO_2-e (Greenhouse Gas Pollution Pricing Act, 2018). Using this price, the equivalent value of SOC was then calculated as follows:

$$SOC\ Value\ \left(\frac{CAD}{ha}\right) = \left(\frac{\$80\ CAD}{t\ CO_2\text{-}e}\right) * \left[SOC\ stock\ \left(\frac{t\ C}{ha}\right)\right] * \left[\frac{44\ t\ CO_2\text{-}e}{12\ t\ C}\right].$$

2.3.8. Statistical Analysis

Differences in soil properties were quantified on a per-depth layer or ESM interval basis, with no comparisons between separate layers or intervals. Soil property data for each interval was checked for normality and homoscedasticity using Shapiro-Wilk and Levene tests, respectively. Where assumptions of homoscedasticity and normality were met, generalized linear models (GLMs) and generalized linear mixed-effects models (GLMMs) were fitted to soil property data, and the model with the more negative corrected Akaike information criterion value was selected as the best model. Where these assumptions did not hold, soil property data were fitted using GLMMs. For both the GLM and GLMM models, treatments were defined as the combination of crop rotation system and associated fertility treatment (Check, Manure, or NPKS) for the Classical Plots, and solely as the cropping system for the Hendrigan Plots (singular fertility levels). Resulting treatments were considered the fixed effects, while replicate plots were considered the random effects.

To test specific hypotheses about the data, a series of predefined linear contrasts were applied to the models mentioned above, with p-values adjusted using the Tukey method to account for the total number of linear contrasts applied. For all models, linear contrast hypothesis tests had their degrees of freedom estimated via the Kenward-Roger method for small sample sizes. Specific hypothesis tests were organized as follows, using the treatments listed in Table S1. Firstly, an initial test was conducted to evaluate the historical impact of fallow on soil C stocks; this comparison contrasted the CG and WF-NPKS treatments. Secondly, the influence of variable fertility was assessed within each of the WF and 5Y treatment regimens. This assessment entailed comparisons among the WF-Check, WF-NPKS, and WF-Manure treatments, respectively, while a separate set of contrasts compared the 5Y-Check, 5Y-NPKS, and 5Y-Manure treatments. Thirdly, the specific contribution of the inclusion of forages in varying temporal intervals (0, 2/5-yr, 3/8-yr, or all years) was conducted by comparing the following: CG, 5Y-NPKS, 5Y-Manure, 8Y, and CF. Finally, a separate set of contrasts was used to determine the relative economic value of SOC either lost or accrued in all treatments, as specifically compared to the CG treatment. This valuation was achieved by contrasting all other treatments against the CG treatment and converting the differences in SOC stock into a dollar amount using current Canadian federal government C pollution prices. For these analyses, the CG treatment was considered the business-as-usual treatment due to its widespread representation on agricultural lands in the region. This treatment was considered representative of regional cropping systems strictly in the sense that it is a fully annual system. Additionally, both the 5Y-Manure and 5Y-NPKS were included in the latter to facilitate comparisons to the 8Y and CF treatments.

All statistical tests and analyses were completed using R and RStudio versions 4.3.1 (Posit team, 2023; R Core Team, 2023). Linear contrasts were computed using the emmeans package (Wickham, 2016). Data visualizations were completed using the ggplot2 and ggpubr packages, and data tables were generated using the knitr and kableExtra packages (Kassambara, 2023; Xie, 2015; Zhu, 2021). All GLM and GLMM model fits were generated using the lme4 package (Bates et al., 2015).

2.4. Results

For the two shallowest fixed depth intervals (0-7.5 cm and 7.5-15 cm), significant differences were found in soil BD between treatments (Fig. 1), with BD declining in treatments having manure addition and/or the inclusion of forage. Additionally, linear contrast analysis (Appendix 2.2, Appendix 2.3) revealed apparent effects on SOC stocks due to fallowing (Fig. 2), fertilizer treatments (Fig. 3), the inclusion of perennial forages (Fig. 4), and agroecological practices as typified by the 8Y cropping system (Fig. 4). However, the extent of the treatment effects was modulated by the overall cropping system they were associated with, together with the specific soil layers wherein differences in SOC stocks manifested. Furthermore, significant differences in the value of accrued SOC were found between the CG treatment and other treatments (Fig. 5).

2.4.1. Soil BD Responses

Differences ($P < 0.05$) in soil BD were observed for the 0-7.5 cm and 7.5-15 cm layers (Fig. 1), with differences most pronounced in the 0-7.5 cm layer. The CF, 8Y, and 5Y-Manure treatments led to the lowest BDs within this layer, while the WF system had the highest BDs. For the 7.5-15 cm layer, few significant differences were present. However, in this layer, the WF-Check treatment had the highest BD, which was significantly higher than either amended 5Y treatment or the 8Y treatment. Similarly, in this layer, the CF and 5Y-Check treatments had higher BDs than the 8Y treatment, which had the lowest overall BD. In contrast, soil BD did not differ among treatments for the 15-30 cm layer (Fig. 1).

2.4.2. SOC Responses to Fallow

Differences in SOC stocks were pronounced between the WF-NPKS and the CG treatment throughout the soil profile, with the CG treatment having a total SOC stock (78 Mg ha^{-1}) 97% larger than the WF-NPKS treatment (39 Mg ha^{-1}) over the whole profile (i.e., from 0-10.6 Tg ha^{-1}) (Appendix 2.2, Fig. 2). In comparison to the CG treatment, SOC stocks within the 0-0.7.5 Tg ha^{-1}, 0.7.5-1.5 Tg ha^{-1}, 1.5-3 Tg ha^{-1}, and 5-

7.5 Tg ha^{-1} layers of the WF-NPKS treatment were found to be reduced at $P < 0.05$ (Fig. 2A). Similarly, the SOC stocks in 3-5 Tg ha^{-1} and 7.5-10.6 Tg ha^{-1} layers were reduced, albeit at $P < 0.10$ (Fig. 2A). All cumulative soil mass layers had higher SOC stocks at $P < 0.05$ within the CG treatment compared to the WF-NPKS treatment, with the difference in SOC stock progressively increasing with sampling depth (Fig. 2B).

2.4.3. SOC Response to Soil Fertility Amendments

Differences in SOC stock ($P < 0.05$) were found between the fertility treatments for each of the WF and 5Y systems (Appendix 2.2, Fig. 3). However, the two systems differed notably from one another in their SOC stock responses to the applied soil amendments.

Within the WF system, a significant amendment response was only observed in the Manure treatment, with the WF-NPKS and WF-Check treatments having similar SOC stocks to one another over the entire soil profile (Appendix 2.2, Fig. 3A). The SOC stock response to manuring was most pronounced in the upper soil profile, as reflected by the significant SOC enrichment within the 0-0.75 Tg ha^{-1} ($P < 0.05$) and 0.75-1.5 Tg ha^{-1} ($P < 0.10$) layers of the WF-Manure treatment compared to the WF-Check and WF-NPKS treatments (Fig. 3A). In the 0-0.75 Tg ha^{-1} layer, the WF-Manure treatment had a SOC stock (19 Mg ha^{-1}) roughly 108% and 176% larger than that of the WF-NPKS (8.9 Mg ha^{-1}) and WF-Check (6.7 Mg ha^{-1}) treatments, respectively. Similarly, in the 0.75-1.5 Tg ha^{-1} soil layer, the WF-Manure treatment had a SOC stock (14 Mg ha^{-1}) 125% and 90% higher than the WF-NPKS (5.2 Mg ha^{-1}) and WF-Check (6.2 Mg ha^{-1}) treatments, respectively. Due to this SOC enrichment in the topsoil, the WF-Manure treatment had a greater ($P < 0.05$) cumulative SOC stock throughout the measured soil profile, with no differences present in the bottom four individual soil mass layers (Fig. 3C). Despite SOC stock differences only being present in the topsoil, the WF-Manure treatment had a whole-profile SOC stock (63 Mg ha^{-1}) 59% and 54% greater than the WF-NPKS (39 Mg ha^{-1}) and WF-Check (41 Mg ha^{-1}) treatments, respectively.

Within the 5Y cropping system, SOC stock responded to amendments in both the 5Y-NPKS and 5Y-Manure treatments, with the response being most prominent in the top two soil layers (Appendix 2.2, Fig. 3B). For both the 5Y-NPKS and 5Y-Manure treatments, SOC stock responded positively ($P < 0.05$) in the 0-0.75 Tg ha^{-1} layer, with the 5Y-Check treatment SOC stock (17 Mg ha^{-1}) being 35% and 30% lower than that in the 5Y-NPKS (22 Mg ha^{-1}) and 5Y-Manure (21 Mg ha^{-1}) treatments, respectively. Despite the absence of differences in SOC stock within the bottom three soil layers ($P > 0.10$), the total (i.e., cumulative) SOC stocks for amended 5Y treatments increased. For both of the amended 5Y treatments, SOC stock increased ($P < 0.05$) for all cumulative soil layers, except for the 0-5 Tg ha^{-1} and 0-10.6 Tg ha^{-1} layers of the 5Y-NPKS treatment, which were significant at $P < 0.10$ instead (Fig. 3D). Overall, the whole-profile SOC stocks for the 5Y-Manure (79 Mg ha^{-1}) and 5Y-NPKS (75 Mg ha^{-1}) treatments were higher ($P < 0.05$ and $P < 0.1$, respectively) than for the 5Y-Check (64 Mg ha^{-1}) treatment, an increase of 24% and 18%, respectively.

2.4.4. Inclusion of Perennial Forages and Green Manuring

Differences in SOC stocks were found between the conventionally fertilized, manured, and non-fallowed treatments but varied notably with depth, as proxied by soil mass (Appendix 2.2, Fig. 4). Overall, variation between SOC stocks was most significant in the uppermost soil layer and diminished with soil depth, with no significant differences present among treatments within the deepest soil mass layer.

For the 0-0.75 Tg ha^{-1} layer, SOC stocks for the 8Y and CF treatments were greater than all other treatments at $P < 0.05$, except for the 8Y and 5Y-NPKS treatments, which differed at $P < 0.10$. Furthermore, within this layer, the CF treatment had a larger SOC stock than the 8Y treatment ($P < 0.05$). Also within this layer, the 5Y-NPKS and 5Y-Manure treatments both had a larger SOC stock than the CG treatment at $P < 0.05$ and $P < 0.10$, respectively. SOC stock differences remained evident ($P < 0.05$) between treatments down to the 5-7.5 Tg ha^{-1} layer, with only the 7.5-10.6 Tg ha^{-1} layer lacking differences in SOC stocks. With the exception of the cumulative 0-7.5 Tg ha^{-1} layer, the CG treatment had SOC stocks that were either not different or were significantly higher than the 5Y treatments in all other individual layers. Due to SOC enrichment in the topsoil, the 8Y and CF treatments had higher ($P < 0.05$) cumulative SOC stocks than all other treatments throughout the soil profile, while the 5Y and CF treatments were not significantly different from one another except in the cumulative 0-7.5 Tg ha^{-1} layer (Fig 5B).

Whole-profile SOC stocks were greater ($P < 0.05$) in the 8Y (98 Mg ha^{-1}), and CF (95 Mg ha^{-1}) treatments compared to the 5Y-Manure (79 Mg ha^{-1}), 5Y-NPKS (75 Mg ha^{-1}), and CG (78 Mg ha^{-1}) treatments. In contrast, the 5Y-Manure, 5Y-NPKS, and CG treatments had similar whole-profile SOC stocks.

2.4.5. Valuation of SOC Degradation and Accrual

Marked differences were found between the valuation (in CAD) of SOC stored throughout the soil profile (i.e., from 0-10.6 Tg ha^{-1}) in the CG treatment compared to the other treatments examined (Fig. 5). Specifically, the 8Y and CF treatments had significantly higher ($P < 0.05$) values of SOC stored in their whole soil profiles compared to the CG treatment, amounting to an additional \$5836 CAD and \$5066 CAD worth of CO_2-e storage, respectively. On the other hand, the WF-Check and WF-NPKS treatments had significantly lower ($P < 0.001$) values of SOC stored in their soil profiles compared to the CG treatment, amounting to a loss of \$10,866 CAD and \$11,190 CAD worth of CO_2-e, respectively. The WF-Manure, 5Y-Check, 5Y-NPKS, and 5Y-Manure treatments did not demonstrate significant differences in the value of SOC stored in their soil profiles compared to the CG treatment, even though the mean value of stored CO_2-e within three of these four treatments trended lower than that of the CG treatment.

2.5. Discussion

2.5.1. Topsoil BD Decreases with Manure Application, Increased Forage Inclusion and SOC Stocks

The BD of the topsoil (i.e., the top 15 cm of soil) differed markedly between the observed cropping treatments. Notably, where either manure amendment was applied, forages were included for longer intervals in the rotation, or SOC stock increased, soil BD decreased. This effect was most evident in the uppermost (0-7.5 cm) soil layer, in which the 5Y-Manure, 8Y, and CF treatments had the lowest BDs. Critically, these treatments are all forage-inclusive and had the greatest SOC stocks in the uppermost soil layer. The only exception to this pattern was the 5Y-Manure treatment, which had a marginally lower SOC stock than the 5Y-NPKS treatment in the uppermost soil layer despite receiving manure amendment. These results agree well with other observations that BD tends to have an inverse relationship with SOM, with manure addition generally increasing SOM while decreasing BD, and plowing of perennial stands tending to decrease SOM and increase BD (Johnston et al., 2009). As such, the increased SOM in the perennial-inclusive or manured treatments likely led to reductions in BD.

Notably, the CF treatment had the highest SOC stock, the longest interval of forage inclusion (continuous), a no-till management regime, and the lowest BD (in the topsoil). While having the same amount of forage inclusion as the 5Y-Manure treatment, the 5Y-NPKS and 5Y-Check treatments had higher BD, despite the 5Y-NPKS treatment having a marginally higher mean SOC stock than the 5Y-Manure treatment in the 0-0.75 Tg ha^{-1} layer. This implies that the combined effects of higher SOC and amendment with manure in the 5Y-Manure treatment collectively led to its lower soil BD. These results align well with the concept that SOM and BD have an inverse relationship, with manuring having a distinct effect of decreasing BD (Johnston et al., 2009). Further, the exceptionally low BD in the surface layer of the CF treatment agrees well with observations that no-till and reduced tillage tend to decrease soil BD through mechanisms such as improved soil structure, increased crop residue retention, and reduced compaction from tillage machinery (Bai et al., 2018; Engell et al., 2022; You et al., 2017). As such, perennial-forage-inclusive systems at the Breton Plots likely reduced BD by directly increasing crop biomass inputs and reducing tillage.

Providing further support for this trend, the CG treatment had a BD in the 0-7.5 cm layer that was marginally lower than the 5Y-Check treatment, but higher than that of the CF, 8Y and fertilized 5Y treatments. In contrast, the CG treatment had a lower SOC stock than the CF, 8Y and amended 5Y treatments in the 0-0.75 Tg ha^{-1} layer but had a marginally greater SOC stock than the 5Y-Check treatment in this layer. Finally, the family of WF treatments typically had the highest BDs while simultaneously having the lowest SOC stocks; the WF also lacked perennial forage inclusion and green manuring. Overall, similar trends were also apparent in the 7.5-15 cm layer. These observations reinforce the concept that BD is inversely related to SOM content. Further, the results here agree with research demonstrating that fallow increases BD compared to green manuring (Mandal et al., 2003) or continuous cropping, especially continuous cropping under no-till management (Aase & Pikul Jr., 1995; Feng et al., 2011). In light of these trends, it is most likely that a combination of high overall SOC stock, amendment with manure or green manure, as well as the inclusion of perennial forages and consequent reductions in tillage, is what led to the relatively low BDs observed in the topsoil of the CF and 8Y treatments, as well as the 5Y system in general.

Given the significant differences in BD found in the topsoil, examining soil properties via ESM intervals instead of fixed depth intervals was warranted. Further, the presence of significant treatment effects on soil BD observed in this study has important implications, as BD impacts many measures of soil function. For example, excessively high BD can indicate soil compaction and negatively impact soil hydrological properties, such as water infiltration rate (Basset et al., 2023; Gumbs & Warkentin, 1972; Nawaz et al., 2013). High BDs and soil compaction can also limit soil aeration (Stepniewski et al., 1994) and reduce plant rooting efficiency (Lipiec & Hatano, 2003), together with seedling emergence rates (Nasr & Selles, 1995). As a result, the effects of agricultural management strategies on soil BD are important to consider. Overall, the findings here indicate that forage inclusion, tillage reduction, green manuring, and amendment with animal manure can be valuable tools to increase SOC stocks and reduce soil BD in the topsoil.

2.5.2. Alternate Year Fallow Promotes SOC Degradation

The negative effects of alternate year fallow on SOC stock were clearly evident by comparing the WF-NPKS and CG treatments. Despite receiving the same type of fertilizer at the same rate during cropped years, having only annual cropping components, and being subjected to similar amounts of tillage, the WF-NPKS and CG treatments exhibited considerable differences in SOC stocks. Given that the most notable difference between these two treatments was the application of fallow on alternate years in the WF-NPKS treatment, it is likely that this practice is responsible for the majority of the 51% decrease in its whole-profile SOC stock compared to the CG treatment. Furthermore, the observed reduction in SOC storage was not concentrated in the upper soil profile but instead extended through to the deepest soil mass layer. This reduction in SOC at depth indicates that fallow has systemic negative impacts that meaningfully impact both topsoil and subsoil C storage. Importantly, the non-manured (i.e., WF-Check, WF-NPKS) treatments within the WF system were found to have experienced not only a relative loss of soil C compared to the CG treatment but also an absolute soil C loss since the initiation of the Classical Plots (Appendix 2.1).

Fallow is well-known to encourage soil erosion and lead to reduced plant biomass inputs during fallow periods, which, in turn, can lead to considerable degradation of SOC (Baumhardt et al., 2015; Nielsen & Calderón, 2011; Tiefenbacher et al., 2021). The results of this study provide further support for this well-known phenomenon and demonstrate how extreme this fallow-induced degradation can become over the long-term, with the WF-NPKS treatment having roughly half the SOC stock of the otherwise comparable CG treatment after four decades. Given that the CG treatment had a much smaller SOC stock than the 8Y and CF treatments, the additional limitation of SOC stock in the WF system represents an additional opportunity cost in terms of lost SOC accrual potential. Considering the broad importance of SOM (and, in turn, SOC) to soil health, the observed effect of fallow limiting SOC stock accumulation reinforces the need to exercise caution when considering this practice. While the use of fallow to maintain cropping system stability and profitability on the Great Plains of North America has been questioned since at least the 1990s (Aase & Pikul Jr., 1995), it is still considered a viable means to maintain farm profitability and production in some drier regions, such as parts of Australia (e.g., Cann et al., 2020) and China (e.g., Li et al., 2014). These considerations must be carefully weighed against evidence from long-term studies, such as that shown here, that regular fallow leads to considerable soil degradation over time.

2.5.3. Soil Amendment Effects on SOC Are Dependent on Cropping System

While soil amendments positively affected SOC stock in both the 5Y and WF systems (Fig. 3), the comparison of the various fertility treatments in these systems revealed important interplays between the overall cropping system and the effect of amendments. Importantly, conventional fertilizer application only improved SOC stock significantly in the 5Y system, whereas manure application enhanced SOC stock in both cropping systems. Furthermore, it should be noted that, while manuring effects were significant in both cropping systems, the magnitude of the increase in SOC stock was much larger in the WF system compared to the 5Y system, increasing SOC stock by 54% and 24% compared to the respective Check treatments.

The positive impact of conventional fertilizer on SOC stock in the 5Y system was likely derived primarily from enhanced crop growth and subsequent increases in crop biomass inputs to the soil (Congreves et al., 2017; Tiefenbacher et al., 2021), as documented in previous studies at the Breton Plots (Dyck et al., 2012; Dyck & Puurveen, 2020). While a similar enhancement of crop biomass production in response to conventional fertilization has also been observed in the WF system (Dyck et al., 2012; Dyck & Puurveen, 2020), it is reasonable to assume that this translated into a negligible long-term change in SOC stock due to ongoing soil erosion during fallow periods. However, another potential factor limiting SOC stocks in the WF system could be a relative increase in the mining of existent SOC due to microbial C limitations arising from a paucity of fresh plant litter. To elaborate, in C-limited systems, the potential for microbial mining of stabilized microbial SOM (i.e., necromass) is increased (Kästner et al., 2021). Further, while fungal residues are important to SOC accrual (Li et al., 2015), and generally have slower turnover times compared to bacterial residues (Dippold et al., 2019; Gunina et al., 2017), the former are particularly prone to destabilization under nutrient-limited conditions (Xue et al., 2024). As such, regular microbial C-limitations induced by a lack of crop biomass inputs during fallowed years would be expected to destabilize persistent SOC (i.e., fungal necromass), limiting long-term gains of SOC (i.e., through fungal necromass accumulation) within the WF system. In contrast, the 5Y system may have been more likely to retain these increased biomass inputs as SOC primarily due to the increased soil coverage and crop biomass inputs provided by the absence of fallow periods and the inclusion of a regular perennial forage phase. Importantly, including perennial phases in rotations has been shown to limit soil erosion and increase soil C storage compared to annual-only cropping systems through mechanisms such as decreased tillage frequency and increased root biomass inputs (Khanal, 2023; King & Blesh, 2018).

On the other hand, the manure-induced increase in SOC stock evident in both the WF and 5Y systems is likely due to a combination of enhanced crop productivity due to nutrient addition, paired with the direct import of C and organic matter within the manure. The direct addition of C via manure is likely the primary reason why the WF system had a positive SOC stock response to manure application but not conventional fertilizer. Manure-associated soil C additions would not only directly increase SOC but would address microbial C-limitations, limiting SOC destabilization and increasing the proportion of biomass inputs converted to stable microbial SOM (Blagodatskaya et al., 2014; Kästner et al., 2021; Sinsabaugh et al., 2013), ultimately increasing long-term SOC storage. Such an outcome would align with various studies indicating that manure application can significantly increase SOC stocks (in some cases more than doubling SOC), with as much as a third of applied manure C retained as SOC (Bhattacharya et al., 2016; Gerke, 2021; Johnson et al., 2007). Providing further support for this concept, previous research has indicated that manure application can reduce or even eliminate SOC declines caused by fallow (Nielsen & Calderón, 2011).

Overall, the results of this study indicate that SOC can be improved through proper nutrient management, and that manuring is a superior means to build SOC as opposed to conventional fertilizer, particularly for rotations that include fallow periods. This dynamic is embodied in the observation that the non-manured WF (i.e., WF-Check, WF-NPKS) treatments have lost soil C since the initiation of the Classical Plots, while the WF-Manure treatment and all amended 5Y system treatments have experienced gains in soil C (Appendix 2.1). Furthermore, compared to either their own initial C contents or that of the CG treatment, the manured treatments of both the WF and 5Y systems, as well as the 5Y-NPKS treatment, have increased C content at an average annual rate of more than 4‰, while the WF-NPKS treatment lost soil C at a similar rate (Appendix 2.1). These relative changes in soil C content show that manure and conventional fertilizer amendments can be used to achieve sustainability goals such as those set by the UN's "4 per 1000 initiative", which aims to increase C in agricultural soils by 4‰ per year to combat climate change, improve soil health and ecosystem services, and increase food security (Minasny et al., 2017). However, where SOM is a limiting factor, manure appears more able to achieve these goals.

2.5.4. SOC Increases with Forage Inclusion and Green Manure Plow-Down

The two treatments with the highest SOC stock were the 8Y and CF treatments, which exceeded even the CG and amended 5Y treatments on a whole-profile basis and across most individual soil mass layers (Fig. 4). It is notable that the 8Y and 5Y-Manure treatments are broadly similar in management, other than the inclusion of green manuring of faba beans, an additional year of perennial forage growth, and higher average applied manure rates (equalling 46 kg N ha^{-1} yr^{-1} vs. 35 kg N ha^{-1} yr^{-1}) in the 8Y system. However, despite the extra year of consecutive perennial forage growth in the 8Y system, the overall proportion of time spent under forage in the 8Y system (37.5% of the time) is marginally less than in the 5Y system (40% of the time). Critically, the 8Y and CF treatments had the longest consecutive periods of perennial forage growth (3 years and continuous, respectively). Perennial crops continue to expand their root systems over multiple years and, as a result, tend to produce greater below-ground biomass—with more root mass at depth—the longer a stand is maintained (Bolinder et al., 2002; Houde et al., 2020; Sakiroglu et al., 2020; F. Zhang et al., 2023). As previously mentioned, perennial crops also reduce soil erosion compared to annuals by increasing soil cover and decreasing tillage frequency. In particular, no-till systems have been found to promote high SOC levels (Bai et al., 2018; Engell et al., 2022; You et al., 2017). As a result of such benefits, the inclusion of perennial forage crops, when grown continuously or as components of a rotational cropping system, have been shown to produce significantly larger SOC stocks than systems with greater proportions of annual cropping (Khanal, 2023; King & Blesh, 2018; Olmstead & Brummer, 2008). Similar to the inclusion of perennials, the use of green manure has also been shown to increase SOC stocks (Bhattacharya et al., 2016) and is, therefore, likely a factor contributing to the further increase in SOC within the 8Y treatment, which exceeded that of the 5Y treatment, which also received manure.

Ultimately, it is likely that the substantial increase in SOC stock within the 8Y and CF treatments compared to all other treatments is largely due to the longer consecutive perennial cropping interval and reduced tillage, coupled with the green manure plow down in the 8Y treatment. Compared to the amended 5Y treatments, both the 8Y and CF treatments had higher SOC stocks at depths extending down as deep as the 5-7.5 Tg ha^{-1} layer, despite the 5Y system being under perennial forages for a greater proportion of its rotation than the 8Y treatment. This is consistent with the notion that the longer the consecutive period that perennials are allowed to grow, the greater the root biomass inputs are likely to be, including at deeper depths. Additionally, the particularly large SOC stock in the 8Y treatment is likely receiving significant contributions from the plow down of faba beans as green manure, and its singularly high average manure rate. As a result of these factors, compared to their own baseline C contents or that of the CG treatment, the 8Y and CF systems have experienced the greatest soil C accrual since the initiation of the Breton Plots, with soil C increases proceeding at average rates of more than 4‰ annually (Appendix 2.1). These results indicate that perennial forage cultivation and legume green manuring are valuable tools for increasing SOC storage and overall agricultural sustainability.

2.5.5. Implications of SOC for C Valuation Policies

The current differences in SOC stocks associated with different cropping systems have substantial economic implications when C pricing is considered. The CG treatment was chosen as the baseline against which to compare the SOC stock values of the other treatments as of 2021, as this treatment is highly representative of annual cash cropping systems that are common both internationally and in Canada. Globally, the Food and Agriculture Organization (FAO) classifies approximately 87.5% of cropland as arable land, which it states is primarily used to produce annual cash crops (FAO, 2021b). Similarly, Canada has approximately 38.4 Mha of cropland, with 28.3 Mha estimated to be seeded to annual grains or oilseeds in 2023-2024 (Agriculture and Agri-Food Canada, 2024; Zafiriou et al., 2023). As a result, annual cash cropping systems represent a common “business-as-usual” scenario for contemporary intensive agriculture in general.

When compared to the CG treatment, the opportunity cost of SOC lost and not accrued within the non-manured WF (i.e., WF-Check, WF-NPKS) treatments was sizable. Both non-manured WF treatments had approximately \$11,000 CAD ha^{-1} worth of SOC less than the CG treatment. On the other hand, the 8Y and CF treatments had accrued more than \$5000 CAD ha^{-1} of additional SOC compared to the CG treatment. Given that the average size of Canadian farms is ~327.6 ha (Statistics Canada, 2022), at the average farm scale, this would amount to more than \$3.6 million CAD worth of SOC lost to fallow, and more than \$1.6 million CAD worth of SOC added in from the use of the 8Y and CF systems, compared to continuous annual cropping. Assuming that these changes in SOC stock developed over a period of 41 years (the period since the initiation of the Hendrigan Plots), this puts the relative ‘farm-level’ SOC cost of fallow at approximately \$90,000 CAD yr^{-1}, or approximately \$40,000 CAD yr^{-1} if developed over the 91 years that the Classical Plots have been managed. In contrast, the added value of SOC stemming from the management practices in the 8Y and CF systems calculated in this manner amounts to more than \$40,000 CAD yr^{-1}, on average.

The considerable differentials in the value of SOC seen here suggest there is an opportunity for governments to incentivize agricultural producers to shift their management by either compensating them for additional SOC stored, or by taxing them for SOC lost due to management changes. Various studies have suggested that global climate change mitigation efforts would benefit from including such soil C valuation policies and have described pathways toward implementing sensible and effective versions of these policies (Amelung et al., 2020; Mooney et al., 2004). Overall, providing direct monetary incentives for increasing SOC in agricultural soils remains a potentially powerful tool for accelerating the shift toward more sustainable soil management.

2.6. Conclusions

The various agricultural management strategies evaluated at the Breton Plots produced significant variation in SOC stock after 41 years. Notably, the use of alternate-year fallow produced the lowest SOC stocks, which were somewhat mitigated by amendment with manure but not by conventional fertilizer application. Soil organic carbon stocks were also found to have a positive response to the inclusion of perennial forages, particularly those with longer intervals of forage growth, as demonstrated by the 8Y and CF treatments having higher SOC stocks and a deeper extent of SOC enrichment compared to all other treatments. Additionally, the use of green manuring within the 8Y treatment was also identified as a likely contributor to its high SOC stock. Gains in SOC were also accompanied by reductions in soil BD, highlighting the importance of using ESM to assess SOC differences.

Changes in SOC stock led to marked differences in the potential value of C stored as SOC among the various treatments. Within the first 10.6 Tg ha^{-1} of soil, alternate year fallow without manure amendment led to approximately \$11,000 CAD ha^{-1} in lost SOC compared to the continuous annual cropping. In contrast, continuous perennial forage cropping or agroecological practices (i.e., diversified rotation including perennial forages, legume green manuring, and animal manure application), led to more than \$5000 CAD ha^{-1} of additional SOC stored in these soils compared to continuous annual cropping.

Importantly, nearly all examined management practices led to average increases in topsoil C of more than 4‰ per year, even when compared to conventionally fertilized continuous annual cropping (Appendix 2.1). These results indicate that global sustainability goals, such as the UN’s 4 per 1000 initiative, are attainable through sensible changes in agricultural management practices. In particular, this study shows that increased use of perennial forages, legumes, organic amendments (i.e., animal and green manure), and no-till management in cropping systems are effective ways to build SOC and enhance agricultural sustainability.

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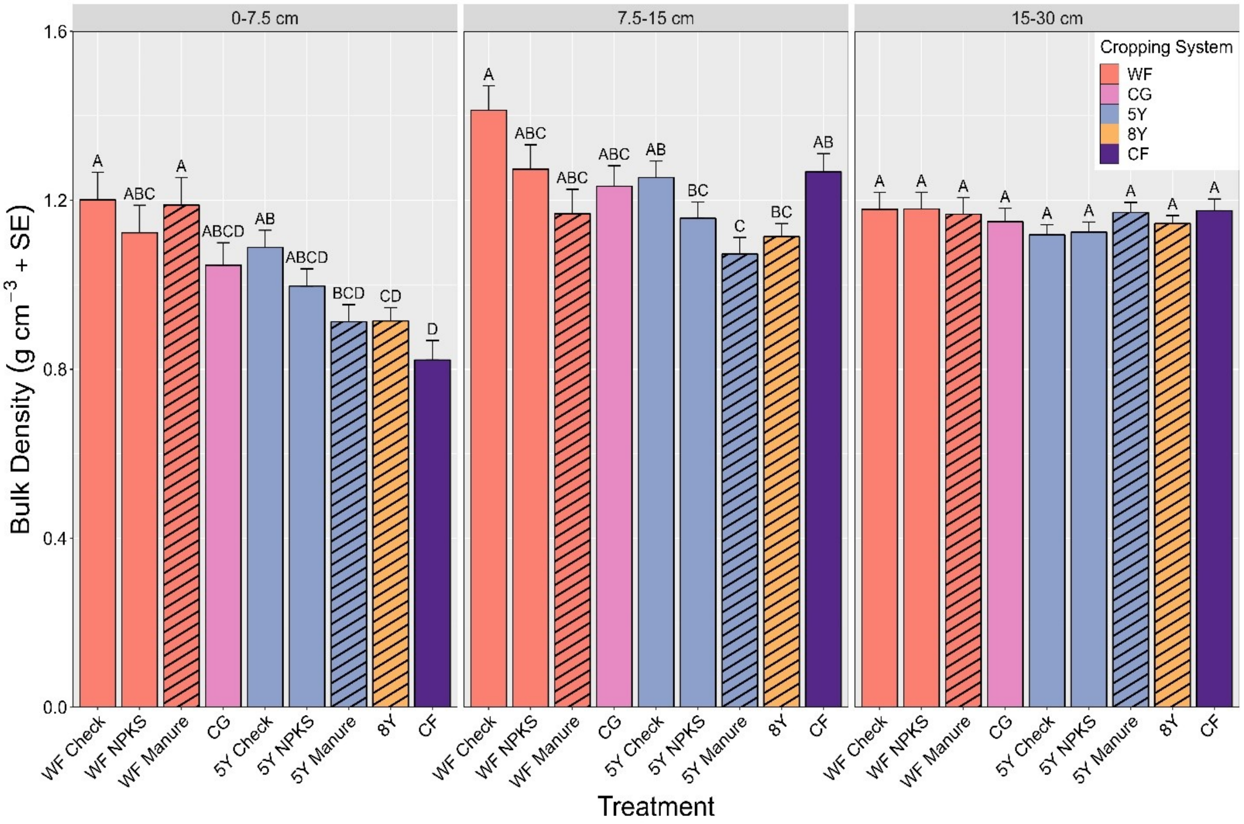

Figure 2.1. Soil bulk density for the upper three fixed depth intervals (0-7.5 cm, 7.5-15 cm, 15-30 cm) sampled. Panels indicate fixed depth interval; bar colour represents overall cropping system; and bar hatching indicates treatment with manure. Lettering indicates significant differences in group means at $P < 0.05$.

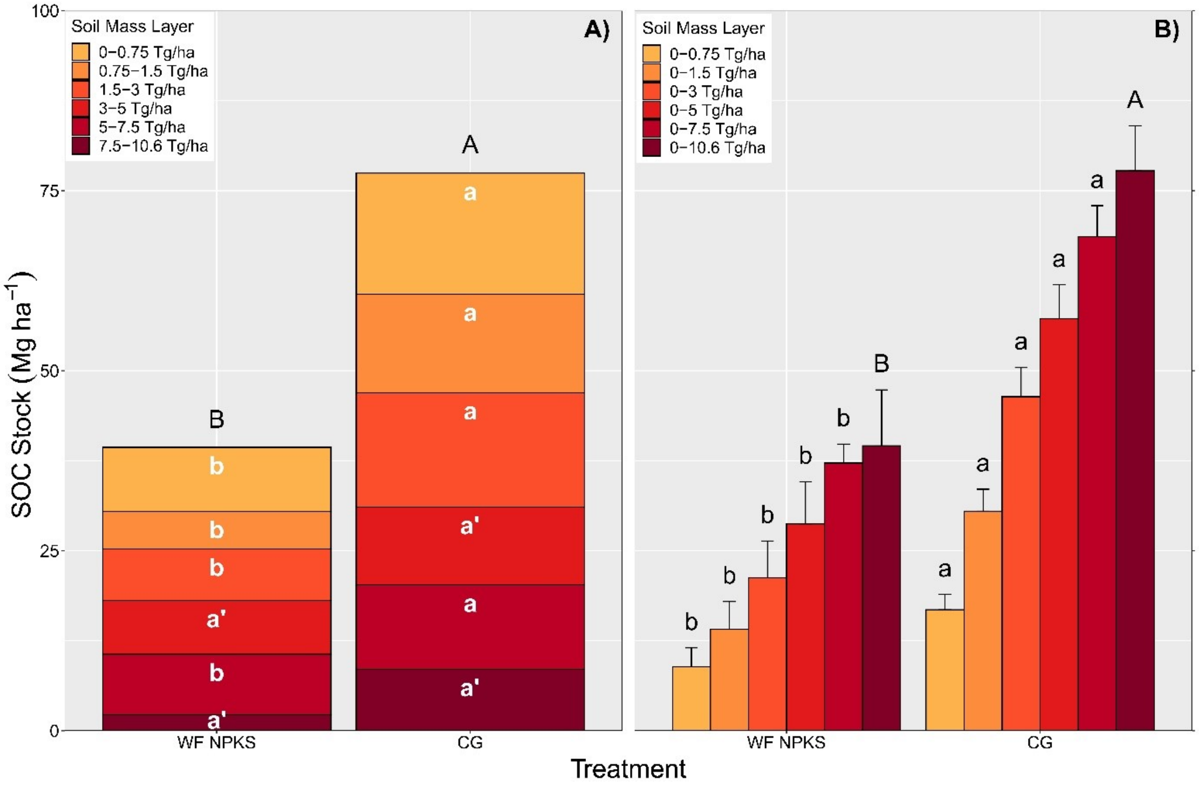

Figure 2.2. SOC stocks for the WF-NPKS and CG treatments, including: **A)** per-layer stocks and **B)** cumulative stocks. Uppercase letters denote significant differences between whole soil profile SOC stocks, while lowercase letters denote differences within a layer. Shared group letters appended with an apostrophe (‘) indicate differences at P < 0.1, all other groups represent differences at P < 0.05. Error bars represent standard error.

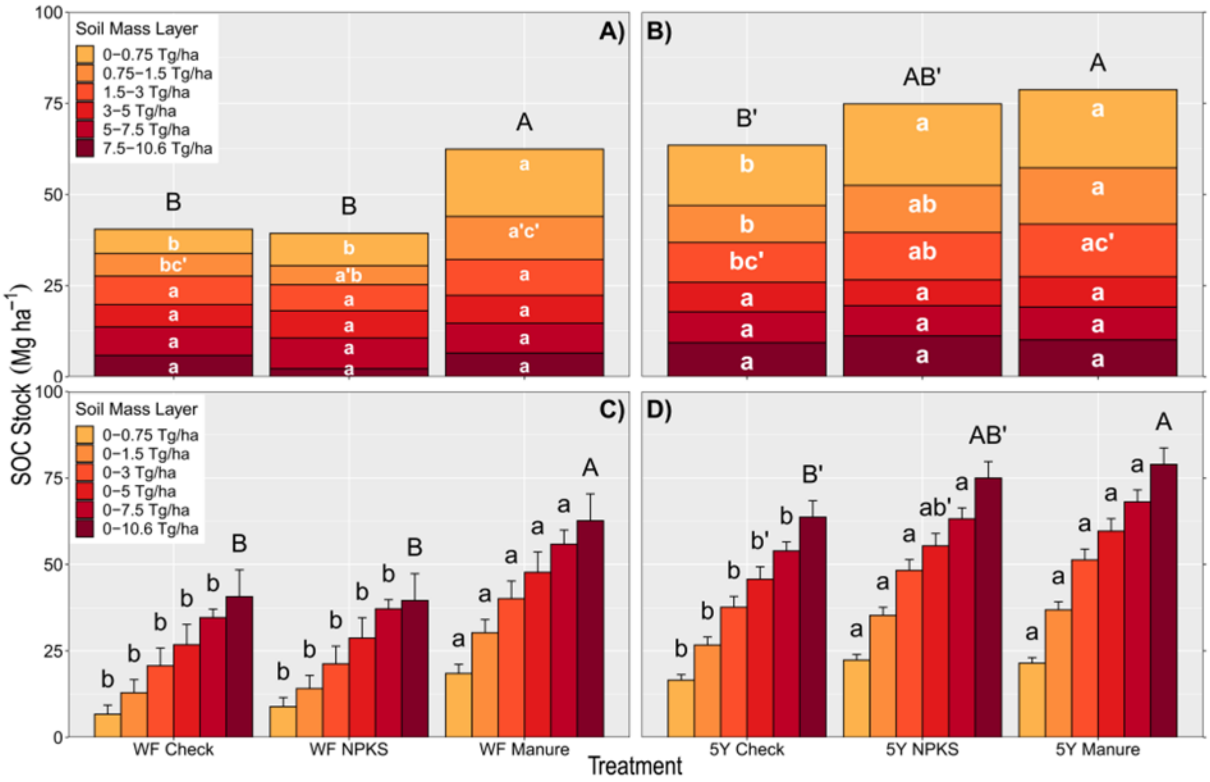

Figure 2.3. SOC stock by applied soil amendment for the WF and 5Y cropping systems: **A)** and **B)** per-layer stocks; **C)** and **D)** cumulative stocks. Uppercase letters denote significant differences between whole soil profile SOC stocks, while lowercase letters denote differences within a layer. Shared group letters appended with an apostrophe (‘) indicate differences at $P < 0.1$, all other groups represent differences at $P < 0.05$. Error bars represent standard error.

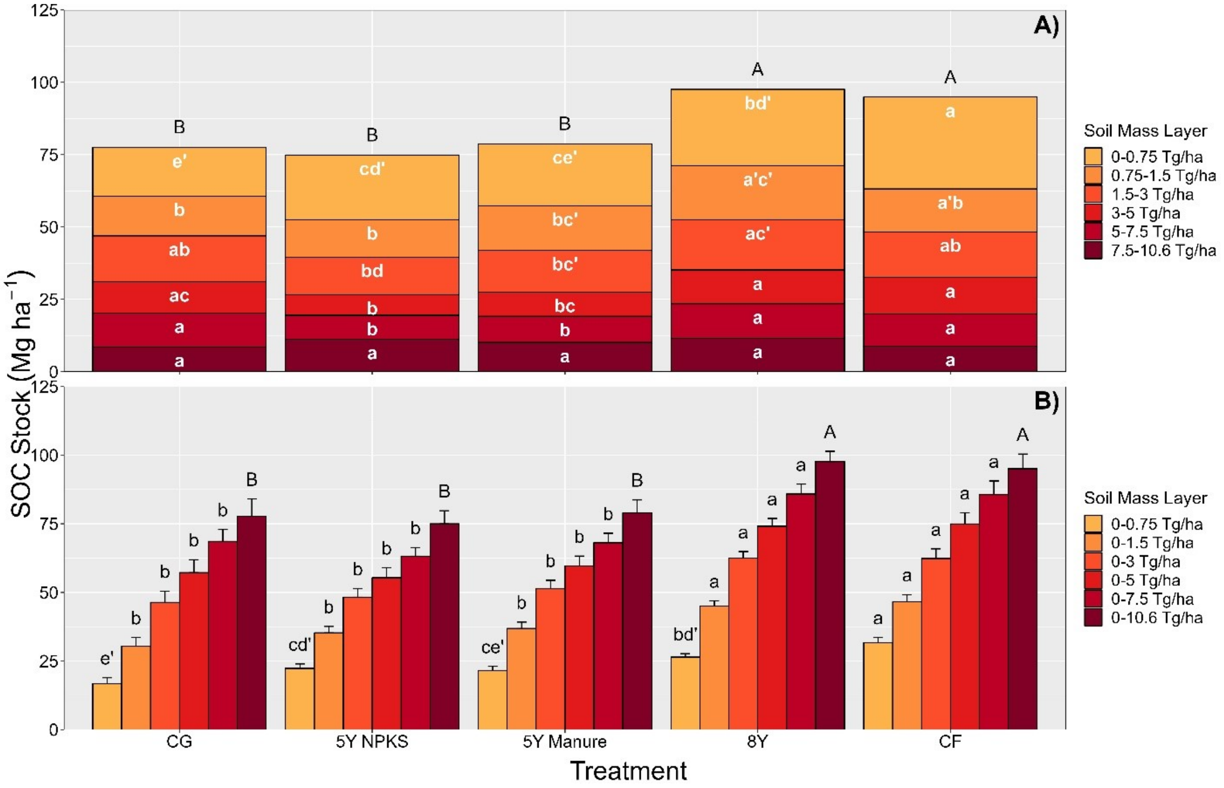

Figure 2.4. SOC stocks for all fertilized, non-fallowed treatments: **A)** per-layer stocks and **B)** cumulative stocks. Uppercase letters denote significant differences between whole soil profile SOC stocks, while lowercase letters denote differences within a layer. Shared group letters appended with an apostrophe (‘) indicate differences at $P < 0.1$, all other groups represent differences at $P < 0.05$. Error bars represent standard error.

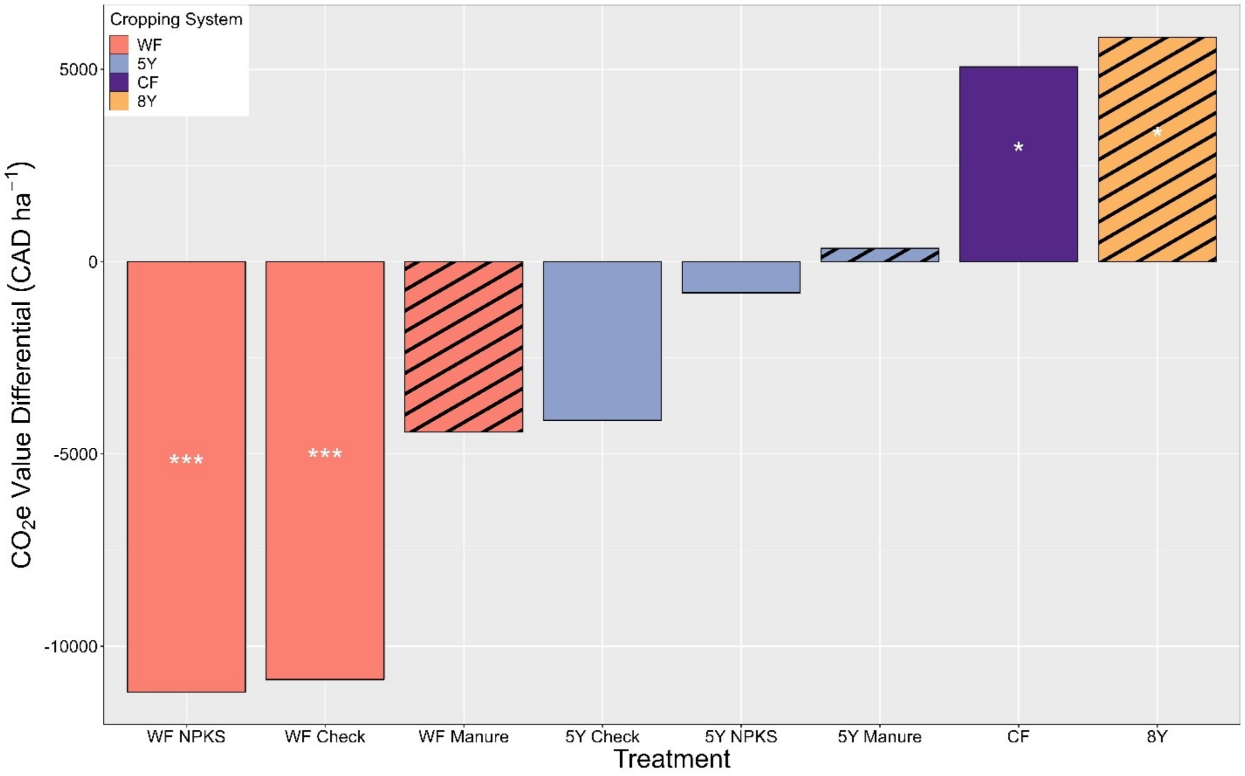

Figure 2.5. The total value of C lost or accrued in the 0-10.6 Tg ha^{-1} soil mass layer for each treatment compared to the CG treatment. The CG cropping system was chosen as a baseline as it most closely represents annual cash cropping systems, which are widespread both regionally and globally. Bar colour represents overall cropping system and bar hatching indicates manured treatments. Significant differences between the displayed treatments and the CG treatment are represented by one, two, or three asterisks (*) for $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

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2.9. Appendix 2

Appendix 2.1. Soil C changes since the initiation of the Classical and Hendrigan Plots experiments. Final values represent soil C content and stock at the time of sampling in 2021. TC = total soil carbon content, ΔTC = change in total soil C content.

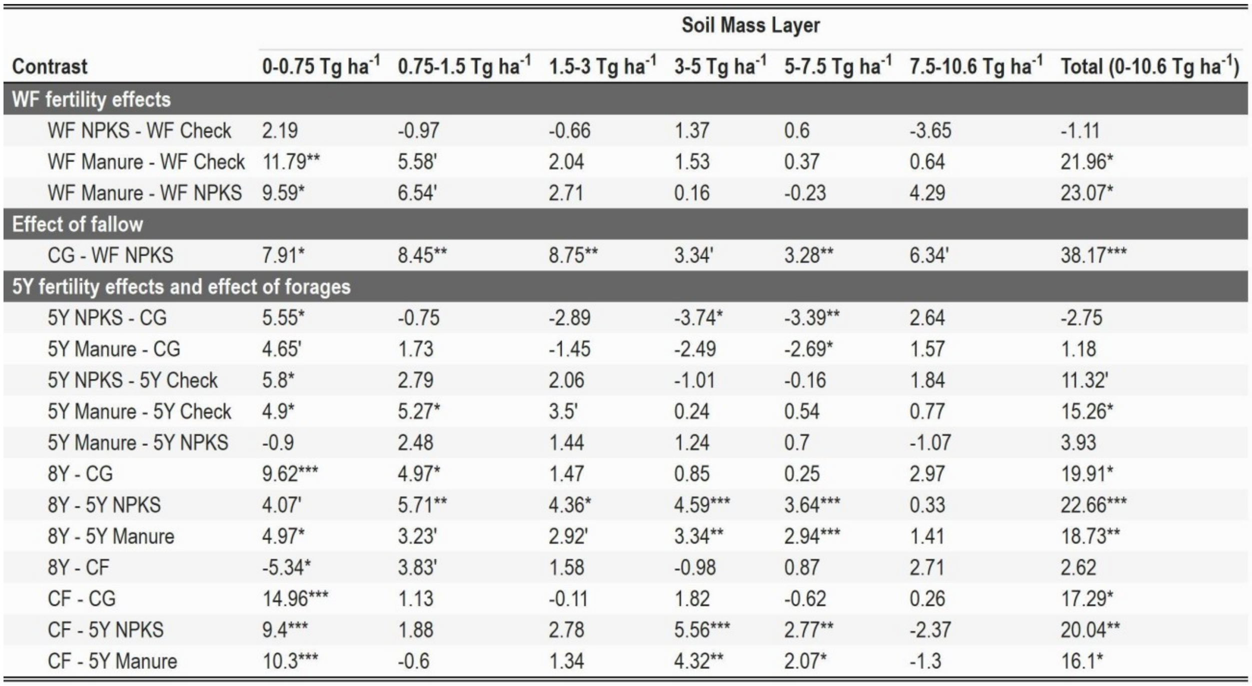
Treatment	Depth (cm)	Initial TC* (mg/g)	Final TC (mg/g)	ΔTC (mg/g)	Annualized ΔTC** (‰)	Relative Annualized ΔTC*** (‰)
WF-Check	0-15	13.9	8.7	-5.2	-5.6	-575.0
WF-NPKS	0-15	13.9	9.7	-4.2	-4.2	-504.0
WF-Manure	0-15	13.9	21.1	7.2	4.9	70.2
5Y-Check	0-15	12.0	15.8	3.8	3.3	-189.0
5Y-NPKS	0-15	12.0	24.3	12.3	8.3	162.3
5Y-Manure	0-15	12.0	27.0	15.0	9.6	254.4
CG	0-15	15.8	20.3	4.5	6.1	N/A
8Y	0-15	15.8	34.8	19.0	19.4	316.3
CF	0-15	15.4	32.9	17.5	18.7	271.4

*Initial TC and C Stock values for the Classical Plots are from 1936, as this is the earliest year for which data is available. Initial TC and C stock values for the Hendrigan Plots are from 1980 (i.e., the true initiation of the experiment).

**Calculated by assuming a linear change in TC each year and averaging the annual change over the experimental period.

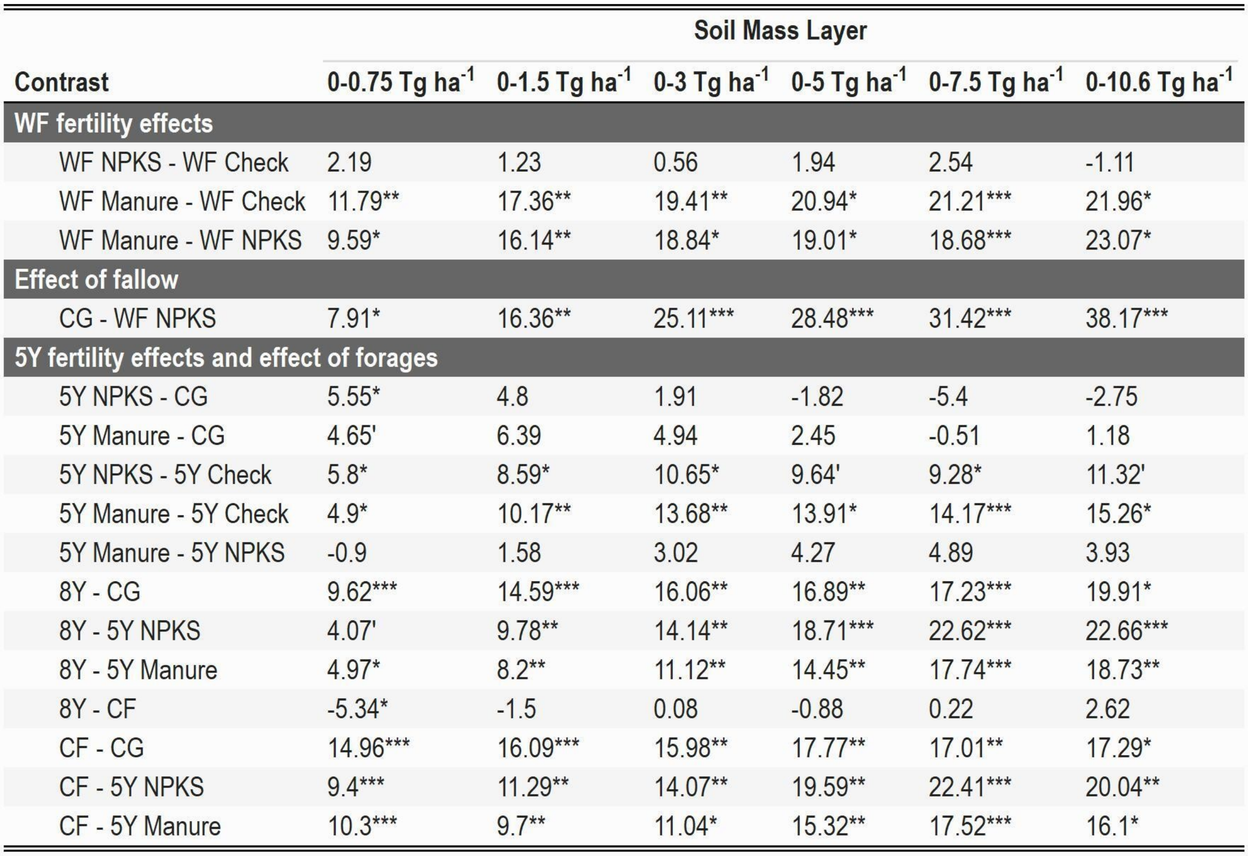
***Average annual soil C change relative to the CG cropping system.

***Appendix 2.2**.* Layer-by-layer linear contrast estimates of differences in mean SOC stocks (Mg C ha^{-1}). All contrasts are tested against the null hypothesis that there is no difference in means. Symbols indicate significant differences between treatment means at the following p-values: '=P<0.1, *=P<0.05, **=P<0.01, ***=P<0.001.



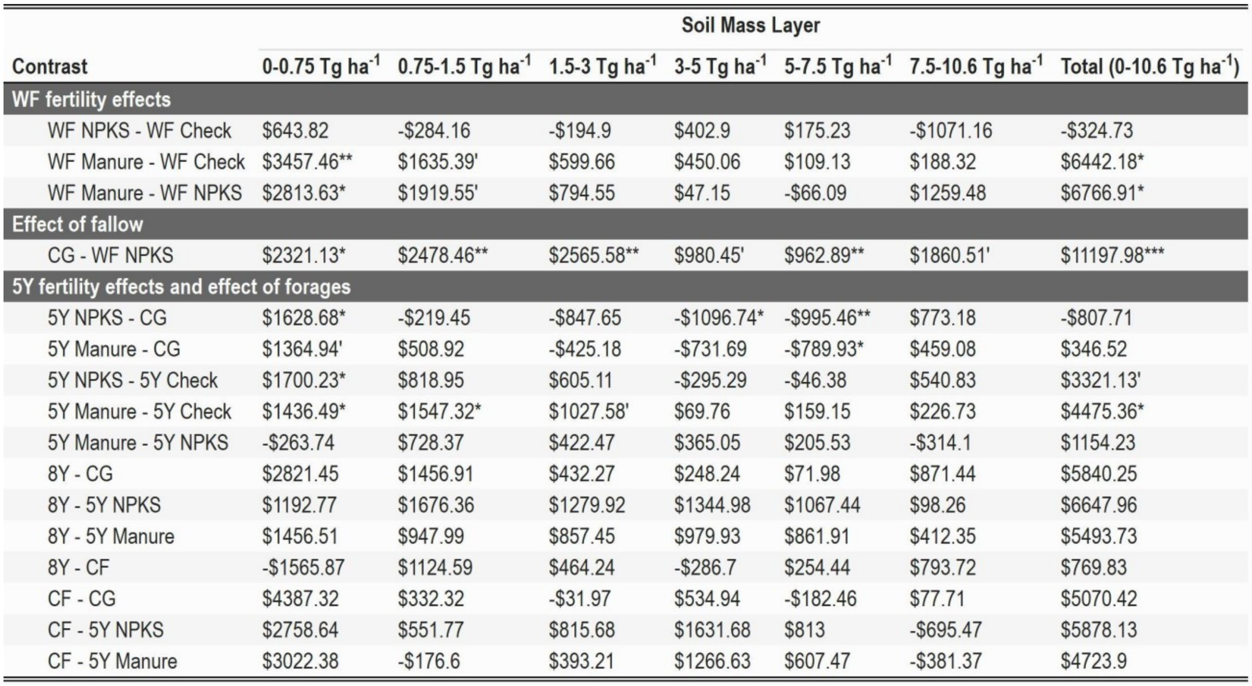
	Soil Mass Layer						
Contrast	**0-0.75 Tg ha^{-1}**	**0.75-1.5 Tg ha^{-1}**	**1.5-3 Tg ha^{-1}**	**3-5 Tg ha^{-1}**	**5-7.5 Tg ha^{-1}**	**7.5-10.6 Tg ha^{-1}**	**Total (0-10.6 Tg ha^{-1})**
WF fertility effects							
WF NPKS - WF Check	2.19	-0.97	-0.66	1.37	0.6	-3.65	-1.11
WF Manure - WF Check	11.79**	5.58'	2.04	1.53	0.37	0.64	21.96*
WF Manure - WF NPKS	9.59*	6.54'	2.71	0.16	-0.23	4.29	23.07*
Effect of fallow							
CG - WF NPKS	7.91*	8.45**	8.75**	3.34'	3.28**	6.34'	38.17***
5Y fertility effects and effect of forages							
5Y NPKS - CG	5.55*	-0.75	-2.89	-3.74*	-3.39**	2.64	-2.75
5Y Manure - CG	4.65'	1.73	-1.45	-2.49	-2.69*	1.57	1.18
5Y NPKS - 5Y Check	5.8*	2.79	2.06	-1.01	-0.16	1.84	11.32'
5Y Manure - 5Y Check	4.9*	5.27*	3.5'	0.24	0.54	0.77	15.26*
5Y Manure - 5Y NPKS	-0.9	2.48	1.44	1.24	0.7	-1.07	3.93
8Y - CG	9.62***	4.97*	1.47	0.85	0.25	2.97	19.91*
8Y - 5Y NPKS	4.07'	5.71**	4.36*	4.59***	3.64***	0.33	22.66***
8Y - 5Y Manure	4.97*	3.23'	2.92'	3.34**	2.94***	1.41	18.73**
8Y - CF	-5.34*	3.83'	1.58	-0.98	0.87	2.71	2.62
CF - CG	14.96***	1.13	-0.11	1.82	-0.62	0.26	17.29*
CF - 5Y NPKS	9.4***	1.88	2.78	5.56***	2.77**	-2.37	20.04**
CF - 5Y Manure	10.3***	-0.6	1.34	4.32**	2.07*	-1.3	16.1*

Appendix 2.3. Linear contrast estimates of differences in cumulative mean SOC stocks (Mg C ha^{-1}). All contrasts are tested against the null hypothesis that there is no difference in means. Symbols indicate significant differences between treatment means at the following p-values: '=P<0.1, *=P<0.05, **=P<0.01, ***=P<0.001.



	Soil Mass Layer					
Contrast	**0-0.75 Tg ha^{-1}**	**0-1.5 Tg ha^{-1}**	**0-3 Tg ha^{-1}**	**0-5 Tg ha^{-1}**	**0-7.5 Tg ha^{-1}**	**0-10.6 Tg ha^{-1}**
WF fertility effects						
WF NPKS - WF Check	2.19	1.23	0.56	1.94	2.54	-1.11
WF Manure - WF Check	11.79**	17.36**	19.41**	20.94*	21.21***	21.96*
WF Manure - WF NPKS	9.59*	16.14**	18.84*	19.01*	18.68***	23.07*
Effect of fallow						
CG - WF NPKS	7.91*	16.36**	25.11***	28.48***	31.42***	38.17***
5Y fertility effects and effect of forages						
5Y NPKS - CG	5.55*	4.8	1.91	-1.82	-5.4	-2.75
5Y Manure - CG	4.65'	6.39	4.94	2.45	-0.51	1.18
5Y NPKS - 5Y Check	5.8*	8.59*	10.65*	9.64'	9.28*	11.32'
5Y Manure - 5Y Check	4.9*	10.17**	13.68**	13.91*	14.17***	15.26*
5Y Manure - 5Y NPKS	-0.9	1.58	3.02	4.27	4.89	3.93
8Y - CG	9.62***	14.59***	16.06**	16.89**	17.23***	19.91*
8Y - 5Y NPKS	4.07'	9.78**	14.14**	18.71***	22.62***	22.66***
8Y - 5Y Manure	4.97*	8.2**	11.12**	14.45**	17.74***	18.73**
8Y - CF	-5.34*	-1.5	0.08	-0.88	0.22	2.62
CF - CG	14.96***	16.09***	15.98**	17.77**	17.01**	17.29*
CF - 5Y NPKS	9.4***	11.29**	14.07**	19.59**	22.41***	20.04**
CF - 5Y Manure	10.3***	9.7**	11.04*	15.32**	17.52***	16.1*

Appendix 2.4. Linear contrast estimates of differences in the mean value of CO_2-e accrued in different treatments. All contrasts are tested against the null hypothesis that there is no difference in means. Symbols indicate significant differences between treatment means at the following p-values: '=P<0.1, *=P<0.05, **=P<0.01, ***=P<0.001.



	Soil Mass Layer						
Contrast	**0-0.75 Tg ha^{-1}**	**0.75-1.5 Tg ha^{-1}**	**1.5-3 Tg ha^{-1}**	**3-5 Tg ha^{-1}**	**5-7.5 Tg ha^{-1}**	**7.5-10.6 Tg ha^{-1}**	**Total (0-10.6 Tg ha^{-1})**
WF fertility effects							
WF NPKS - WF Check	$643.82	-$284.16	-$194.9	$402.9	$175.23	-$1071.16	-$324.73
WF Manure - WF Check	$3457.46**	$1635.39'	$599.66	$450.06	$109.13	$188.32	$6442.18*
WF Manure - WF NPKS	$2813.63*	$1919.55'	$794.55	$47.15	-$66.09	$1259.48	$6766.91*
Effect of fallow							
CG - WF NPKS	$2321.13*	$2478.46**	$2565.58**	$980.45'	$962.89**	$1860.51'	$11197.98***
5Y fertility effects and effect of forages							
5Y NPKS - CG	$1628.68*	-$219.45	-$847.65	-$1096.74*	-$995.46**	$773.18	-$807.71
5Y Manure - CG	$1364.94'	$508.92	-$425.18	-$731.69	-$789.93*	$459.08	$346.52
5Y NPKS - 5Y Check	$1700.23*	$818.95	$605.11	-$295.29	-$46.38	$540.83	$3321.13'
5Y Manure - 5Y Check	$1436.49*	$1547.32*	$1027.58'	$69.76	$159.15	$226.73	$4475.36*
5Y Manure - 5Y NPKS	-$263.74	$728.37	$422.47	$365.05	$205.53	-$314.1	$1154.23
8Y - CG	$2821.45	$1456.91	$432.27	$248.24	$71.98	$871.44	$5840.25
8Y - 5Y NPKS	$1192.77	$1676.36	$1279.92	$1344.98	$1067.44	$98.26	$6647.96
8Y - 5Y Manure	$1456.51	$947.99	$857.45	$979.93	$861.91	$412.35	$5493.73
8Y - CF	-$1565.87	$1124.59	$464.24	-$286.7	$254.44	$793.72	$769.83
CF - CG	$4387.32	$332.32	-$31.97	$534.94	-$182.46	$77.71	$5070.42
CF - 5Y NPKS	$2758.64	$551.77	$815.68	$1631.68	$813	-$695.47	$5878.13
CF - 5Y Manure	$3022.38	-$176.6	$393.21	$1266.63	$607.47	-$381.37	$4723.9

Chapter 3 – Soil Necromass Carbon Displays Complex Relationships with Soil Carbon and Nitrogen Pools

3.1. Abstract

Maintaining or increasing soil organic matter (SOM) in agricultural systems is essential to avoiding soil degradation and associated losses in crop productivity, and can help mitigate agricultural greenhouse gas emissions. Given that soil microbial necromass has been shown to be a major contributor to SOM formation and persistence, understanding the response of necromass to agricultural management will be crucial to efforts to maintain and increase SOM in agricultural soils. However, soil microbial necromass responds slowly to changes in management, meaning long-term datasets are required for high-precision analysis of necromass responses to management practices. Here, surficial soil samples (0-7.5 cm) from the Breton Plots research facility were used to test the effects of various long-term agricultural practices on topsoil physicochemical properties and necromass. Samples were gathered from the Classical Plots (est. 1930) comparing a wheat-fallow rotation and a 5-year rotation comprising three years of annual cash crops and two consecutive years of perennial forage hay. These rotations include non-amended, conventionally fertilized, and manured sub-treatment permutations. Additionally, samples were collected from the Hendrigan Plots (est. 1980), which compare three cropping systems: a no-till continuous forage system; a continuous grain system; and a complex 8-year agro-ecological rotation comprising annual cash crops, leguminous green manure, and three consecutive years of perennial forage hay. Soil physicochemical properties [carbon (C) content, nitrogen (N) content, C:N ratio, and $\delta^{13}C$] were determined for the whole soil and three particle size and density fractions, namely, light fraction organic matter (LFOM; $\rho < 1.7$ g cm^{-3}), coarse mineral-associated organic matter (CMAOM; $\rho > 1.7$ g cm^{-3}, d > 53 μm), and fine mineral-associated organic matter (FMAOM; $\rho > 1.7$ g cm^{-3}, d < 53 μm). The pH and bulk density values were also quantified for the whole soil. Additionally, soil fungal, bacterial, and microbial (fungal+bacterial) necromass C were analyzed along with their associations with soil physicochemical properties and their contribution to, and ability to predict, total SOC.

Compared to continuous annual cropping, alternate fallow raised soil $\delta^{13}C$ and reduced soil C content, N content, and C:N ratio in the whole soil and mineral fractions. Fallow inclusion also decreased bulk soil fungal and microbial necromass C content, and increased the proportion of necromass derived from bacteria, signalling extreme microbial processing of SOM and mining of fungal necromass by soil bacteria. Manure amendment uniformly increased bulk soil C and N, primarily through increased contributions from the FMAOM and CMAOM fractions, which were likely linked to observed increases in contributions from both fungal and bacterial necromass. Conventional fertilizer application consistently increased bacterial necromass C but had inconsistent effects on other soil physicochemical properties that depended on the overall cropping system in which it was applied. Perennial grass-legume forage mixtures increased C and N content in all physical soil fractions and necromass pools in proportion to the consecutive periods for which they were grown in a cropping system, indicating the sensitivity of these properties to increased plant biomass inputs. No-till contributed to a singularly high LFOM-derived C content with a high C:N ratio, and a resultantly high contribution of relatively stable fungal residues to total necromass. In contrast, legume green manure contributed to a low LFOM fraction C:N ratio and increased the relative contribution of generally less stable bacterial residues to total necromass. Finally, various correlations between necromass C pools and soil physicochemical properties indicated close associations between necromass and mineral-associated organic matter formation, as well as between necromass formation and high-quality (i.e., low C:N ratio) plant litter, particularly for bacterial necromass. These results provide insight into how soil necromass accumulation can be modified by agricultural practices to maximize SOM formation and persistence while clarifying the physicochemical mechanisms through which these practices impact soil necromass.

3.2. Introduction

Soil organic carbon (SOC) is the largest terrestrial carbon (C) reservoir, storing 2 and 2.8 times more C than the terrestrial biomass and atmospheric C pools, respectively (Lal, 2004). Furthermore, agricultural soils globally have the potential to store an additional 0.6-1.2 Gt C year^{-1} through improved management, considerably mitigating net anthropogenic C emissions (Lal et al., 2007). Primarily, it is soils that have been moderately to severely degraded through land-use change or contemporary agricultural production practices (e.g., abundant tillage), leading to sizable declines in their initial SOC pools, that have the potential to sequester additional C (Lal, 2021). This is also important, considering that SOC is essential to soil function, playing an important role in enhancing soil properties such as buffering capacity, soil aggregation, and water-holding capacity (Johnston et al., 2009). According to the Food and Agriculture Organization (FAO), 33% of all soils are moderately to highly degraded (FAO, 2015), implying considerable potential to increase soil health globally via restoration. Increasing SOC storage would help address at least two major challenges of the modern era: reducing net anthropogenic greenhouse gas (GHG) emissions while simultaneously meeting the increasing global demand for material, food, and fuel via improved soil health. However, in order to increase global SOC storage, it is necessary to understand how SOC fundamentally forms and is retained in the soil, and thereby to predict which agricultural practices will increase SOC.

Contemporary understanding of soil organic matter (SOM) generation and persistence emphasizes the contribution of soil microbial organic matter (OM), along with plant and faunal detritus and waste, to the overall SOM pool (Lehmann & Kleber, 2015). However, living microbial biomass is ephemeral, often exhibiting considerable spatiotemporal variability, even on relatively small temporal and spatial scales (Grzyb et al., 2022; Liu et al., 2010; Piotrowska & Długosz, 2012; Piotrowska-Długosz et al., 2019; Ye et al., 2019), and generally accounts for only 1% to 5% of total SOM (Anderson & Domsch, 1989; Dalal, 1998). Despite this fact, multiple studies have inferred that microbially-generated compounds can remain stable in the soil after cell death and may contribute more than 50% of total SOM via long-term accumulation (Miltner et al., 2012;

Simpson et al., 2007; Wang et al., 2021). Taken together, these findings imply that measurements of (living) microbial biomass are unlikely to be representative of the long-term impact of microbial activity on SOM. The limited ability to elucidate the long-term impacts of microbial communities on SOC makes microbial biomass problematic as a direct predictor of SOM dynamics across extended time scales.

As an alternative, the quantification of (non-living) soil microbial necromass is one potential method for more robustly evaluating the long-term contributions of soil microbial communities to SOM formation. This approach involves the identification of specific, microbe-associated residues—such as particular amino sugars—that persist in the soil after cell death, via techniques such as gas chromatography (GC) or high-performance liquid chromatography analyses, followed by back-conversion to dry-weight biomass (Liang et al., 2012, 2020; Mou et al., 2021; Salas et al., 2023). Recent analyses of necromass C have found that this C pool often makes up more than 50% of SOC (Kallenbach et al., 2015; Liang et al., 2019, 2020; Wang et al., 2021). Furthermore, the microbial necromass C (MNC) pool may be more stable than other C pools, such as plant residue C, due to factors such as adsorption with the soil mineral fractions, leading to enhanced physical protection from microbial breakdown (Buckeridge et al., 2020; Kleber et al., 2015; Miltner et al., 2012). Buckeridge et al. (2020) used stable isotope labelling to demonstrate that necromass adsorbs to mineral surfaces, and Mitner et al. (2012) directly imaged mineral-adsorbed cells via scanning electron microscopy. However, there is limited understanding of how the necromass fraction of SOM responds to different land management strategies, particularly on long timescales.

The response of necromass to amendments tends to differ for organic and inorganic amendments (Li et al., 2020; Tang et al., 2023; Xia et al., 2021; Zhou et al., 2023). For example, a meta-analysis by Zhou et al. (2023) found that, on average, nitrogen (N) fertilization increased MNC by 12%, whereas manure and straw amendments both increased MNC by 21%. This analysis also indicated that the necromass fungal-to-bacterial ratio (henceforth, fungi:bacteria ratio) was generally lower under manure addition (Zhou et al., 2023). Similarly, Li et al. (2020) found that amino sugar production increased by 19.7% from fertilization, and 82.9% to 107% from manure, compared to control treatments after 30 years of annual amendment. This same study showed that relative to control treatments, the phospholipid fatty acid (PLFA) fungi:bacteria ratio was increased by fertilizer amendment and unchanged by manure amendment but was decreased by the combination of fertilizer and manure amendment (Li et al., 2020). Another 30+ year long-term agroecosystem experiment (LTAE) found that the combined treatments of fertilizer and manure increased both microbial biomass and necromass by up to 1.5 times compared to treatment with fertilizer alone (Xia et al., 2021). In this same investigation, combined manure and fertilizer treatment reduced bulk soil PLFA fungi:bacteria ratio compared to both the control treatment and the treatment solely receiving fertilizer (Xia et al., 2021).

Studies have also indicated that soil necromass content is sensitive to differing systems of vegetation and levels of soil C inputs (Gilmullina et al., 2023; Hu et al., 2023; Li et al., 2015, 2023; Rui et al., 2022; Zhou et al., 2023). For instance, Gilmullina et al. (2023) observed that after 13 years of continuous treatment, bare fallow had a significantly lower concentration of amino sugars compared to an unmanaged grassland site (i.e., taken out of agricultural production at the beginning of the study period). Gilmullina et al. (2023) also observed a lower amino sugar concentration in the bare fallow treatment than in pastures subjected to mowing or either low or high-intensity grazing, albeit these differences were statistically non-significant. Similarly, another study comparing amino sugar contents under various cropping systems within a Mollisol soil found that treatments of bare parent material and natural fallow (i.e., idled land left to revegetate naturally) had lower amino sugar content compared to an alfalfa treatment under no-till management (Li et al., 2015). This same study also found that fungal amino sugars responded more than bacterial amino sugars to variation in vegetation management and, ultimately, dominated the microbial contribution to SOC content despite bacteria being the predominant component of live microbial biomass (Li et al., 2015).

Further consistent with these patterns, increasing biomass C inputs through cover cropping, the utilization of perennial rather than annual crop species, or improved grazing management, have all been shown to increase necromass C (Hu et al., 2023; Li et al., 2023; Rui et al., 2022; Zhou et al., 2023). For example, a meta-analysis by Zhou et al. (2023) showed that cover cropping increased soil necromass C by 12%. Similarly, Hu et al. (2023) found that the inclusion of non-legume and legume cover crops within an orchard system increased SOC by 2% and 23%, respectively, with differences in microbial necromass being the most important explanatory factor. Li et al. (2023) found that after only two years of treatment, grasslands sown with perennial grasses led to greater bacterial necromass C (BNC), fungal necromass C (FNC), and total MNC, relative to those either sown yearly to a mix of annual grass and legumes, as well as those sown strictly to annual grasses. Gilmullina et al. (2023) found a decrease in soil amino sugar content as more plant biomass was removed from pasture systems by annual grazing or mowing, or where biomass inputs were eliminated due to bare fallow. In contrast, after 13 years, an unmanaged site that had been removed from agricultural production at the initiation of the experiment had the highest amino sugar content, which was followed by treatments of low-intensity grazing, high-intensity grazing, and mowing with straw removal, with bare fallow having the lowest overall amino sugar content (Gilmullina et al., 2023). A study of Mollisol soils in the north central United States found that perennial pastures managed via rotational grazing had significantly higher amino sugar content compared to continuous maize cropping after 29 years (Rui et al., 2022).

Despite much interest in understanding the impact of microbial necromass on SOC formation and persistence, there is still a paucity of research linking different land management strategies to specific soil necromass responses. Therefore, to increase SOC stores and benefit soil health while limiting agricultural GHG emissions, it is necessary to improve our quantitative understanding of the long-term influence of specific agricultural management practices on soil microbial contributions to SOC.

This work reports on the effects of various long-term cropping systems on fungal, bacterial, and microbial (fungal + bacterial) necromass C after more than four decades of stable treatments at the University of Alberta’s Breton Plots in central Alberta, Canada. Here, the effects of fallow, fertilizer and manure amendments, together with varying temporal intervals of perennial grass-legume forage inclusion, on soil necromass C were directly quantified. Further, the associations between soil necromass C pools and other soil C and N pools were examined. Necromass C responses to management were evaluated by 1) quantifying the specific effect of fallowing on BNC, FNC, and MNC, 2) examining the differential impacts of fertilizer and manure amendments on soil necromass C contents and ratios relative to a no-amendment control, and 3) analyzing how different intervals of perennial forage inclusion (none, vs. 2/5 year rotation, vs. 3/8 year rotation, vs. continuously for 40 years) influenced necromass C dynamics.

Relationships between different soil C pools were examined via pairwise comparison of necromass C pools, soil physical fraction C and N pools, and fraction ^{13}C:^{12}C ratios (i.e., $\delta^{13}C$). Additionally, the overall contribution of MNC to SOC storage was examined by evaluating the ratio of necromass C pool size to total SOC. Finally, bacterial, fungal, and total microbial (fungal+bacterial) necromass were tested as predictors of total SOC. An improved understanding of soil necromass C dynamics will help clarify how different agricultural management strategies can be used to increase necromass C accumulation generally, as well as elucidate the specific contribution of FNC or BNC pools to agricultural C storage.

3.3. Materials and Methods

3.3.1. Study Site Description

Samples were gathered from LTAEs at the University of Alberta’s Breton Plots, located outside the town of Breton (53°09’N, 114°44’W) in west central Alberta, Canada. The site was originally a boreal forest ecosystem but was converted to agricultural production in 1919-1920, with the first experimental plots established in 1930 (Dyck et al., 2012). The Breton Plots are located in the grey-wooded soil zone of Alberta, and the soils present at the site are classified as either Luvisols, Orthic Dark Gray Luvisols, or Typic Cryoboralfs, in accordance with the World Reference Base for Soil Resources, the Canadian System of Soil Classification, or the United States Department of Agriculture, respectively (Izaurralde et al., 2001; Soil Classification Working Group, 1998). All treatments examined are summarized in Table S1 and discussed in detail below.

In 1930, the original experiment at the Breton Plots was established and has remained in continuous operation up to the present. This experiment, titled the ‘Classical Plots’, was initially composed of a continuous wheat (*Triticum aestivum* L.) system and a four-year rotation consisting of three years of cereal and one year of legumes (Dyck et al., 2012). However, in 1938, the four-year rotation was modified into a five-year (5Y) rotation that maintained three years of cereals—namely, wheat, oats (*Avena sativa* L.), and barley (*Hordeum vulgare* L.)—while the single-year legume phase was converted into a two-year perennial forage hay phase consisting of alfalfa (*Medicago sativa* L.) and downy brome (*Bromus tectorum* L.) (Dyck et al., 2012). Similarly, in 1941, the continuous wheat system was converted to an alternating wheat-fallow (WF) rotation (Dyck et al., 2012). Both the WF and 5Y systems test various soil amendments, including: 1) no amendment (Check), 2) amendment with conventional fertilizer (NPKS), and 3) amendment with manure (Manure). In 1980, the applied amendment rates were increased to more closely reflect contemporary intensive farming practices (Dyck et al., 2012). Additionally, beginning in 2000, straw was no longer removed during harvest and was instead retained in the plots (Dyck et al., 2012). No further changes to plot management have been made up to this point. The exact amendment rates and crop rotation patterns for the Classical Plots are described in Tables S2 and S3, respectively.

In 1980, another experiment, titled the ‘Hendrigan Plots’, was established at the Breton site on previously cropped land (Dyck et al., 2012). The Hendrigan Plots consist of three cropping systems, namely a continuous perennial forage (CF) system, a complex eight-year (8Y) rotation, and a continuous grain (CG) system. Within the CF system, a polyculture of white Dutch clover (*Trifolium repens* L.), creeping red fescue (*Festuca rubra* L.), and tall fescue (*Lolium arundinaceum* (schreb.) Darbysh.) are grown, and small amounts of conventional (NPKS) fertilizer (see Table S2 for rates) are applied annually (Dyck et al., 2012). This system is meant to reflect a livestock-exclusive agricultural system that is strictly mechanically harvested, with the only external source of N coming from fertilizer amendment (Dyck et al., 2012). The crop rotation patterns for the 8Y rotation have varied slightly since 1980 (see Table S1). However, the 8Y rotation has historically included annual grains, faba beans as a green manure crop (harvested for seed in recent years), and ended with three consecutive years of perennial forage hay (Dyck et al., 2012; Ross et al., 2008). Unlike the CF system, the 8Y system loosely mimics a livestock-inclusive, rotational grazing system in the sense that N is recycled via animal waste (manure) at the start of each rotation cycle (Dyck et al., 2012). To achieve this goal, manure is applied to replace 70% of the N removed with the above-ground plant matter during the harvest of the perennial forage crop (Dyck et al., 2012). Finally, in the CG system, barley is grown every year, and all N is imported via conventional fertilizer amendment (Dyck et al., 2012). All soil amendment rates for the Hendrigan Plots can be found in Table S2.

In addition to the differences discussed above, each cropping system undergoes unique amounts of tillage. Both systems in the Classical Plots, as well as the CG and 8Y systems, are tilled once in the spring and fall, except for when perennials are present in the 5Y (years 4-5) and 8Y (years 6-8) cropping systems (Grant et al., 2001; Ross et al., 2008). In contrast, the CF system is never tilled but occasionally undergoes stand rejuvenation via broadcast seeding of white Dutch clover seed at a rate of 1 kg ha^{-1} (Ross et al., 2008). Additionally, when present in any system, forage crops are harvested twice per season (Dyck et al., 2012; Ross et al., 2008).

3.3.2. Soil Sampling

Soil cores were extracted between October 12 and 15, 2021, from both the Classical and Hendrigan Plots, 41 years after the start of the Hendrigan plots, using a truck-mounted hydraulic Gidding soil coring unit equipped with a 120 cm long by 6 cm diameter sampling tube. Each plot was sampled in three random locations by submersing the sampling tube to 90 cm below the mineral soil surface and removing the extracted soil core. For each plot, the 0-7.5 cm layer was collected from two of these cores and bulked, while the 0-7.5 cm layer for the third core was gathered and bagged separately for bulk density (BD) analysis. At random locations in each plot, an additional two cores were taken down to 30 cm, and the 0-7.5 cm layer was separated and added to the bulked soil for that plot.

3.3.3. Laboratory Analyses

3.3.3.1. Soil Processing

All samples were air-dried and sieved to less than 2 mm. Samples used for elemental [e.g., total C (TC) and total N (TN) quantification] and necromass analyses were finely ground for 2 minutes using a Retsch MM 400 Mixer Mill (Retsch GmbH, Haan, Germany) at 30 Hz to pulverize and homogenize the soil. Soil samples were also analyzed for BD and pH. Samples retained for BD were oven-dried at 65°C for at least 24 hours, and the oven-dried weight was divided by the initial bulk sample volume to determine BD:

$$BD\ \left(\frac{g\ soil}{cm^3\ soil}\right) = \frac{oven\ dried\ soil\ weight\ (g)}{bulk\ soil\ volume\ (cm^3)}.$$

Soil pH was measured in a 1:2 soil-to-water ratio as described in Miller et al. (2013). Samples were allowed to settle for 30 minutes before being measured for pH using an Fisherbrand accumet XL200 pH and conductivity meter (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA).

3.3.3.2. Soil Fractionation

Air-dried and sieved (< 2 mm) samples were separated through a combination of density and size fractionation into the following three components: light fraction OM (LFOM; density < 1.7 g cm^{-3}), coarse mineral-associated OM (CMAOM; density > 1.7 g cm^{-3}; diameter > 53 μm), and fine mineral-associated OM (FMAOM; density > 1.7 g cm^{-3}; diameter < 53 μm) following the protocol described by Samson et al. (2020). For each sample, 15 g of soil was placed into a 50 mL centrifuge vial with ten glass beads. Vials were loaded onto a reciprocating shaker for 30 min to disperse soil aggregates. Following this, 40 mL of sodium iodide (NaI) was added, and vials were shaken vigorously to suspend the soil before being centrifuged at 4220 RCF for 120 min. The supernatant solution was then decanted into a vacuum filtration apparatus by pouring through a 0.45 μm porosity polyvinylidene fluoride filter membrane to collect the LFOM. Residual NaI was removed from the LFOM using the vacuum filtration apparatus and rinsed with ~400 mL of ultrapure water. The LFOM was left to air dry for at least 24 hr, then carefully removed from the membrane using a razor blade, placed in a tin, and set aside for oven drying.

To remove residual NaI from the remaining mineral-associated OM (MAOM) fractions, the centrifuge vials were refilled with ~40 mL of ultrapure water. Next, vials were placed back on the reciprocating shaker for 60 min, removed, vortexed and stirred to re-suspend the remaining MAOM fractions. After re-suspension, vials were centrifuged for 60 min at 4220 RCF, and the supernatant decanted. At least two additional rinses were completed until the solution regained a clear hue. After the final rinse, vials were filled to the 50 mL line with ultrapure water and redispersed using the shaker and stirring as above. The soil solution was passed through a 53 μm sieve to separate the FMAOM from the CMAOM. All three collected fractions were placed into an oven to dry at 55 °C for at least 24 hr. Lastly, all three dry fractions were weighed and packaged for elemental analysis.

3.3.3.3. TC, TN, Organic C, and δ^{13}C Measurement

Soil samples (whole soil and all three fractions) from the top 7.5 cm of mineral soil underwent flash combustion analysis (AOAC International, 2000) to determine their TC and TN concentrations. SOC concentration was also determined for the whole soil samples following carbonate removal with hydrochloric acid (HCl). These analyses were completed by the Natural Resources Analytic Laboratory in the University of Alberta's Department of Renewable Resources using a Thermo FLASH HT Plus 2000 Organic Elemental Analyzer (Thermo Fisher Scientific Inc., Milan, Italy). The C content (LFOM-C, CMAOM-C, FMAOM-C) and N content (LFOM-N, CMAOM-N, FMAOM-N) contained in each of the three soil fractions on a gram of soil basis were calculated from the C and

N concentrations multiplied by the ratio of the respective fraction weight to total soil weight. δ^{13}C was measured simultaneously by chromatographically separating C dioxide (CO_2) and N gas (N_2) and analyzing them with a ConFlo IV continuous flow isotope ratio mass spectrometer (Thermo Fischer Scientific Inc., Bremen, Germany) calibrated against the Vienna Peedee Belemnite (VPDB) international reference scale.

3.3.3.4. Amino Sugar Analysis and Conversion to Necromass C and N

Whole soil samples were analyzed for their contents of glucosamine, galactosamine, muramic acid, and mannosamine using GC detection of derivatized samples, following the method described by Liang et al. (2012). In brief, samples underwent a four-stage laboratory process involving acid extraction with 6M HCl, sample purification following the addition of myo-inositol as an internal standard, aldononitrile acetate derivatization, and quantification using a GC flame ionization detector equipped with a 30 m long, 0.25 mm internal diameter, 0.25 μm film thickness, stationary phase (5%-phenyl)-methyl-polysiloxane nonpolar column with hydrogen as the carrier gas, at 0.5 mL min^{-1} constant flow. Soil glucosamine and muramic acid contents (mg g soil^{-1}) were then used to determine fungal, bacterial, and total microbial (fungal+bacterial) necromass C and N, as described by Liang et al., (2019). In brief, the bacterial glucosamine content was calculated by multiplying the soil muramic acid content by 1.43, and the fungal glucosamine content was calculated as the total muramic acid content minus the bacterial muramic acid content. The BNC and FNC contents were then estimated by multiplying the soil muramic acid content by 44.66 and the fungal muramic acid content by 9.39, respectively (Liang et al., 2019).

3.3.4. Statistical Analysis

Statistical analyses were completed in R and RStudio versions 4.3.1 (Posit team, 2023; R Core Team, 2023). Levene and Shapiro-Wilk tests were run on all soil response data to check for homoscedasticity and normality, respectively. Datasets found to be homoscedastic and normal were fit to generalized linear models (GLMs). In contrast, datasets that did not display these characteristics (e.g., muramic acid content, necromass fungi:bacteria ratio) were fit using generalized linear mixed-effects models (GLMMs). The combination of amendments applied and contrasting cropping systems were used to define treatments as fixed effects (as described in Table S1). Plot replicates were defined as random effects.

Predefined linear contrasts were then applied to the GLMs and GLMMs, with p-value significance of the resulting comparisons adjusted using a Tukey correction to account for the total number of comparisons made; degrees of freedom were estimated using the Kenward-Roger method for small sample size. Linear contrasts were applied to test a set of specific hypotheses about treatment effects on the necromass C variables (bacterial, fungal, microbial, fungi:bacteria), including the following: 1) the effect of fallow was tested by contrasting the WF-NPKS and CG treatments; 2) amendment effects were assessed by separately contrasting the three primary amendment treatments (Check, NPKS, Manure) within each of the WF and 5Y systems; and 3) the impact of various intervals of consecutive forage growth (none, two years, three years, continuous) was tested by contrasting the CG, 5Y-NPKS, 5Y-Manure, 8Y, and CF treatments. Additionally, associations between several soil variables were tested in two ways. First, linear regression models (LMs) were applied to assess the pairwise relationship between SOC and each of bacterial, fungal, and total MNC. The Pearson r^2 was used as a goodness-of-fit measurement for these three associations. Second, a pairwise correlation matrix for all soil and soil fraction C and N pools was determined. A non-parametric Spearman's rank correlation coefficient was used to describe the degree of correlation between each variable pair.

The emmeans package was used to complete linear contrast analyses (Lenth, 2023). The ggplot2, ggcorrplot, and ggpubr packages were used for data visualization (Kassambara, 2023b, 2023a; Wickham, 2016). All LM, GLM, and GLMM model fits were generated using the lme4 package (Bates et al., 2015).

3.4. Results

3.4.1. Soil Physicochemical Properties

Significant treatment effects were found in all observed soil physicochemical properties of the top 7.5 cm of mineral soil (Table 3.1). Cropping system SOC content followed the trend CF > 8Y > 5Y > CG > WF, with SOC responding to amendment treatments within the WF and 5Y systems following the trend: Manure > NPKS > Check (unamended). Individual C and N contents derived from each measured soil fraction, and the C:N ratio for the whole soil and each fraction, followed the same rank pattern among the five treatments, except for FMAOM-C, TN, and FMAOM-N, each of which was greatest within the 8Y treatment. BD followed a reverse trend, with the WF treatments having the highest BDs, and the CF treatment having the lowest BD. Soil pH values were generally lowest in the fertilized treatments (WF- and 5Y-NPKS, CG, CF). Finally, δ^{13}C values were notably more positive within the WF-Check and WF-NPKS treatments, but otherwise varied in a more complex way depending on management factors and vegetation cover types within each treatment.

3.4.2. Necromass C Response to Fallow

Microbial necromass C content in the top 7.5 cm of soil was significantly different (z = 2.713, P = 0.0067) between the WF-NPKS and CG treatments, with the latter having 64% more MNC than the former (Fig. 3.1A). Similarly, FNC also differed significantly (z = 2.948, P = 0.0032) between the WF-NPKS and CG treatments, with the CG treatment having 86% more FNC. In contrast, BNC did not differ between the WF-NPKS and CG treatments (P = 0.8844), with the CG treatment having only 1% more BNC on average. Not surprisingly, the necromass fungi:bacteria ratio differed significantly (z = 3.296, P = 0.0010) between the WF-NPKS and CG treatments, with the ratio 86% higher for the CG treatment as compared to the WF-NPKS treatment (Fig. 3.1B).

3.4.3. Necromass C Response to Soil Amendments

Microbial, bacterial, and FNC responded positively to the addition of soil amendments within both the WF and 5Y cropping systems (Figs. 3A and 3B). However, amendment responses varied notably between the two systems. Specifically, each necromass pool in the WF system responded more strongly to individual amendments than the response of the necromass C pools in the 5Y system. In contrast, the necromass fungi:bacteria ratio did not respond significantly to amendments in either cropping system (Figs. 3C and 3D).

For the WF system, the bacterial, fungal, and total MNC contents were generally significantly higher for the WF-NPKS treatment than the WF-Check treatment, and were also higher for the Manure treatment than the NPKS treatment. More specifically, MNC was 87% higher ($z = 2.976$, $P = 0.0029$) for the WF-NPKS treatment than for the WF-Check, 247% higher ($z = 4.660$, $P < 0.0001$) for the WF-Manure compared to the WF-Check, and 85% higher ($z = 2.865$, $P = 0.0042$) for the WF-Manure compared to the WF-NPKS. BNC varied similarly, with significant differences between all three WF treatments. Relative to the WF-Check treatment, BNC content was 96% ($z = 3.968$, $P < 0.0001$) and 315% ($z = 5.419$, $P < 0.0001$) higher for the WF-NPKS and WF-Manure, respectively, and was 112% higher ($z = 3.839$, $P = 0.0001$) for the WF-Manure compared to the WF-NPKS. FNC also differed significantly between all WF treatments. Specifically, FNC was 84% higher ($z = 2.561$, $P = 0.0104$) for the WF-NPKS compared to the WF-Check, 224% higher ($z = 4.050$, $P = 0.0001$) for the WF-Manure than the WF-Check, and 76% higher ($z = 2.365$, $P = 0.0180$) for the WF-Manure than for the WF-NPKS. Finally, while the necromass fungi:bacteria ratio did not respond significantly to soil amendments in the WF system, this ratio trended lower within the NPKS and Manure amended treatments relative to the WF-Check treatment.

Within the 5Y cropping system, total MNC content again responded positively to soil amendments (relative to the 5Y-Check treatment) with conventional fertilizer and manure. However, no significant difference in MNC was present between the 5Y-Manure and 5Y-NPKS treatments ($z = 1.309$, $P = 0.1904$). For the 5Y system, MNC content was 23% ($z = 1.700$, $P = 0.0892$) and 45% ($z = 2.895$, $P = 0.0038$) greater for the 5Y-NPKS and 5Y-Manure treatments compared to the 5Y-Check treatment, respectively. Similarly, for the 5Y system, BNC content increased in response to soil amendment with fertilizer and manure. BNC was 41% (z = 3.419, P = 0.0006) and 55% (z = 4.120, P < 0.0001) higher for the 5Y-NPKS and 5Y-Manure treatments relative to the 5Y-Check, respectively. In contrast, FNC content only responded positively to manure amendment, with FNC 19% higher (z = 2.449, P = 0.0143) for the 5Y-Manure treatment compared to the 5Y-Check treatment. No significant differences or trends were present for the necromass fungi:bacteria ratio within the 5Y system.

3.4.4. Necromass C Response to Perennial Forages

Bacterial necromass C, FNC, and total MNC in the top 7.5 cm of mineral soil differed significantly among the amended and non-fallowed treatments containing variable perennial forage inclusion, with an increasing trend as the length of perennial forage inclusion increased (Fig. 3.3A). The necromass fungi:bacteria ratio also differed significantly within this family of treatments (Fig. 3.3B).

Microbial necromass C content was lowest for the CG treatment, and in comparison, was 17% (z = 0.880, P = 0.3786), 37% (z = 2.003, P = 0.0452), 61% (z = 3.440, P = 0.0006), and 86% (z = 3.638, P = 0.0003) greater for the 5Y-NPKS, 5Y-Manure, 8Y, and CF treatments, respectively, although the difference between the CG and 5Y-NPKS treatments was not significant. In addition, the 8Y and CF treatment had significantly greater MNC than the 5Y-NPKS treatment, with MNC content 38% (z = 2.888, P = 0.0039) and 60% (z = 3.199, P = 0.0014) greater for the 8Y and CF treatments, respectively. Finally, MNC content in the CF treatment was 36% greater (z = 2.251, P = 0.0244) than in the 5Y-Manure treatment but was not different from that of the 8Y treatment.

Bacterial necromass C followed similar trends, with all treatments having greater BNC than the CG treatment. Compared to the CG treatment, BNC content was 60% (z = 4.041, P = 0.0001), 75% (z = 4.648, P < 0.0001), 133% (z = 6.871, P < 0.0001), and 103% (z = 5.040, P < 0.0001) greater within the 5Y-NPKS, 5Y-Manure, 8Y, and CF treatments, respectively. Additionally, the 8Y and CF treatments had greater BNC contents than the 5Y-NPKS treatment, with BNC content being 46% (*z* = 3.776, *P* = 0.0002) and 27% (*z* = 2.168, *P* = 0.0302) greater in the 8Y and CF treatments, respectively. Finally, BNC content was 33% (*z* = 2.860, *P* = 0.0042) greater in the 8Y treatment compared to the 5Y-Manure treatment, while the former was not significantly different from that in the CF treatment.

Fungal necromass C content also varied similarly to the above, although fewer differences were present. Fungal necromass C was greater in the 8Y and CF treatments compared to the CG, 5Y-NPKS, and 5Y-Manure treatments. Relative to the CG treatment, FNC content for the 8Y and CF treatments was 47% (*z* = 2.413, *P* = 0.0158) and 83% (*z* = 3.125, *P* = 0.0018) greater, respectively.

Finally, the necromass fungi:bacteria ratio varied significantly among treatments having different levels of perennial forage inclusion, with the 5Y-NPKS and 8Y treatments having a lower ratio than the CG and CF treatments. The fungi:bacteria ratio also differed between the 5Y-Manure and CG treatments. Relative to the CG treatment, the necromass fungi:bacteria ratio was 33% (*z* = -2.721, *P* = 0.0065), 21% (*z* = -1.910, *P* = 0.0561), and 37% (*z* = -2.894, *P* = 0.0038) lower within the 5Y-NPKS, 5Y-Manure, and 8Y treatments, respectively. Similarly, the necromass fungi:bacteria ratio for the 5Y-NPKS and 8Y treatments was 27% (*z* = -2.721, *P* = 0.0065) and 31% (*z* = -2.894, *P* = 0.0038) lower than for the CG treatment, respectively. Lastly, compared to the 5Y-Manure treatment, the necromass fungi:bacteria ratio for the 8Y treatment was 20% lower (*z* = -1.718, *P* = 0.0858).

3.4.5. Necromass C Contribution to Total SOC

The proportion of SOC composed of FNC and total MNC did not differ significantly between treatments (Fig. 3.4). In contrast, the proportion of BNC responded significantly ($\chi^2 = 46.7$, P < 0.0001) to treatment. Notably, the WF-Manure treatment had the highest mean proportion of BNC among all treatments at 15 ± 2%, though it did not differ from the other WF treatments, nor the 5Y-Manure and 8Y treatments. In contrast, the CG and CF treatments had the lowest proportion of BNC at 7.9 ± 0.3% and 7.7 ± 0.5%, respectively, but did not differ significantly from any other treatments except for the WF-Manure and WF-NPKS. For five out of nine treatments, mean MNC exceeded 50% of total SOC, with an average contribution of necromass C between all treatments of 49 ± 2% and a range of 39.6-57.5%.

3.4.6. Relationship of Necromass C to Other Soil C and N Pools

Bacterial necromass C, FNC, and total MNC were all found to correlate significantly ($P < 0.05$) with various other measured soil properties (Fig. 3.5). More specifically, all three necromass C pools were better correlated ($P < 0.05$) with FMAOM-C (the amount of FMAOM fraction C per gram of soil) than CMAOM-C. CMAOM-C, in turn, correlated better with necromass C than LFOM-C. Furthermore, BNC and total MNC were both associated more closely with FMAOM-C than total SOC. The same trend can also be seen between necromass C content and LFOM-N, CMAOM-N, and FMAOM-N. However, for all but BNC, TN is more closely associated with necromass C than FMAOM-N. A similar trend in correlations between necromass C content (for all necromass pools) and $\delta^{13}C$ values for each soil fraction can be seen. More specifically, the strongest associations were between the C contents for each necromass pool and $\delta^{13}C$ values for the FMAOM fraction, with the aforementioned associations being stronger than those with total soil $\delta^{13}C$. The BNC pool was an exception to this trend and was most strongly associated with LFOM fraction $\delta^{13}C$. In contrast, the C contents of the various necromass pools correlated more strongly with the C:N values of the LFOM and CMAOM fractions than with the C:N values for the FMAOM fraction; the lone exception to this pattern was for BNC content, which correlated best with LFOM followed by the FMAOM C:N values.

As shown in Fig. 3.6, each necromass C pool was related to total SOC to a different extent. Bacterial necromass C content had the weakest relationship with total SOC ($r^2 = 0.448$; Fig. 3.6A), followed by FNC ($r^2 = 0.635$; Fig. 3.6B), and then total microbial necromass ($r^2 = 0.654$; Fig. 3.6C). Additionally, Fig. 3.6 demonstrates how the different treatments cluster and exhibit characteristic amounts of variation among the replicates of each treatment. For all three necromass pools, the non-manured WF (i.e., WF-Check, WF-NPKS) treatments create a cluster with low SOC and low necromass C compared to the WF-Manure treatment, the latter of which was considerably higher in both measures. This manure-induced increase in necromass C was particularly pronounced for the BNC pool. Additionally, the 8Y treatment showed greater degrees of variation in total SOC than in necromass C and displayed the greatest overall variation in total SOC across replicates of all treatments. On the other hand, the CF treatment tended to be high in both necromass C and SOC while displaying relatively low variation in either of these metrics.

3.5. Discussion

3.5.1. Soil Physicochemical Properties

3.5.1.1. Soil C, N, BD, and pH

Much research has indicated that practicing bare fallow can contribute to reductions in SOM and, in turn, soil C and N (Baumhardt et al., 2015; Li et al., 2014; Nielsen & Calderón, 2011; Plaza-Bonilla et al., 2015; Ramesh et al., 2019; Rodgers et al., 2021; Tiefenbacher et al., 2021). Bare fallow is generally recognized for decreasing soil C and N through mechanisms such as increased erosion and reduced plant biomass inputs to the soil (Baumhardt et al., 2015; Nielsen & Calderón, 2011; Plaza-Bonilla et al., 2015; Ramesh et al., 2019; Tiefenbacher et al., 2021), and disrupting microbial community function (Hirsch et al., 2017; Nielsen & Calderón, 2011; Rodgers et al., 2021). These observations align well with the present work, as the fallowed treatments generally had the lowest topsoil SOC and TN contents. Bare fallow systems have also been shown to promote high BD (Aase & Pikul Jr., 1995; Feng et al., 2011; Mandal et al., 2003), which is mirrored by the results of this study. As such, alternating fallow at the Breton Plots appears to have negatively impacted soil C storage, fertility, and soil health (through increased BD). Despite the general tendency for the fallowed treatments to be relatively low in C and N content and high in BD (Table 3.1), these deleterious impacts were effectively mitigated by manure amendment. This mitigation is probably due to the manure directly bolstering nutrient levels and maintaining overall SOM content at sufficient levels to limit erosion and promote soil function and crop productivity. Given that SOM is critical to soil aggregation, water-holding capacity, nutrient availability and other important soil functions (Johnston et al., 2009), it is possible that SOM was a limiting resource in the WF system and that intermittent manuring overcame this limitation, at least based on the measured levels of SOC.

In contrast, conventional fertilizer and manure amendments are both well-documented to increase SOM—as well as overall soil C and N—levels in agricultural systems (Bai et al., 2018; Beillouin et al., 2023; Crews & Rumsey, 2017; Hou et al., 2020; Johnston et al., 2009; Plaza-Bonilla et al., 2015; Ramesh et al., 2019; Tiefenbacher et al., 2021). Soil C generally increases through increased crop biomass production, which in turn, is stimulated by fertilizer or manure amendment, while manure amendment can also increase soil C through the direct addition of C within manure (Bai et al., 2018; Beillouin et al., 2023; Crews & Rumsey, 2017; Hou et al., 2020; Johnston et al., 2009; Plaza-Bonilla et al., 2015; Ramesh et al., 2019; Tiefenbacher et al., 2021). Soil N increases with fertilization and manure amendment primarily due to the direct addition of N with these amendments (Johnston et al., 2009). Soil amendments can also substantially impact soil pH (Tian & Niu, 2015; S. Zhang et al., 2023). Conventional fertilizers often lower pH over time due to proton generation during nitrification of added ammonia and the removal of base cations (Chadwick & Chorover, 2001; Guo et al., 2010). On the other hand, manure amendment often increases soil buffering capacity and pH via the addition of OM and base cations, neutralizing acidic species and constraining overall soil pH toward neutral values (Liu et al., 2020; Ning et al., 2020). These dynamics are reflected here, with the various fertilized treatments (WF-NPKS, CG, 5Y-NPKS, CF) consistently having the lowest pH values and the unamended (WF-Check, 5Y-Check) or manured (WF-Manure, 5Y-Manure, 8Y) treatments having generally higher pH values (Table 3.1). While both amendments improved soil C storage and fertility at the Breton Plots, conventional fertilizer negatively impacted soil health by causing soil acidification, whereas manure amendment avoided this pitfall.

Increasing perennial inclusion in cropping systems has generally been shown to increase SOM, C, and N levels through mechanisms such as increasing root biomass inputs (Beillouin et al., 2023; Bolinder et al., 2002; Glover et al., 2010; King & Blesh, 2018; Ledo et al., 2020; Mosier et al., 2021; Tiefenbacher et al., 2021) and reducing erosion (Durán Zuazo & Rodríguez-Pleguezuelo, 2008; Pimentel et al., 1987). Related to this, no-till practices have also been shown to improve SOC and soil N levels, increase topsoil C:N ratios, and decrease BD (Bai et al., 2018; Engell et al., 2022; You et al., 2017). Legume inclusion benefits soil by increasing soil N levels through biological N fixation and the provision of high-quality litter (Jensen et al., 2012; Stagnari et al., 2017). Legume inclusion has also been shown to have mixed but generally positive effects on SOC accumulation (Jensen et al., 2012; King & Blesh, 2018; Plaza-Bonilla et al., 2015; Ramesh et al., 2019; Stagnari et al., 2017; Tiefenbacher et al., 2021). Similarly, green manuring has been shown to improve soil C and N content through increased soil cover and the direct addition of large quantities of biomass to the soil (Stagnari et al., 2017; Tiefenbacher et al., 2021). The results of this study agree with such observations, as soil C and N content showed a distinct increasing trend with increasing perennial grass-legume forage inclusion, with the fully perennial (CF) and green-manure-inclusive (8Y) systems having the highest overall C and N contents and low BDs (Table 3.1). In summary, perennial grass-legume forage inclusion increased soil C storage and fertility, and benefitted soil health by decreasing BD.

Notably, the 8Y and CF treatments had the highest SOC and TN contents, likely due to their high degrees of perennial forage and legume inclusion, as well as the green manuring of faba beans within the 8Y rotation. However, it is unclear whether there was a greater impact on soil C and N at the Breton Plots due to the incorporation of longer consecutive periods of perennial forage growth, as opposed to having perennials make up a larger proportion of the total crops grown in a rotation. To elaborate, the 5Y system spends two years (40%) of its rotation cycle under perennial forage growth, whereas the 8Y treatment spends three years (37.5%) of its rotation under perennial forage growth. Despite the 8Y rotation spending a lower proportion of its time under perennial forages, the latter had greater soil C and N than any of the 5Y treatments (including the 5Y-Manure), which may indicate that the length of consecutive perennial growth in these cropping systems was more impactful than the proportion of time the system spent under perennial crops. However, differences between the 5Y system and the 8Y treatment could also be due to other factors, including green manuring in the 8Y treatment, which involved the incorporation of considerable OM into the soil. In any case, the importance of the consecutive length of perennial cultivation has previously been demonstrated by research showing that perennial crops can continue to accrue increasing root biomass for many years in the absence of disruption to the perennial stand (Bolinder et al., 2012; Houde et al., 2020; Sakiroglu et al., 2020; F. Zhang et al., 2023), which could ultimately increase SOC accrual.

3.5.1.2. Soil C:N Ratios

Soil C:N ratios can be altered by soil amendments, microbial activity, and tillage. Manure amendment often increases soil C:N values, as manure tends to have relatively high C:N ratios (~20:1) compared to soil (~14:1) (Cleveland & Liptzin, 2007). In contrast, conventional fertilizers tend to lower soil C:N ratios due to the direct addition of large amounts of N (Congreves et al., 2017; Li et al., 2022). Soil microbial biomass C:N ratios generally range between 4 and 14, most commonly approaching 8 (Cleveland & Liptzin, 2007; Griffiths et al., 2012; Horwath, 2007; Manral et al., 2023; Moore et al., 2000). In contrast, crop biomass C:N ratios range widely between crop types and tissues (e.g., from 9 to 132), but are typically between 11 (i.e., legumes) and 50 (Curtin et al., 1998; Finney et al., 2016; Gan et al., 2011; Jílková et al., 2020). This disparity in C:N ratios means that as plant inputs and overall SOM become more progressively processed by microbes, soil C:N values tend to decrease, approaching those of microbial biomass. On the other hand, reduced and no-tillage systems have been shown to increase soil C:N ratios (Wulanningtyas et al., 2021; You et al., 2017).

These effects on C:N ratios can be seen in the topsoil of the treatments observed here (Table 3.1). Similar to its low δ^{13}C values, soils of the WF system generally had low C:N values, likely due to the system's SOM pool being proportionally more microbially processed due to the reduction of fresh OM inputs into the C-limited system.

Further, soils in the WF system are relatively enriched in bacterial necromass, and bacteria have lower tissue C:N ratios than fungi (Wang & Kuzyakov, 2024), which may skew the bulk soil C:N ratio to a lower value relative to the other cropping systems. Overall, the low bulk soil C:N ratios in the WF system reinforce the idea that this system is highly degraded in SOC, signalling its low C storage potential. Finally, C:N ratio responses to amendments were less apparent, though the WF system also had a lower C:N ratio due to fertilization and an increase due to manure amendment. However, this was notably not mirrored in the other cropping systems. The positive impact of forgoing tillage can be seen in the CF treatment, which has the highest C:N ratio, likely due to a build-up of relatively high C:N ratio plant litter. This high C:N ratio provides further evidence of this cropping system's high soil C storage potential, indicating that tillage reduction can be an effective tool for promoting soil C storage.

3.5.1.3. Stable C Isotopes

Similar to the C:N ratios, soil ^{13}C:^{12}C ratios (i.e., $\delta^{13}C$) respond to a variety of factors, including manure amendment and microbial activity. Generally, manure amendment tends to lower (i.e., make more negative) soil $\delta^{13}C$ (Gerzabek et al., 1997; Senbayram et al., 2008; Treasure et al., 2016) due to the depletion of ^{13}C in animal manure relative to that in crops and soil (Bol et al., 2005). Compared to fertilization and no amendment, the addition of manure appears to have contributed to lower $\delta^{13}C$ values in the WF system (Table 3.1). Notably, this effect of manure was not observed in the 5Y system. In contrast, microbial decomposition of SOM preferentially releases ^{12}C as $^{12}CO_2$ since it is lighter and more energetically favourable for metabolic use, leading to the enrichment of ^{13}C and, in turn, increased (i.e., more positive) $\delta^{13}C$ values in the remaining substrate (Blaser & Conrad, 2016; Conrad, 2007; Werth & Kuzyakov, 2010). This microbe-driven enrichment in ^{13}C is likely the reason for the high $\delta^{13}C$ values observed in the non-manured treatments of the WF system (i.e., WF-Check, WF-NPKS), indicating a high degree of microbial SOM processing. Microbial processing stabilizes SOM by facilitating mineral associations and soil aggregation (Cotrufo et al., 2013), and fallowing is known to promote soil C degradation (Baumhardt et al., 2015; Li et al., 2014; Nielsen & Calderón, 2011; Plaza-Bonilla et al., 2015; Ramesh et al., 2019; Rodgers et al., 2021; Tiefenbacher et al., 2021). More specifically, fallow has been shown to greatly decrease LFOM content (Gregorich et al., 2006; Janzen et al., 1992). Given these observations, the high $\delta^{13}C$ values measured in the WF system likely indicate that the soil C pool remaining in this treatment has been enriched in relatively stable MAOM-C as the more labile LFOM-C is preferentially lost. Further, the mining of microbial necromass by living soil microbes increases in efficiency as nutrient limitations increase in the soil (Kästner et al., 2021). As such, the recycling of necromass C by soil microbes, which would further enrich SOM in ^{13}C during periods of fresh plant-OM limitations (i.e., fallowed years), likely contributes to the WF system's high $\delta^{13}C$ values. Ultimately, it appears that the use of fallow at the Breton Plots has resulted in the destabilization of soil C through the loss of the majority of labile C, and increased mining of the ostensibly more stable soil C pools.

3.5.1.4. Physical Soil Fractions

Soil physical fractions have unique relationships with soil amendments. For example, increasing conventional fertilizer rates tends to preferentially increase the total amount of crop residue stored in the soil as LFOM and CMAOM—collectively known as particulate OM (POM); these changes occur through mechanisms such as increasing crop residue inputs and modifying decomposition dynamics (Janzen et al., 1992; Kauer et al., 2021; Semenov et al., 2023; Silveira et al., 2013; Zou et al., 2023). Conventional fertilizer application has also been shown to increase the C and N contents within the POM and FMAOM fractions (Dou et al., 2016; Kauer et al., 2021; Yan et al., 2007). The results presented here generally agree with these observations, as the C and N contributions from the LFOM and FMAOM fractions increased with the occurrence of conventional fertilization within both the WF and 5Y systems (Table 3.1). Despite this, the LFOM fraction experienced greater proportional increases in C and N under fertilization. Given that fertilization increased C and N in both the most labile (LFOM) and most stable (FMAOM) soil fractions, its overall effect on SOM stability is unclear. However, the proportionally greater increase in LFOM-derived, compared to FMAOM-derived, C and N implies that SOM stability may be marginally decreased by conventional fertilizer.

In contrast, liquid dairy manure application has been shown to preferentially increase the total size and contribution of MAOM, but not LFOM, to the total soil C and N pools (Maillard et al., 2015; Samson et al., 2020). Solid organic amendments, such as straw and farmyard manure, are known to increase both POM- and FMAOM-derived soil C and N content (Kauer et al., 2021; Semenov et al., 2023; Yan et al., 2007). While Yan et al. (2007) found significant increases in POM-derived C and N in response to manure amendment, these treatment-induced increases were non-significant for the LFOM fraction in isolation, suggesting that manuring preferentially enriches C and N within the CMAOM fraction. These effects also seem to be reflected here, as there were increases in C and N derived from the CMAOM and FMAOM fraction following manure amendment in the WF system (Table 3.1). Importantly, the 5Y system also showed increased FMAOM-derived C and N, while LFOM-C did not respond in either system, and LFOM-N only increased with manuring in the WF system. In light of these relatively greater increases in MAOM compared to LFOM, it appears that manure amendment at the Breton Plots has increased SOM stability.

Individual soil physical fractions are differently associated with microbial communities. For instance, as summarized in a review by Lavallee et al. (2020), POM is primarily plant-derived but turns over rapidly and thus is an important source of nutrients for soil microbes. More specifically, the minerally uncomplexed (LFOM) component of POM is the most closely associated with fresh plant residues, having high proportions of aliphatics and carbohydrates (Gregorich et al., 2006). While the minerally complexed CMAOM component of POM appears to be highly similar to LFOM in terms of chemical makeup, it has been observed to be relatively rich in microbially derived compounds, suggesting it may largely represent LFOM that has undergone substantial microbial processing and mineral complexing (Li et al., 2013). It has also been posited that CMAOM acts as a precursor to FMAOM genesis (Robertson et al., 2019). Unlike CMAOM, FMAOM is primarily composed of microbially-derived compounds but is physically protected from turnover by close association with soil silt and clay particles, making it less available to soil microbial communities (Cotrufo et al., 2013; Lavallee et al., 2020; Samson et al., 2020).

Overall, the findings reported here corroborate these previous observations (Table 3.1). More specifically, the C:N ratios for each treatment were significantly lower in the CMAOM and FMAOM fractions compared to the LFOM fraction, implying microbial enrichment (Cleveland & Liptzin, 2007; Curtin et al., 1998; Finney et al., 2016; Griffiths et al., 2012; Horwath, 2007; Jílková et al., 2020; Kauer et al., 2021; Manral et al., 2023; Moore et al., 2000). However, the FMAOM fraction generally had a higher C:N ratio than the CMAOM fraction, which contraindicates its status as, ostensibly, the most highly microbially processed fraction. This peculiarity may be explained by the fractionation process itself. During the centrifugation phase of fractionation, the FMAOM fraction is the last to be removed from suspension and, as a result, is preferentially lost if 100% of the soil material is not recovered. Further, as shown in Table S4, N was preferentially lost compared to C during the fractionation process. These two factors likely resulted in artificially high C:N ratios in both the CMAOM and FMAOM fractions, particularly the latter. These two factors could also explain why the whole soil C:N ratio appears lower in some treatments (e.g., WF-Manure, 5Y-Manure) than would be expected by completing a weighted average of the individual fraction C:N ratios.

As expected from highly microbially processed OM (Blaser & Conrad, 2016; Conrad, 2007; Werth & Kuzyakov, 2010), the FMAOM fraction's δ^{13}C values were uniformly the highest of the three fractional pools for all treatments (Table 3.1). Once again, the δ^{13}C values for the whole soil diverge from what would be expected from taking a weighted average of the three fractional pools, with most treatments having whole soil δ^{13}C values that are less negative than any of the fractional pools. This outcome could be due to the loss of low molecular weight compounds, such as microbial amino acids, as dissolved organic C and N during the liquid suspension and NaI rinsing phases of the fractionation process. Such losses of low molecular weight compounds would be consistent with those demonstrated during the extraction of dissolved organic C and N in a radioisotope labelling study, which found that more than 90% of ^{14}C labelled amino acids and glucose were lost after 15 minutes of shaking in 20

°C distilled water or a 0.5 M potassium sulphate (K_2SO_4) solution (Rousk & Jones, 2010).

Plant communities and vegetation management affect soil fractions heterogeneously. In agricultural environments, systems that include greater occurrences of perennial vegetation have been shown to increase, whereas greater proportions of bare fallow have been found to decrease proportionally, total LFOM and POM content, together with their contributions to soil C and N (Gosling et al., 2013; Haynes, 2005; Janzen et al., 1992; Kim et al., 2022; van der Pol et al., 2022; Winck et al., 2014). These dynamics are largely driven by differences in total litter biomass inputs, as demonstrated by consistent increases in POM content as more litter is retained in litter-manipulation studies (Lavallee et al., 2020; Samson et al., 2020). The influence of such biomass input gradients can be seen here, as LFOM- and CMAOM-derived C and N were much lower in the WF-NPKS treatment than in the comparable CG treatment (Table 3.1), which differs primarily in its lack of an alternate-year fallow phase. Further, there appears to be a uniform increase in LFOM- and CMAOM-derived C and N as the perenniality of vegetation within treatments increased (e.g., for the fertilized treatments: CF > 5Y-NPKS > CG > WF-NPKS).

The influence of vegetation on soil MAOM accumulation and C and N content appears less clear. For example, Kim et al. (2022) and van der Pol et al. (2022) both examined the impacts of perennial grain crops compared to annual grain crops on MAOM-derived C, with the former finding significant increases in MAOM, and the latter finding no increase, under perennial grains. However, as summarized in a review article by Moukanni et al. (2022), various studies have provided observational and theoretical support for the idea that belowground biomass—due to generally higher lability, proximity to the soil microbial community, and closer association with the rhizosphere—is a more important driver of C accumulation in the MAOM fraction than aboveground biomass. This means that more highly perennialized systems. which generally produce greater root biomass inputs (Beillouin et al., 2023; Bolinder et al., 2002; Glover et al., 2010; King & Blesh, 2018; Ledo et al., 2020; Mosier et al., 2021; Tiefenbacher et al., 2021), should increase MAOM content compared to systems relying more heavily on annual crop inputs. This concept aligns well with the present work (Table 3.1), as FMAOM-derived C and N displayed an increasing trend with vegetation perenniality (e.g., CF > 5Y-NPKS > CG > WF-NPKS). As such, perennial crops have apparently increased SOM stability at the Breton Plots by increasing storage in the FMAOM fraction.

Finally, the quality of OM inputs also plays a critical role in determining if OM will be preferentially retained as POM or MAOM (Gan & Drinkwater, 2024; Lavallee et al., 2020; Moukanni et al., 2022; Zhang et al., 2022). As summarized by Lavallee et al. (2020), various litter-manipulation studies have found that high-quality litter (i.e., that with a low C:N ratio) results in preferential accumulation of MAOM, whereas low-quality litter (i.e., that with a high C:N ratio) results in preferential accumulation of POM. This concept is in alignment with findings that, compared to other crop residue types, leguminous residues tend to preferentially stimulate C and N accumulation in the MAOM fraction (Gan & Drinkwater, 2024; Moukanni et al., 2022; Zhang et al., 2022). The observations presented here support this concept, as the FMAOM-derived C and N were, on average, highest in the treatments that included leguminous perennial forages (Table 3.1). Furthermore, the greatest mean FMAOM-derived C and N contents were found in the complex 8Y system. This treatment had the lowest mean LFOM C:N ratio of all treatments, indicating that its litter inputs were of particularly high quality. In addition, the 8Y treatment historically included a legume green manure phase, resulting in large quantities of high-quality litter being moved belowground over time, where it would directly stimulate FMAOM formation. Overall, this suggests that legumes, particularly when used as green manure, have contributed to SOM stabilization at the Breton Plots by favouring FMAOM formation.

3.5.2. Fallow Promoted Necromass C Loss and Favoured Bacterial Necromass Accumulation

The fallow-induced reduction in soil MNC presented here aligns well with other studies of necromass responses to plant biomass inputs. Gilmullina et al. (2023) established contrasting levels of biomass inputs on previously homogenous soil systems, finding that after 13 years, continuous bare fallow had lower amino sugar content than ungrazed, mowed, and both low- or high-intensity grazed pasture. Further, this same study found that the amount of amino sugars in the soil was linearly related to the amount of plant biomass directly retained in the treatment (Gilmullina et al., 2023). The observed halving of MNC in the alternate-year fallow (WF) system compared to the continuous annual grain (CG) system (Fig. 3.1A) would align with such a relationship, as the alternate-year fallowing fundamentally cut in half the crop biomass inputs entering the system (even if crop yields remained comparable). Similarly, a study of a Mollisol soil by Li et al. (2015) found that after eight years of consistent management, a bare parent material treatment, as well as a treatment allowed to revegetate naturally, both had lower amino sugar content than an alfalfa treatment as well as fertilized annual cropping treatments with partial or complete residue retention. Given that alternate-year bare-fallow in the WF system results in the absence of crop biomass inputs half the time, it is unsurprising that this management strategy produced a dramatic reduction in necromass C. In turn, this reduced necromass C content contributes to the overall low C storage afforded by the WF system.

Li et al. (2015) also found that fungal amino sugar contents responded more significantly to variations in crop biomass inputs. This aligns well with the results of the present study, as FNC content was lower and BNC content was not significantly different between the WF-NPKS and CG treatments (Fig. 3.1A). Given that the WF-NPKS and CG treatments are broadly similar, containing only annual grains and being fertilized at the same rate in cropped years, the difference in crop biomass inputs is the most likely reason for these differences. Furthermore, soil bacteria are known to have lower C:N ratios, higher growth rates, and more efficient uptake of low-molecular-weight (LMW) organic compounds than soil fungi (Wang & Kuzyakov, 2024). These factors presumably gave bacteria a competitive edge over fungi within the WF system. Compared to fungi, bacteria would become C-limited less quickly (lower C:N ratio) and would also be able to more effectively capitalize on periodic OM pulses (higher growth rates), giving them an advantage in the WF system, which is characterized by periodic pulses of plant-OM followed by periods of C-limitation. Additionally, during fallow periods, LMW compounds would increase in proportion as the more complex plant-OM compounds from the previous year break down in the soil. Given the competitive advantage bacteria have over fungi in utilizing LMW compounds, this increase would further favour bacterial community dominance. This view is supported in the present work by the much lower necromass fungi:bacteria ratio found in the WF-NPKS treatment relative to the CG treatment (Fig. 3.1B).

As de Vries et al. (2006) summarized, higher biomass fungi:bacteria ratios are often considered more environmentally sustainable due to fungi-dominated soil communities increasing soil N retention and crop nutrient uptake, relative to bacteria-dominated communities. If this concept holds true, then the lower necromass fungi:bacteria ratio due to fallow seen here may also indicate an increase in N leaching potential and reduced crop nutrient uptake efficiency. In light of these drawbacks, this fallow-induced lowering of the fungi:bacteria ratio emphasizes the importance of avoiding fallow periods and ensuring fields are covered by vegetation each year. However, Xue et al. (2024) concluded that in the case of microbial metabolism limitations, fungal necromass is more likely than bacterial necromass to be released from mineral surfaces and become susceptible to degradation. As such, the high frequency of microbial nutrient limitation presumably induced by bare fallow in the WF system, would favour bacterial necromass accumulation, and therefore the necromass fungi:bacteria ratio observed here may not reflect that of the living biomass. Such a discrepancy would not be unprecedented. For example, Li et al. (2015) found that, while bacteria dominated the living biomass in a Mollisol soil, the necromass was dominated by fungal residues. Other studies have shown that fungal residues generally turn over more slowly (i.e., are more stable) in the soil than bacterial residues (Dippold et al., 2019; Gunina et al., 2017), which may indicate that when nutrient limitations are not extreme, a higher necromass fungi:bacteria ratio is indicative of a more stable necromass C pool. Given that fallow led to such dramatic reductions in the necromass fungi:bacteria ratio, this suggests that fallow may also be destabilizing SOM, providing additional motivation to avoid the practice in favour of continuous cropping.

3.5.3. Necromass C Responses to Amendments Were Modulated by Overall Cropping System

Necromass C responses to amendments were particularly pronounced in the WF system compared to the 5Y system (Fig. 3A-B). However, in both systems, manure led to larger necromass C increases than conventional fertilizer. This aligns with a meta-analysis by Zhou et al. (2023) that found conventional fertilization increased MNC by 12% on average, compared to an average increase of 21% due to manure amendment. Li et al. (2020) found similar results, with annual amendments of fertilizer and manure increasing amino sugar production by 19.7%, and 82.9% to 107%, respectively. However, the increases in MNC seen in the WF system due to fertilizer (87%) and manure (247%) were considerably greater in scale than in either of these prior studies. In contrast, the MNC increases seen in the 5Y system due to fertilizer (23%) and manure (45%) amendments are much closer in scale to those observed in the previous studies. This unusually extreme response of the WF system to soil amendments may be due to the exceptionally severe soil microbial nutrient limitations induced by fallowing in the system, as described below.

The discrepancies in MNC responses within the WF and 5Y systems (Fig. 3.2A-B) may relate to each system's scale and variety of resource limitations. In the WF-Check treatment, it appears there are considerable C and N limitations to microbial growth. Thus, while conventional fertilization significantly increases MNC by alleviating microbial N-limitation, the overall C-limitation remains as no C is imported with the fertilizer amendment. On the other hand, the manure application addresses both the C and N limitations within this agricultural soil, leading to a significantly greater necromass C increase than that observed from the conventional fertilizer amendment. In contrast, the 5Y system appears to have much milder deficits in C and N, which results in more modest improvements with either fertilizer or manure amendment. Because of this, a significant increase in necromass C is evident only when the beneficial effects of the C and N amendments are both present (i.e., via manure amendment). This is perhaps not surprising, as reducing microbial C-limitations allows soil microbes to maintain active growth for longer periods (Blagodatskaya et al., 2014), thus reducing the proportion of C lost to cellular maintenance (Sinsabaugh et al., 2013). These results suggest that manure amendment can considerably increase soil C storage compared to conventional fertilizer, particularly in degraded soils.

The observed responses of the individual necromass C pools within the WF and 5Y systems (Fig. 3.2A-B) allow for a more robust examination of the potential limiting factors addressed by the fertilizer and manure amendments. Given the relatively low C:N ratio of soil bacterial biomass compared to fungal biomass, bacterial communities would be more prone to N limitation, whereas fungal communities would be more prone to C-limitation (Wang & Kuzyakov, 2024). This concept is further supported by observations that high soil fertility (i.e., via high soil N content) and nutrient availability tend to preferentially benefit soil bacteria and decrease the necromass fungi:bacteria ratio (Zhou et al., 2023). In contrast, high C:N ratio amendments such as straw tend to favour fungal growth and increase the soil fungi:bacteria ratio (Zhou et al., 2023). As such, if soil N limitations are addressed by conventional fertilizer amendment alone, one would expect a greater response in bacterial versus FNC content. Indeed, we observed this in response to fertilization within the WF and 5Y systems. In response to fertilization, BNC increased (compared to the relevant Check treatments) by 96% and 42%, while FNC increased by 84% and 19%, in the WF and 5Y systems, respectively.

Alternatively, if there is a soil C limitation uniquely addressed by the manure amendment, FNC is expected to increase more significantly than BNC. By examining how the bacterial and fungal necromass pools changed in the manured treatments compared to the fertilized treatments, it may be possible to decouple the impacts of C and N limitations (assuming that the fertilizer treatment effectively removes N limitations). Compared to the relevant fertilized treatments, manure application increased BNC by 112% and 9%, and fungal necromass by 76% and 19%, within the WF and 5Y systems, respectively (Fig. 3.2C-D). Here, the 5Y system conforms to expectations, while the WF system shows a larger increase in BNC than FNC. However, the latter may be due to the preferential release and decay of FNC in the rather extreme, nutrient-limited environment of the WF system (Xue et al., 2024). Overall, the generally higher necromass fungi:bacteria ratio of the 5Y system compared to the WF system suggests that the 5Y system may have less N leaching and greater crop nutrient uptake potential, as well as higher necromass C stability (De Vries et al., 2006; Dippold et al., 2019; Gunina et al., 2017). These results demonstrate that perennial grass-legume forage inclusion, and continuous cropping more generally, benefitted soil fertility, cropping system sustainability, and soil C stability at the Breton Plots and, ultimately, reduced the need for soil amendments.

3.5.4. Perennial Forages and Organic Amendments Increased Necromass C Accumulation

Overall, soil necromass C content increased proportionately with the extent of perennial forages in rotation, legume inclusion, and organic amendment inputs (i.e., manuring and green manuring; Fig. 3.3A). This conclusion is supported by the observation that both the amended 5Y treatments, along with the 8Y and the CF treatments, had greater necromass C contents than the CG treatment. The CG, 5Y-NPKS, and CF treatments differ from one another primarily on their level of perennial forage inclusion and applied fertilizer rates—averaging 90 kg N ha^{-1} yr^{-1}, 35 kg N ha^{-1} yr^{-1}, and 16 kg N ha^{-1} yr^{-1}, respectively. Given that the CF system received the least fertilizer of these treatments but still had among the highest necromass C, this supports the notion that an increasing time under perennial forages best explains the observed hierarchy in MNC content (CF > 5Y-NPKS > CG). On the other hand, the 5Y-Manure and 8Y treatments distinctly differ from the three previously mentioned treatments in their use of organic amendments, particularly manure addition. Further, the 8Y treatment varies from the 5Y-Manure treatment in that it historically included a green manure phase, and also had three consecutive years of perennial forages rather than two, suggesting these two factors together explain the observed differences between these treatments. It is the superposition of the above factors then, that leads to the overall observed hierarchy of necromass C among all treatments (CF > 8Y > 5Y-Manure > 5Y-NPKS > CG).

Perennial crops generally produce greater root biomass than their annual counterparts while reducing soil erosion, meaning cropping systems that include some degree of perennial crop inclusion usually accrue larger SOC stocks (Khanal, 2023; King & Blesh, 2018; Olmstead & Brummer, 2008). Given that microbial C-limitations have been shown to reduce overall biomass and necromass production (Xue et al., 2024), it is likely that the inclusion of perennials at the Breton Plots has allowed for greater SOC accrual, and simultaneously, greater necromass C accumulation by reducing C-limitations. Besides the benefits granted by higher SOC levels, it is possible that necromass C accumulation in the perennial-inclusive treatments was directly benefitted by increases in root biomass. For example, Xu et al. (2022) showed that Alfisols amended with maize root matter had superior microbial residue accumulation compared to those amended with maize shoot residues after 150 days. These results indicate that, over extended temporal scales, root biomass may be superior to shoot biomass in promoting microbial necromass accumulation, even in the absence of differences in the spatial arrangement of the residues. Including legumes in cropping systems also has important implications for necromass dynamics. High-quality legume residues may have enhanced microbial biomass production and, ultimately, entombment in the perennial forage-inclusive systems at the Breton Plots. This would be consistent with results that have shown that high-quality (i.e., low C:N ratio) plant residues from legumes enhance microbial biomass production (Frasier et al., 2016) and necromass C accumulation (Hu et al., 2023) compared to lower-quality non-leguminous plant residues. Hu et al. (2023) found that necromass C accumulation increased by 23% and 2% due to legume-inclusive and exclusive cover crops, respectively.

The combination of legume- and perennial- inclusion in the 5Y-NPKS treatment has presumably driven the increase in MNC compared to the CG treatment (Fig. 3.3A) through the mechanisms described above. The addition of manure to the 5Y-Manure treatment then further increased the necromass C content through the mechanisms described in Section 3.4.3. Similarly, the 8Y system was further enriched in necromass through the additional presence of legumes (faba beans) for one year each rotation, which were historically used as green manure, thereby adding large quantities of high-quality legume residues to the soil. Further, perennial vegetation stands often continue to increase their root biomass inputs for multiple years in the absence of stand disturbance (Bolinder et al., 2012; Houde et al., 2020; Sakiroglu et al., 2020; F. Zhang et al., 2023). This tendency means that the three-year consecutive forage production period in the 8Y system (compared to the two-year period of the 5Y system) likely contributed to its high necromass C content by enhancing belowground biomass additions. Another important factor to consider is the impact of mechanical tillage. A meta-analysis by Zhou et al. (2023) found that no-till and reduced tillage systems increased MNC by 20% on average. The positive effects of no-till combined with a fully perennial, legume-inclusive cropping system likely resulted in the CF treatment having the highest MNC content. Ultimately, the unique combination of factors in the systems that included perennial grass-legume forages increased necromass C and, in turn, SOC storage compared to forage-exclusive systems at the Breton Plots, and this increase appeared proportional to the length of consecutive forage growth.

The necromass fungi:bacteria ratio appears to have been relatively unaffected by the degree of perenniality in the cropping systems at the Breton Plots, as demonstrated by the similar fungi:bacteria ratios between the fully annual CG treatment and fully perennial CF treatment (Fig. 3.3B). However, it also appears that the inclusion of legumes generally reduced the necromass fungi:bacteria ratio, as the 5Y-NPKS and 8Y treatments were lower than the CG treatment (Fig. 3.3B). This is likely due to the high-quality legume residues favouring bacterial over fungal growth (Wang & Kuzyakov, 2024). On the other hand, the CF treatment’s high necromass fungi:bacteria ratio may also be a result of the absence of tillage maintaining high levels of plant litter in the soil, as evidenced by its singularly high LFOM-derived C content (Table 3.1), which consequently raised the whole soil C:N ratio (Table 3.1). These observations align well with those of Zhou et al. (2023), who found that no-till and reduced tillage preferentially increased the fungal necromass contribution to SOC. In contrast to the findings of de Vries et al. (2006) that lower biomass fungi:bacteria ratios indicate higher soil N loss potential, previous observations of the CG and 8Y treatments at the Breton Plots indicated that the CG treatment suffers from greater annual N leaching (Dyck et al., 2012). However, as discussed previously, the 8Y treatment's low necromass fungi:bacteria ratio may not be representative of the living biomass (Li et al., 2015). Finally, the high necromass fungi:bacteria ratio of the CG and CF systems compared to the other treatments may also indicate relatively higher degrees of C stability, as well as improved N retention and crop nutrient uptake in these two systems (Dippold et al., 2019; Gunina et al., 2017)

3.5.5. Necromass C Estimates Exceeded 50% of Total SOC

In line with a growing body of research indicating the primacy of microbial residues in SOC formation (Kallenbach et al., 2015; Liang et al., 2019, 2020; Wang et al., 2021), MNC was observed to generally make up more than 50% of the SOC at the Breton Plots (Fig. 3.4). While the proportion of SOC made up of fungal and total microbial necromass did not respond significantly to treatment, the proportion of SOC made up of bacterial necromass did respond significantly (Fig. 3.4). This may indicate that BNC was more responsive to the differences in management imposed across the Breton Plots. However, it should be noted that the BNC contribution to SOC was indistinguishable for all but the WF treatments. As such, it seems that the WF system displayed unique dynamics in its bacterial C pool. This aligns with the findings of Xue et al. (2024) that compared to bacterial necromass, fungal necromass is preferentially released from mineral associations when microbial metabolism is resource-limited. Once released, the LMW organic compounds (i.e., cell wall components) comprising the fungal necromass would be preferentially recycled into bacterial necromass due to bacteria outcompeting fungi for such resources (Wang & Kuzyakov, 2024). This, in turn, helps explain the apparent enrichment in bacterially derived SOC of the WF system.

3.5.6. Soil Properties Had Unique Relationships With Necromass C Pools

3.5.6.1. Necromass C and Soil Physicochemical Properties

The close positive association between all three necromass C pools and the FMAOM-derived C pool (Fig. 3.5) likely indicates that the majority of FMAOM-derived C is microbial in origin. By extension, the ratio of plant-derived C to necromass C is likely lowest in the FMAOM fraction, meaning that plant and amendment inputs would have a relatively weak impact on the maintenance of the FMAOM-C pool, which represents the most stable C pool, and an important C stock for long-term storage in agricultural soils. These findings align well with those reviewed by Lavallee et al. (2020), demonstrating that FMAOM is characterized by LMW compounds, largely of microbial origin, while

POM is more closely associated with fresh plant inputs. Further, Samson et al. (2020) demonstrated that CMAOM-C was more sensitive to crop residue addition and organic amendments than FMAOM-C, suggesting a closer relationship between the CMAOM fraction and fresh OM inputs.

Similarly, the relatively strong negative associations between the necromass C pools and FMAOM $\delta^{13}C$ (Fig. 3.5) demonstrate that microbial C processing is strongly altering stable C isotope ratios within the FMAOM fraction, which supports the view that FMAOM is primarily composed of microbial residues. However, the negative sign of the association is puzzling, as it implies that ^{13}C enrichment is most significant where there are low amounts of MNC (implying low microbial biomass). In contrast, it is expected that ^{13}C enrichment should be highest where there is high microbial biomass due to microbial C isotope partitioning during microbial biomass generation through preferential metabolic use of ^{12}C (Blaser & Conrad, 2016; Conrad, 2007; Werth & Kuzyakov, 2010). However, the notable enrichment in ^{13}C within the WF system (Table 3.1), where MNC was lowest, may generate the observed negative association. This enrichment would be an expected effect of the recycling of already ^{13}C-enriched fungal necromass into bacterial necromass (leading to further ^{13}C enrichment) during periods of microbial nutrient limitation within the WF system, as proposed previously.

The observation that all three necromass C pools were more closely associated with TN than any other N (nitrogen) pool (Fig. 3.5) potentially indicates that necromass accumulation depends primarily on overall soil N content. Given that this association was positive, it suggests that soil microbial communities are not overly sensitive to the fraction in which N is stored in the soil (i.e., POM vs. MAOM) so long as it is sufficiently abundant. However, this apparently contradicts previous findings that POM is generally a more readily available nutrient source due to its weaker physicochemical protections than MAOM (Lavallee et al., 2020). If this is the case, a higher POM-derived N content should stimulate higher microbial biomass generation. The strong positive association between the three necromass C pools and FMAOM-derived N further supports the concept that FMAOM primarily comprises necromass-derived OM.

Given the close relationship of LFOM to fresh plant inputs (Gregorich et al., 2006), the negative associations between the necromass C pools and the LFOM fraction C:N ratio (Fig. 3.5) suggests that necromass accumulation increases as the quality of plant biomass inputs increases (i.e., C:N ratio decreases). Furthermore, compared to FNC content, BNC content was more strongly associated with the LFOM fraction C:N ratio. This suggests that high-quality plant inputs preferentially increase bacterial, compared to fungal necromass accumulation. These findings align well with previous research demonstrating that high-quality plant inputs increase microbial biomass and necromass accumulation (Frasier et al., 2016; Hu et al., 2023) and favour bacterial over fungal growth (Wang & Kuzyakov, 2024).

In contrast, the strong positive associations of necromass C pools with the MAOM fractions' C:N ratios (Fig. 3.5) align with observations that these fractions are strongly influenced by the accumulation of necromass on the surfaces of soil mineral particles (Lavallee et al., 2020; Miltner et al., 2012; Samson et al., 2020; Simpson et al., 2007) and thus represent physically stabilized SOM. More specifically, the strong positive association between FNC and the CMAOM fraction suggests that fungal necromass preferentially accumulates in the CMAOM fraction at the Breton Plots. In contrast, bacterial necromass was more strongly associated with the FMAOM fraction C:N ratio, suggesting preferential accumulation in this fraction. The apparent preferential accumulation of fungal and bacterial necromass in different soil physical fractions is likely a result of spatial niche differentiation in microbial biomass. For example, Chiu et al. (2006) demonstrated that fungal biomass dominated in sand-sized (> 53 μm diameter) soil particle fractions (i.e., POM, CMAOM), while microbial biomass dominated in silt- and clay-sized (< 53 μm diameter) fractions (i.e., FMAOM) within forest, grassland, and transitional soils at a site in central Taiwan. Finally, the total MNC pool being most strongly associated with the CMAOM fraction C:N ratio likely reflects that fungal necromass dominates the necromass C pool. Thus, variation in the fungal necromass pool, which appears closely linked to the CMAOM fraction, drives overall variation in MNC of this agricultural soil.

3.5.6.2. Necromass C and SOC

Each necromass C pool was related to total SOC to differing degrees (Fig. 3.6). Bacterial necromass C displayed the weakest overall relationship with SOC ($r^2 = 0.448$), which is not surprising given that it made up the minority of the MNC pool. In contrast, FNC had a notably stronger relationship with SOC ($r^2 = 0.635$) and dominated the overall MNC pool. Regardless of their relative sizes, the FNC pool may have a stronger relationship with SOC than BNC due to the generally higher stability of fungal residues (Dippold et al., 2019; Gunina et al., 2017). As a result of these differences in stability, one would expect that BNC would cycle more quickly and contribute less to stable SOC in the long term. Nevertheless, total MNC provided a marginally stronger relationship with SOC ($r^2 = 0.654$) than BNC or FNC in isolation. This indicates that bacterial necromass still plays a measurable role in determining overall SOC levels at the Breton Plots. Overall, MNC variation explained 65.4% of the variation in SOC at the Breton Plots, underscoring its important role in long-term agricultural SOC accumulation, and its value as a factor regulating total SOC content and storage potential. This conclusion aligns well with the growing body of research indicating that microbial residues make up the majority of soil C in a diversity of ecosystems (Liang et al., 2019, 2020; Miltner et al., 2012; Simpson et al., 2007; Wang et al., 2021).

3.6. Conclusions

At the Breton Plots, fallow was found to contribute to low soil fertility, reductions in soil health metrics (i.e., increased BD and reduced SOM content), and low SOC storage potential. Fallow also reduced the fungal contribution to microbial necromass, potentially reducing C stability and agroecosystem sustainability through increased N leaching and reduced nutrient uptake potential. In contrast, conventional fertilization, manure addition, and perennial and legume inclusion (i.e., perennial forages) increased soil fertility, C storage, and microbial necromass accumulation. All these interventions increased C storage within either the CMAOM or FMAOM fractions, or both, signalling increases in stable SOM pools. More specifically, manure amendment and legume inclusion favoured SOM accumulation in the moderately stable CMAOM and highly stable FMAOM fractions, while fertilization and perennial inclusion preferentially increased SOM accumulation in the fast-cycling LFOM and stable FMAOM fractions. Compared to conventional fertilization, manure amendment also caused generally greater increases in soil C and N and raised soil pH, while fertilizer caused acidification and lowered the necromass fungi:bacteria ratio. These results indicate that, compared to fertilizer, manure amendment was more beneficial to soil fertility, health, C stability, SOM accumulation, and agroecosystem sustainability. Similarly, legumes, which appear to have preferentially increased bacterial necromass accumulation through the provision of high-quality litter, may also have contributed to low soil necromass fungi:bacteria ratios. However, given that legumes increased FMAOM formation and reduced the need for N fertilization, negative impacts on C stability and agroecosystem sustainability were likely minimal to non-existent. Furthermore, strong associations between MAOM-C and N pools, necromass C, and high $\delta^{13}C$ values support the concept that MAOM formation is primarily microbially driven. Further, low LFOM C:N ratios were closely associated with necromass accumulation, particularly in the bacterial pool. Finally, total MNC strongly predicted total SOC, with FNC providing the majority of this predictive power, indicating the importance of microbial necromass to overall SOM accumulation.

3.7. Acknowledgements

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Table 3.1. Soil physicochemical properties of the top 7.5 cm of mineral soil. Soil C and N contents for each fraction represent the content derived from that fraction present in the whole soil (e.g., grams of LFOM-derived C per gram of soil). Lettering indicates significant differences between treatments for each response variable listed by column ($P < 0.05$). See Table S4 for elemental recovery percentages following the fractionation process.

Treatment	**C Content (mg g soil^{-1})**				**N Content (mg g soil^{-1})**				**pH**
	SOC	**LFOM**	**CMAOM**	**FMAOM**	**TN**	**LFOM**	**CMAOM**	**FMAOM**	
WF-Check	9.0^{f}	0.8^{c}	1.1^{c}	5.7^{e}	0.96^{g}	0.03^{g}	0.15^{d}	0.6^{f}	6.9^{ab}
WF-NPKS	12.0^{f}	1.6^{abc}	0.9^{c}	7.5^{de}	1.35^{efg}	0.07^{f}	0.15^{d}	0.78^{e}	6.1^{cde}
WF-Manure	24.9^{cde}	1.2^{abc}	4.7^{ab}	16.5^{bc}	2.40^{cde}	0.06^{e}	0.46^{bc}	1.41^{cd}	7.0^{a}
CG	22.8^{de}	2.4^{ab}	3.0^{b}	13.9^{bc}	2.05^{defg}	0.11^{d}	0.29^{c}	1.27^{bd}	5.6^{cf}
5Y-Check	22.3^{e}	1.4^{b}	4.8^{b}	12.4^{cd}	1.81^{efg}	0.06^{e}	0.46^{c}	1.13^{b}	6.7^{ab}
5Y-NPKS	29.3^{cd}	3.3^{ab}	4.7^{b}	16.1^{b}	2.59^{cd}	0.19^{c}	0.45^{c}	1.45^{d}	5.1^{f}
5Y-Manure	30.1^{bc}	2.8^{ab}	4.9^{b}	22.8^{a}	3.31^{bc}	0.15^{c}	0.45^{bc}	1.92^{ac}	6.5^{bd}
8Y	36.9^{ab}	4.8^{ab}	9.2^{a}	25.4^{a}	4.11^{a}	0.29^{b}	0.88^{a}	2.15^{a}	6.2^{e}
CF	47.2^{a}	12.0^{a}	10.1^{a}	22.4^{a}	3.61^{ab}	0.49^{a}	0.75^{ab}	1.77^{a}	5.4^{f}

Treatment	**C:N**				**δ^{13}C**				**BD (g cm^{-3})**
	Whole	**LFOM**	**CMAOM**	**FMAOM**	**Whole**	**LFOM**	**CMAOM**	**FMAOM**	
WF-Check	9.0^{cd}	24.4^{ab}	7.2^{c}	9.4^{e}	-25.4^{c}	-26.9^{cd}	-26.0^{d}	-25.9^{e}	1.2^{a}
WF-NPKS	8.6^{d}	22.2^{abc}	6.4^{c}	9.6^{de}	-25.9^{bc}	-26.7^{d}	-26.4^{d}	-26.2^{e}	1.1^{abc}
WF-Manure	10.2^{bc}	19.1^{bcd}	10.2^{b}	11.7^{abc}	-27.5^{a}	-27.0^{cd}	-28.6^{abc}	-27.6^{cd}	1.2^{a}
CG	11.4^{b}	22.4^{ab}	10.1^{b}	11.0^{bcd}	-27.5^{a}	-27.9^{abc}	-28.4^{abc}	-27.1^{b}	1.1^{abcd}
5Y-Check	10.3^{bc}	23.7^{a}	10.3^{b}	11.0^{bc}	-27.5^{a}	-28.5^{b}	-28.4^{bc}	-27.5^{d}	1.1^{ab}
5Y-NPKS	10.9^{b}	17.4^{cd}	10.4^{b}	11.2^{abc}	-27.7^{a}	-28.5^{b}	-28.7^{ac}	-27.7^{ac}	1.0^{abcd}
5Y-Manure	10.8^{b}	18.9^{bcd}	11.0^{b}	11.9^{ab}	-27.8^{a}	-28.4^{ab}	-28.7^{abc}	-28.1^{a}	0.9^{bcd}
8Y	10.8^{b}	16.7^{d}	10.5^{b}	11.8^{ab}	-27.8^{a}	-28.3^{ab}	-29.0^{a}	-28.1^{a}	0.9^{cd}
CF	14.3^{a}	23.8^{a}	13.4^{a}	12.6^{a}	-26.9^{ab}	-27.6^{acd}	-28.0^{b}	-27.3^{bd}	0.8^{d}

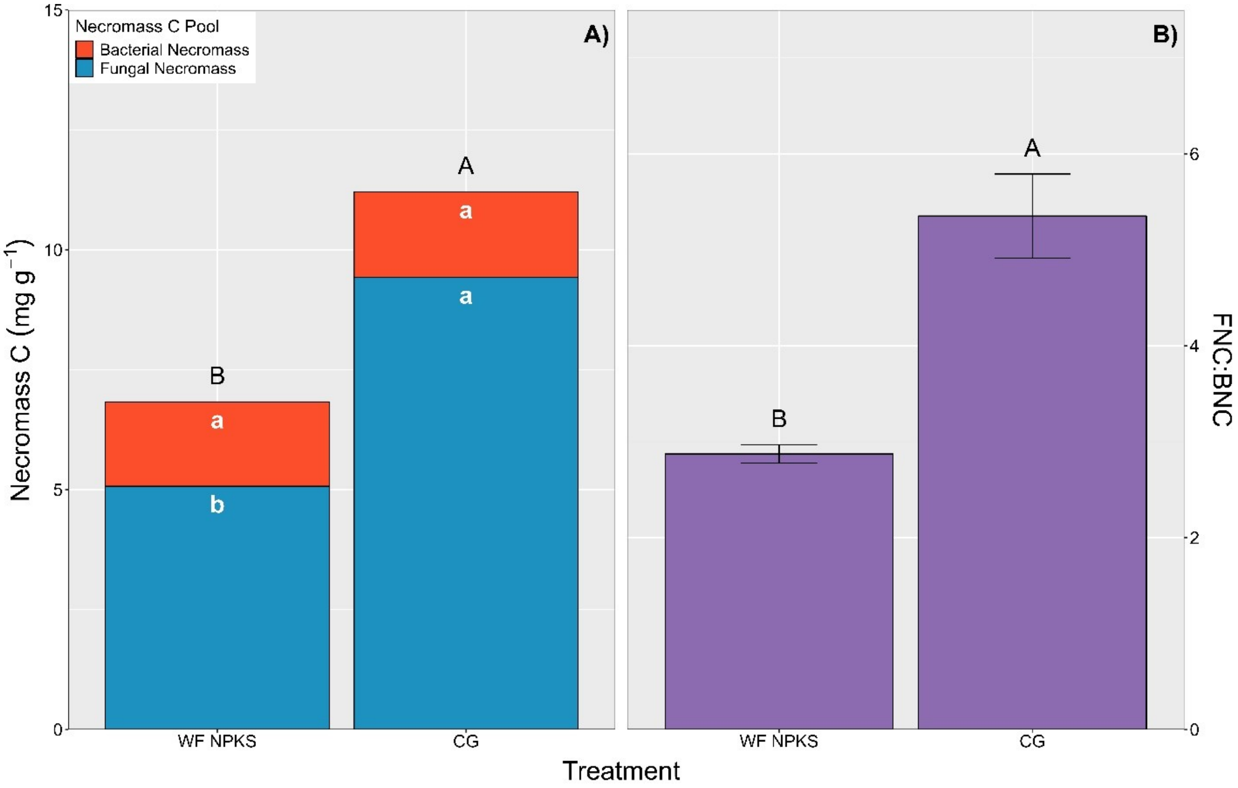

Figure 3.1. **A)** Necromass C for the WF-NPKS and CG treatments within the upper 7.5 cm of mineral soil. Lowercase letters denote significant differences (P < 0.05) within the fungal and bacterial necromass pools. Uppercase letters represent significant differences between the total microbial (bacterial + fungal) necromass pools. **B)** Fungal-to-bacterial necromass ratios for the WF-NPKS and CG treatments. Group letters denote significant differences (P < 0.05). Error bars represent 1 standard error. FNC:BNC = necromass fungi:bacteria ratio.

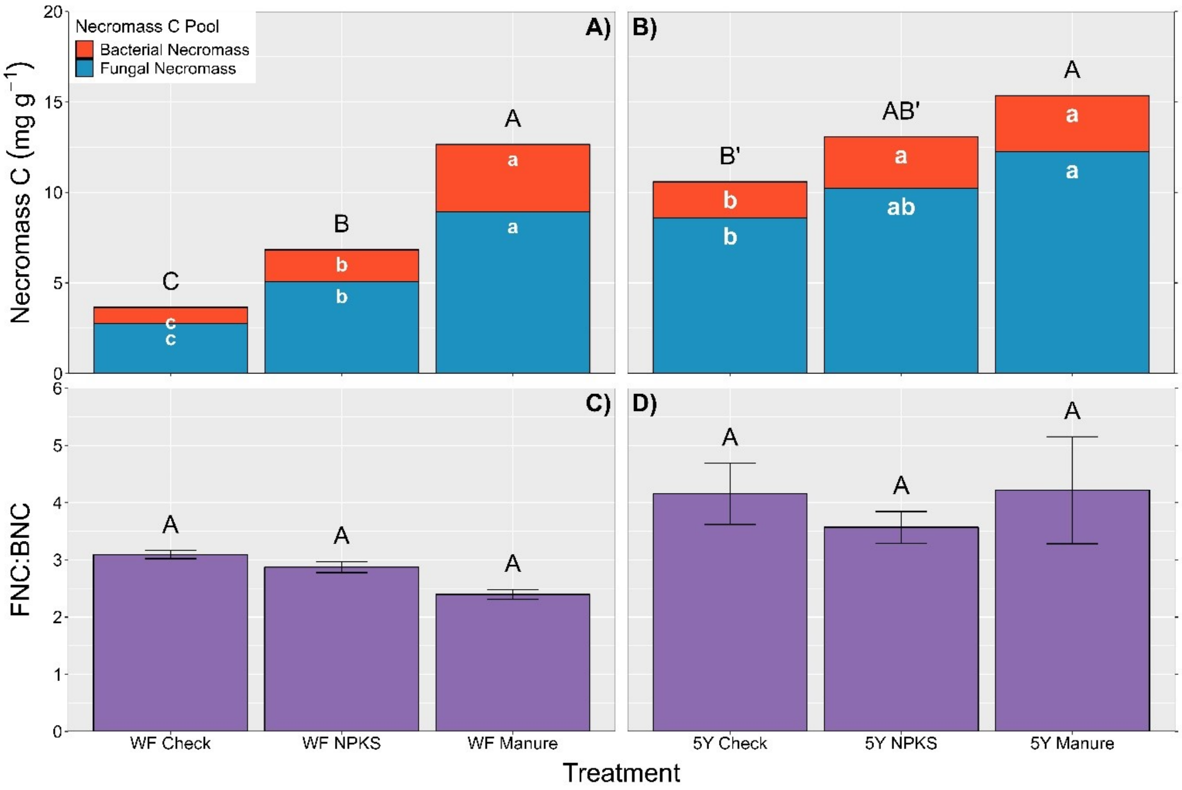

Figure 3.2. Necromass C in the upper 7.5 cm of mineral soil for: **A)** all amendment treatments in the WF system; and **B)** all amendment treatments for the 5Y system. Lowercase letters denote significant differences ($P < 0.05$) within the fungal and bacterial necromass pools, while uppercase letters represent significant differences between the total microbial (bacterial + fungal) necromass pools. Shared group letters appended with an apostrophe (‘) indicate differences at $P < 0.1$. Fungal-to-bacterial necromass ratios for: **C)** all amendment treatments within the WF system; and **D)** all amendment treatments within the 5Y system. Group letters denote significant differences ($P < 0.05$). Error bars represent 1 standard error. FNC:BNC = necromass fungi:bacteria ratio.

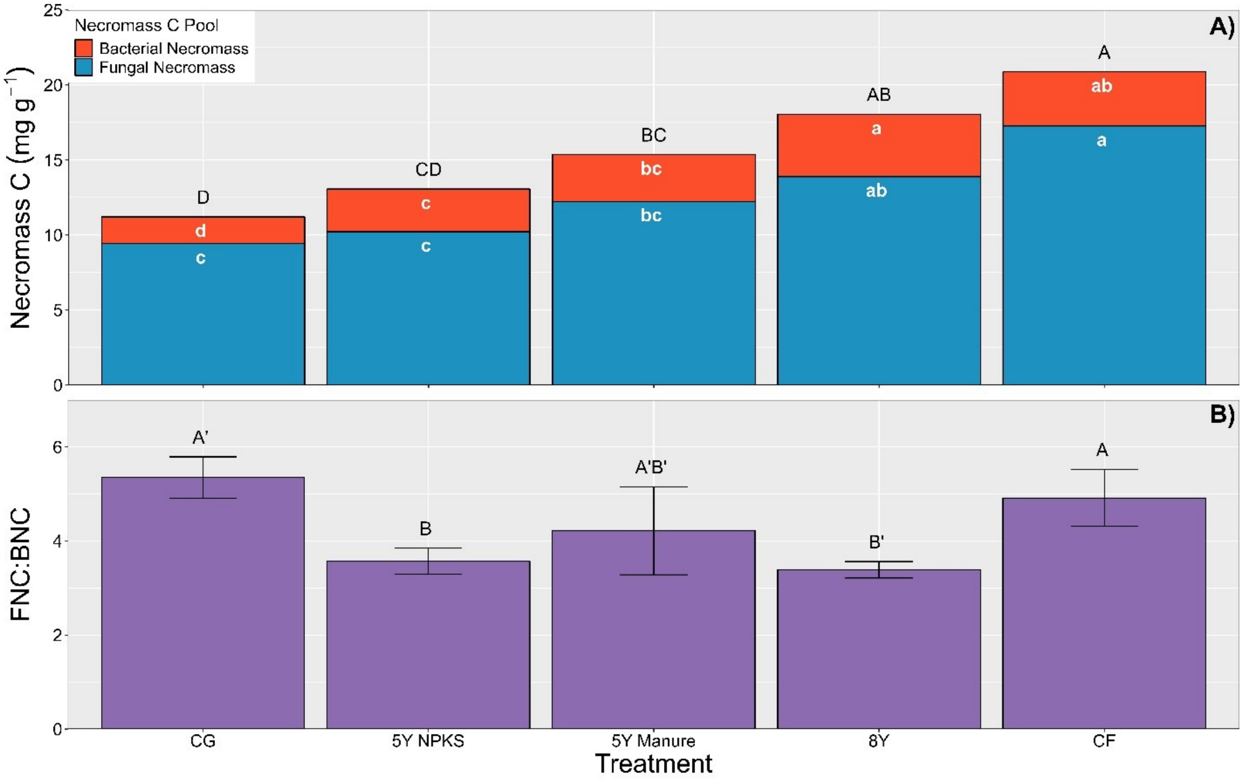

Figure 3.3. **A)** Necromass C in the top 7.5 cm of mineral soil for all amended and non-fallowed treatments containing variable inclusion of perennial forages. Lowercase letters denote significant differences ($P < 0.05$) within each of the fungal and bacterial necromass pools. Uppercase letters represent significant differences between the total microbial (bacterial + fungal) necromass pools. **B)** Fungal-to-bacterial necromass ratios for all amended and non-fallowed treatments. Group letters denote significant differences ($P < 0.05$). Shared group letters appended with an apostrophe (‘) indicate differences at $P < 0.1$ Error bars represent 1 standard error. FNC:BNC = necromass fungi:bacteria ratio.

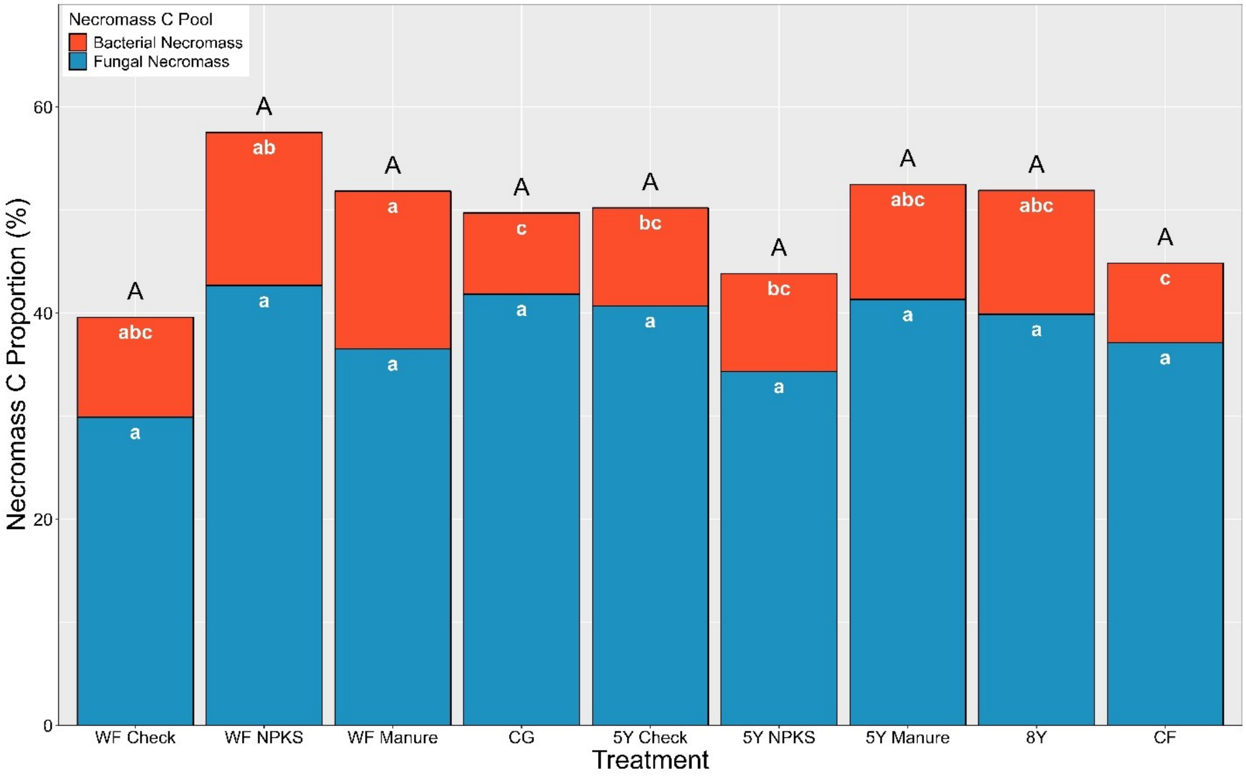

Figure 3.4. Necromass C proportion of total SOC for all treatments in the top 7.5 cm of soil. Lowercase letters denote significant differences ($P < 0.05$) within the fungal and bacterial necromass pools. Uppercase letters represent significant differences between the total microbial (bacterial + fungal) necromass pools.

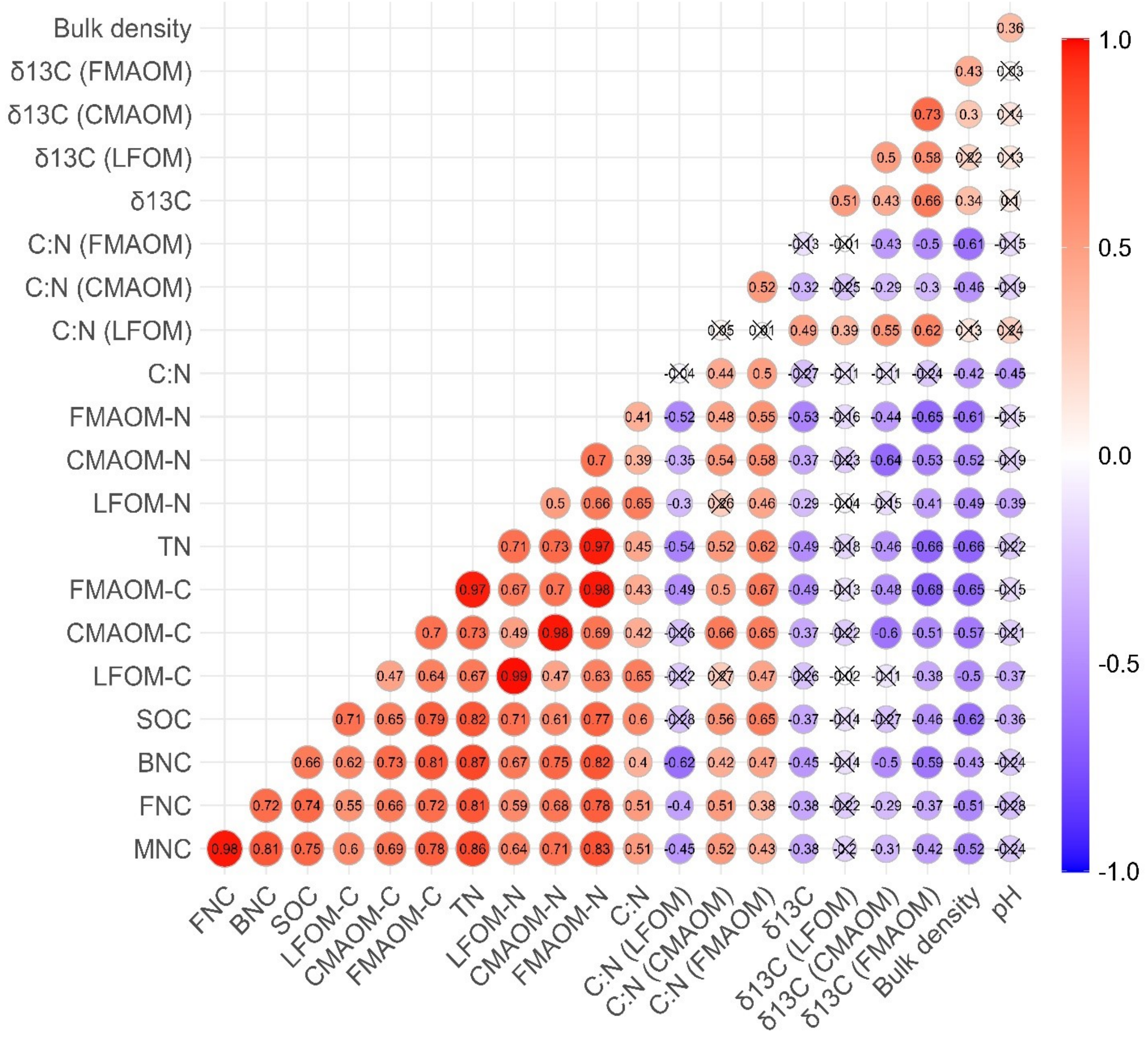

Figure 3.5. Correlation heatmap of soil variables examined for the top 7.5 cm of mineral soil within the various treatments at the Breton LTAE. Values indicated in the bubbles are Spearman correlation coefficients. Bubble size, shade, and colour represent the directionality and strength of the correlation. Crosses represent non-significant correlations ($P > 0.05$).

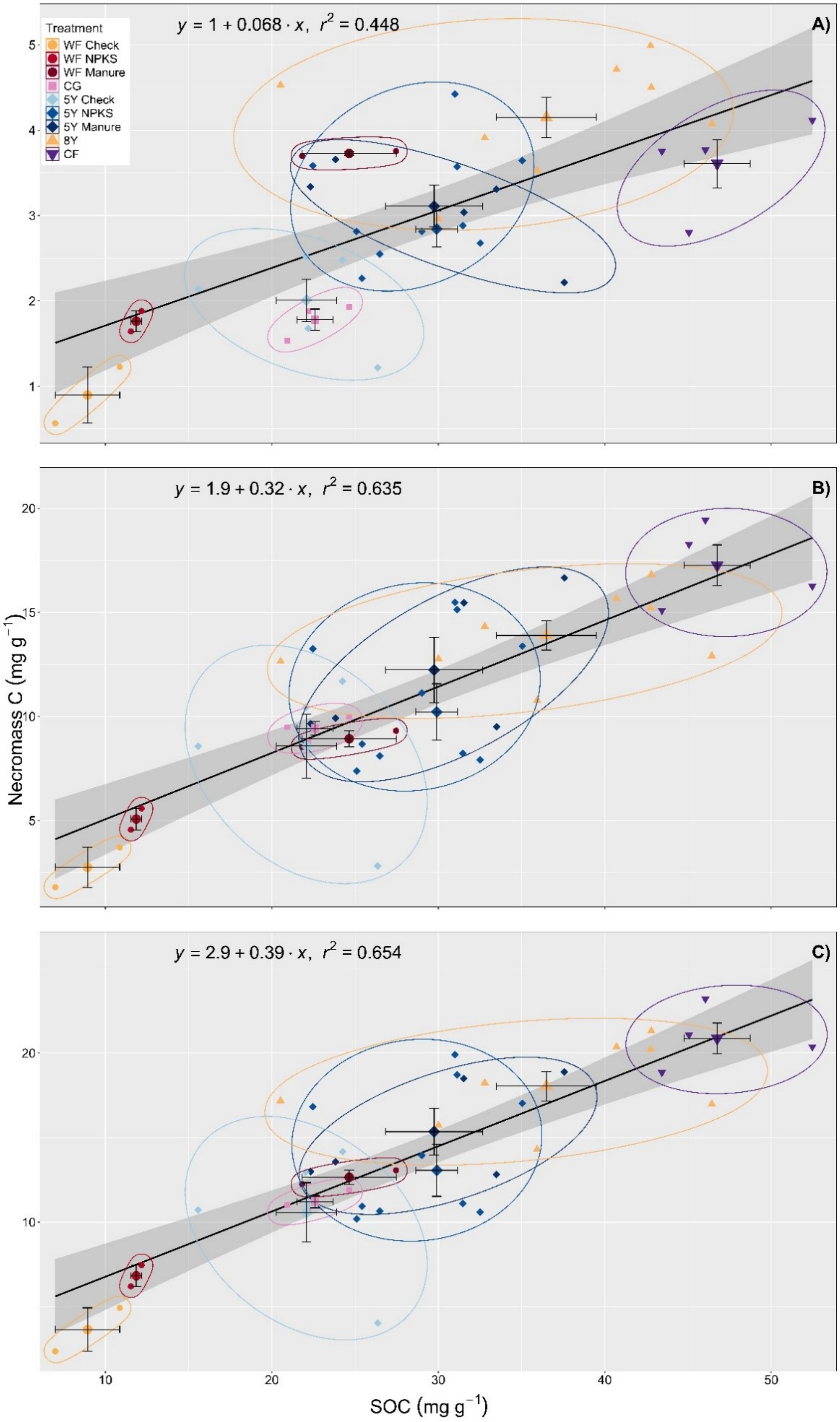
y = 1 + 0.068 · x, r² = 0.448
A)
Treatment
WF Check
WF NPKS
WF Manure
CG
5Y Check
5Y NPKS
5Y Manure
8Y
CF
1
2
3
4
5
y = 1.9 + 0.32 · x, r² = 0.635
B)
5
10
15
20
Necromass C (mg g⁻¹)
y = 2.9 + 0.39 · x, r² = 0.654
C)
10
20
10
20
30
40
50
SOC (mg g⁻¹)

Figure 3.6. Linear regressions of SOC against: **A)** bacterial necromass C; **B)** fungal necromass C; and **C)** total microbial necromass C. Smaller points represent individual data points, and large data points represent treatment means. Error bars represent treatment standard errors. Ellipses indicate treatment clustering. r^2 values are the square of Pearson's correlation coefficient.

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Chapter 4 – Thesis Overview, Significance, and Suggestions for Future Research

4.1. Overview

Overall, this work aimed to leverage long-term experimental data to quantify how various common agricultural management practices impact soil organic matter (SOM) formation and accrual, as well as stabilization (i.e., the slowing of SOM turnover). In particular, management-induced modulations of soil carbon (C) storage potential and microbial-driven SOM dynamics were explored in detail.

4.1.1. Chapter 2

Chapter 2 provided an in-depth exploration of the soil organic C (SOC) stocks at the Breton Plots as of 2021, after more than four decades of continuous management. This analysis aimed to identify cropping systems that could enhance soil C storage to decrease net agricultural greenhouse gas (GHG) emissions and improve soil health. Further, the monetary value of the SOC stock differences between each treatment and the conventionally managed annual grain cropping system (e.g., the “business-as-usual” treatment) were determined. This analysis aimed to put SOC stock differences in monetary terms and provide a basis for agricultural C valuation initiatives.

SOC stocks were examined in various equivalent soil mass (ESM) intervals corresponding to a maximum sampling depth of 90 cm below the mineral soil surface. As a result of the considerable age and variety of the examined treatments, and the sampling depth, both topsoil (i.e., 0-15 cm) and subsoil (i.e., below 15 cm) SOC formation and degradation processes could be probed with a high degree of resolution. This high-resolution analysis allowed the disentanglement of the unique impacts of alternating fallow, conventional (industrial) fertilization, manure amendment, and perennial grass-legume forage inclusion on SOC. The use of alternating fallow was found to limit SOC storage in both the topsoil and subsoil dramatically. Amendment with conventional fertilizer effectively increased SOC stocks in a perennial-forage- and legume-inclusive rotation, though SOC enrichment was limited to the topsoil. However, fertilization did not increase SOC stocks in the alternately fallowed system, neither in aggregate nor in the topsoil or subsoil individually. On the other hand, manure amendment increased SOC stocks in both the fallowed and rotationally cropped systems. This SOC enrichment was restricted to the topsoil for the fallowed system but extended into the subsoil within the rotationally cropped system. Finally, even in the face of decreased nutrient import through fertilizer and manure, the inclusion of perennial grass-legume forages increased SOC levels in proportion to the consecutive duration over which forages were grown, with associated reductions in tillage likely contributing to this effect.

These results clearly showed that, while both fertilizer and manure amendments generally enhanced SOC storage, manure led to greater SOC enhancements than fertilization in organic matter (OM) limited systems. Further, they indicate that fully perennial grass-legume forage systems, and diversified crop rotations that include legumes and perennials, can increase SOC storage to similar extents while reducing total nutrient import needs.

4.1.2. Chapter 3

Chapter 3 examined the impact of agricultural management practices on microbial necromass C (MNC) levels in the topsoil, seeking to clarify the contribution of soil microbial factors to the SOC stocks observed in Chapter 2. Furthermore, this chapter examined the relationships between microbial necromass and the C and nitrogen (N) pools in the bulk soil, as well as in the light fraction OM (LFOM), coarse mineral-associated OM (CMAOM), and fine mineral-associated OM (FMAOM) fractions. This examination was done to illuminate necromass formation pathways and relate necromass C to overall SOM stability. Associations between necromass C, and both bulk and fractional soil $\delta^{13}C$ and C:N values, were also examined as additional proxies for microbial SOM processing and measures of SOM formation dynamics. Finally, bacterial, fungal, and total MNC were tested for their ability to predict total SOC linearly.

In general, MNC responses to agricultural management practices mirrored those of total SOC, albeit with some notable differences. In addition, the impacts of these practices varied for bacterial and fungal necromass. More specifically, alternating fallow greatly reduced total MNC through reductions in fungal necromass C, leading to concomitant decreases in the necromass fungi:bacteria ratio. Fertilization and manuring increased MNC compared to no amendment, and these increases were more dramatic within the fallowed system than the rotationally cropped system. Manure application increased necromass C more than fertilizer application in the alternately fallowed system, whereas either fertilizer or manure amendment led to comparable necromass C increases within the rotationally cropped system. Perennial grass-legume forage polycultures increased MNC in proportion to the consecutive length of perennial forages grown within the rotationally and continuously cropped systems. Increased legume residue addition appears to have lowered the necromass fungi:bacteria ratio, whereas no-till management appears to have increased this ratio. In line with other studies, necromass C approached 50% of total SOC at the Breton Plots, with necromass C accounting for an average of 49 ± 2% of total SOC between all treatments (Li et al., 2023; Liang et al., 2019; Wang et al., 2021). Total MNC was the best predictor of overall SOC, with fungal necromass C providing the majority of this predictive power.

4.2. Significance of Findings

Synthesizing the findings of the two research chapters, it is apparent that the management practices applied at the Breton Plots have had considerable and diverse impacts on SOC stock and SOM formation, degradation, and stability. The observed effects on SOC and SOM, the likely mechanisms that drove them, and their broader significance, can be summarized as follows:

4.2.1. Fallow Caused Soil Degradation and Reduced SOM Stability

Alternating fallow led to SOM degradation in both the topsoil and subsoil, resulting in an overall SOC stock that was considerably (51%) lower than under conventional annual grain cropping. This soil degradation was induced by reducing crop biomass inputs, potentially promoting soil erosion, and causing significant microbial nutrient limitations (particularly C-limitations). These same processes reduced C stability and overall cropping system sustainability by increasing bacterial dominance of microbial communities, priming the breakdown and microbial recycling of soil necromass (as suggested by singularly high ^{13}C enrichment and low bulk soil C:N ratios), and lowering the necromass fungi:bacteria ratio. The loss of SOC at depth is particularly concerning, as subsoil C appears to have considerably longer residence times than that in the topsoil (Rumpel & Kögel-Knabner, 2011), suggesting greater general stability. However, these negative impacts were effectively countered in the topsoil by considerable OM (and C) import as manure, suggesting that OM (and, in effect, C) was a limiting resource in the fallowed system. Importantly, these benefits of manuring were not seen in the subsoil C pool. Furthermore, soil fertility was also reduced by fallow, as evidenced by the singularly low soil N within the fallowed system.

These results further cement the view that long-term use of alternating fallow leads to soil degradation, compromised soil health, reduced cropping system sustainability, and the release of C into the atmosphere. In monetary terms, the value of SOC storage forgone due to fallow amounted to more than \$11,000 CAD ha^{-1} after 90 years of treatment.

4.2.2. Conventional Fertilizer Increased Topsoil C Storage Without Changing SOM Stability

Amendment with conventional (NPKS) fertilizer generally improved SOC stocks, primarily by increasing crop production and subsequent LFOM generation. However, this SOC enrichment was restricted to the topsoil. Conventional fertilizer also benefitted SOC stocks by reducing microbial nutrient limitations and increasing necromass contributions to FMAOM. However, fertilizer did not have an apparent effect on SOM stability. Effects on SOM stability were muddied as this amendment preferentially increased SOM within the least stable (i.e., LFOM) and most stable (i.e., FMAOM) fractions while having little apparent effect on necromass fungi:bacteria ratios. In addition, fertilization also led to soil acidification along with increased soil fertility.

As a result, conventional fertilization increased SOC with little apparent effect on overall C stability and a mixed effect on soil health indices. Soil indices of cropping system sustainability were mostly unchanged by fertilization. Nonetheless, the potential for environmental damage was likely increased within the fertilized treatments relative to the non-fertilized treatments, for example, due to greater N leaching from higher quantities of mineral soil N, as previously observed at the Breton Plots (Dyck et al., 2012). Additionally, the potential for increased GHG emissions due to fertilization may have further reduced the sustainability of these treatments, given that fertilizer application is a primary source of anthropogenic nitrous oxide emissions (Shcherbak et al., 2014).

4.2.3. Manure Increased Soil C More Than Fertilizer and Benefitted SOM Stability

Amendment with manure generally increased SOC stocks to a greater extent than conventional fertilizer, preferentially increasing C storage in the CMAOM and FMAOM fractions. Increased C storage within these fractions was likely a result of manure amendment simultaneously alleviating microbial C and N limitations, leading to greater and more efficient production of microbial biomass and necromass through both the fungal and bacterial pools. Furthermore, within the 5-year rotational system, manure amendment increased SOC in both the topsoil and shallow subsoil, likely due to improved rooting at deeper depths during the perennial phase. This increase in subsoil C additionally benefitted overall SOC stock stability. Manure also generally raised soil pH and consistently raised soil fertility.

Taken together, these effects suggest that manure amendment not only increased total SOC storage but also SOM stability and overall soil health while not reducing cropping system sustainability. However, the increased risk of soil phosphorus (P) loading, leaching, and resultant eutrophication of surrounding waterbodies resulting from manure application must be considered when evaluating the potential for environmental damages from this amendment.

4.2.4. Perennial Forage Polycultures and Legume Green Manure Increased SOC Storage and Promoted Necromass Accumulation

Systems that included perennial forage polycultures (i.e., grass-legume mixtures) increased SOC stocks and decreased soil bulk density (BD) in proportion to the consecutive length of forage inclusion. Further, this C enrichment extended below the immediate topsoil. This dynamic is mirrored and likely enhanced by microbial necromass levels progressively increasing in proportion to the consecutive length of forage growth within a system. However, the effect of forage inclusion was superimposed with the effects of nutrient management via soil amendments, such that even the forage-inclusive treatments with the lowest SOC stocks had similar SOC stocks to the annual grain system. Namely, the amended treatments of the 5-year rotational system had average annual amendment N rates approaching a third of those in the annual grain system while still having similar SOC stocks. On the other hand, forage inclusion appears to have led to considerably higher SOC storage in the 8-year rotational system, where the amendment N rate was similar to the annual grain system. Albeit the incorporation of legume green manure may have contributed to the high SOC storage in the 8-year rotational system.

Summing up, the inclusion of perennial forage polycultures greatly enhanced SOC storage. They also increased SOC stability by increasing subsoil C storage and promoting the formation of MAOM-C through necromass accumulation. Further, forage-induced increases in SOC were valued at more than \$5000 CAD ha^{-1}. However, the exclusion of tillage and the inclusion of legume green manuring appeared to strongly modulate the impact of forages on soil C. Forgoing tillage appears to have contributed to an immensely C-rich surface soil layer, promoted a high LFOM C:N ratio, and established a high topsoil necromass fungi:bacteria ratio, all indicating stabilization of the topsoil C within fungal necromass. On the other hand, legume green manuring appears to have lowered the topsoil necromass fungi:bacteria ratio (potentially lowering topsoil C stability) while increasing C storage deeper in the soil profile (e.g., within the 15-30 cm layer) where C is generally more stable. As such, both management approaches appear to have increased SOC stabilization through different means. In any case, extended temporal intervals of forage inclusion also limited the need for soil amendments, reducing environmental pollution risks associated with their use while maintaining soil fertility and lowering BD. Through these mechanisms, forage inclusion increased environmental sustainability and soil health.

4.3.4. Final Synthesis

By leveraging data from the Breton Plots long-term agroecosystem experiment, this work provides a rare example of an analysis of long-term agricultural management practices that have been ongoing for as long as 90 years. Given the slow pace of many soil processes, this long-term management allows an unusually high-resolution examination of societally important soil processes that support global food security, economic prosperity, and environmental sustainability.

At the Breton Plots, cropping systems with consecutive periods of grass-legume forage polycultures benefitted soil C storage and stability, soil health, and cropping system sustainability compared to those with shorter intervals of perennial forage growth, or those lacking forage inclusion. No-till and annual legume growth and green manuring appeared to have further enhanced these benefits when applied to forage-inclusive systems. Additionally, amendment with manure enhanced soil C storage and stability, soil health, and cropping system environmental sustainability, compared to conventional amendment with fertilizer. Further, the soil C storage increases provided by these practices exceeded the “4 per 1000” target annually, signalling their ability to help achieve the United Nation’s (UN) Sustainable Development Goals (Minasny et al., 2017). The results of this study indicate that, in isolation, shifting cropping systems toward any of the above practices is likely to increase SOM levels and stability, as well as general soil health and cropping system sustainability.

Taken together, these results imply that converting conventional annual grain systems to integrated crop-livestock systems that include perennial forage polycultures (with legumes) could greatly improve SOC storage and soil health, with little need for nutrient import. In turn, minimizing nutrient import would likely limit environmental damages associated with agricultural N and P leaching. The 8-year rotational system at the Breton Plots provides a proof-of-concept for an essentially self-sustaining cropping system based on these principles. This system biologically fixes sufficient levels of N so as not to require any N import—recall manure application in the 8-year system replaces 70% of the N removed with forage hay—while enhancing SOC stocks and stability, soil health, reducing fertilizer-induced N pollution, and maintaining crop yields (Dyck et al., 2012).

While any such shifts away from annual grain production systems are likely to limit crop production profits, at least in the short term, this analysis indicates that C crediting systems could help reduce profit gaps by rewarding producers for increased soil C storage. It was demonstrated here that management-induced soil C storage differences can amount to substantial monetary sums under current C-valuation regimes. However, modulating soil C storage is one of many ways an agricultural practice can alter GHG emissions. For example, concurrent emission reductions stemming from lowered fertilizer use and production of nitrous oxides or reduced tillage and fuel use—which provide direct economic benefits—should also be rewarded by C crediting strategies. More speculatively, as environmental degradation intensifies, governments may seek to internalize less direct environmental externalities, such as the eutrophication of waterbodies surrounding agricultural areas. If such processes can be effectively priced into pollution management frameworks, agricultural producers may find much narrower, or even reversed, profit gaps when implementing the kinds of management practices discussed here.

After more than four decades of diverse management practices at the Breton Plots, amendment with manure, inclusion of legumes, and increasing the temporal interval of perennial forage growth led to enhanced soil C storage, soil health, and environmental sustainability, compared to conventional cropping practices. Additionally, these soil improvements amounted to considerable economic benefits under current C-valuation practices, and their scale is in line with the aspirations of the UN’s “4 per 1000” initiative (Minasny et al., 2017). These results suggest that agricultural practices can be adapted to serve current needs for environmental sustainability while maintaining productivity and economic competitiveness, assuming proper incentive structures are provided by pollution-pricing strategies.

4.3. Suggestions for Future Research

While this study provides evidence that various agricultural management practices can improve SOM stocks, benefit soil health and productivity, and increase agricultural sustainability, the adoption of these practices will depend upon their economic feasibility. Future studies at the Breton Plots should focus on precisely quantifying the economic impacts of these practices and how they relate to SOM levels and other soil health metrics. In this case, differences in agronomic input costs and yields would be key factors to examine. Further, exploring relationships between crop yields and SOM content could capture evidence of additional economic benefits from changing agricultural practices.

Additionally, given the critical role of microbes in determining topsoil SOM dynamics seen in this work, it would be wise to examine microbial necromass in the subsoil to illuminate how microbial-driven SOM dynamics change with depth. Further, given the varied associations between microbial necromass pools and soil physical fractions, directly measuring the necromass within individual soil fractions could provide further insight into necromass dynamics at the Breton Plots. If paired with subsoil necromass measurements, this could provide information on any depth-dependent differences in fraction-specific SOM formation that may be present.

Following the suggestions of Whalen et al. (2022), it would also be beneficial to measure the Gram(+):Gram(-) bacteria ratio and a series of plant- (e.g., cutin, suberin, and lignin monomers) and microbe-specific (e.g., extracellular polymeric substances) biomarkers, perhaps using a molecular fingerprinting type analysis. The Gram(+):Gram(-) bacteria ratio could be used to constrain microbial necromass calculations further, and the resulting biomarkers could allow independent analysis of the direct contribution of plant-OM to SOM and provide separate indices to estimate the microbial contribution. These estimates could then be used to evaluate and affirm the reliability of the amino sugar method for estimating necromass.

Further, it would be interesting to compare fungi:bacteria ratios within living microbial biomass to those in necromass at the Breton Plots, as the biomass and necromass ratios may have important implications for agroecosystem sustainability (De Vries et al., 2006) and SOM stability (Dippold et al., 2019; Gunina et al., 2017), respectively. If these ratios are consistently similar, one can confidently be used as a proxy for the other. If the ratios are divergent, however, this may provide evidence for differences in necromass formation or persistence due to the origin of microbial residues.

Finally, given that the perennial forage crops examined here were grass-legume mixtures, it may be beneficial to complete similar explorations in systems that explicitly separate legumes and perennial grasses to better ascertain their individual effects on the soil. Similarly, no-till and legume green manuring practices may have substantially impacted the soil properties measured here, but these practices were confounded by the simultaneous use of legumes and perennial grasses. As such, it may be worthwhile to test the effects of these specific practices more explicitly on the soil variables seen here, as well as understand their impacts on SOC storage and soil function.

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Appendix

Table S1. Examined cropping treatments and their management characteristics.

Treatment	Experiment	Fallow	Fertilizer	Manure	Legume Green-manure	Rotation Length (yr)	Forage Interval (yr)	Reps	Age* (yr)
WF-Check	Classical	✓	X	X	X	2	0	2	91
WF-NPKS	Classical	✓	✓	X	X	2	0	2	91
WF-Manure	Classical	✓	X	✓	X	2	0	2	91
5Y-Check	Classical	X	X	X	X	5	2	5	91
5Y-NPKS	Classical	X	✓	X	X	5	2	5	91
5Y-Manure	Classical	X	X	✓	X	5	2	5	91
CG	Hendrigan	X	✓	X	X	Continuous	0	3	41
8Y	Hendrigan	X	X	✓	✓	8	3	8	41
CF	Hendrigan	X	✓	X	X	Continuous	Continuous	4	41

* Age of the of the treatment at the time of sampling in 2021

Table S2. Crop rotation patterns and fertilization rates for all examined treatments at the Breton Plots

Treatment	Phase	N (kg ha^{-1})	P (kg ha^{-1})	K (kg ha^{-1})	S (kg ha^{-1})***
WF-Check	Wheat	0	0	0	0
	Fallow	0	0	0	0
WF-NPKS	Wheat	90	22	46	20
	Fallow	0	0	0	0
WF-Manure	Wheat	90	Depends on manure elemental makeup		
	Fallow	0	0	0	0
5Y-Check	Wheat	0	0	0	0
	Oats	0	0	0	0
	Barley	0	0	0	0
	Hay	0	0	0	0
	Hay	0	0	0	0
5Y-NPKS	Wheat	50	22	46	20
	Oats	75	22	46	20
	Barley	50	22	46	20
	Hay	0	22	46	20
	Hay	0	22	46	20
5Y-Manure*	Wheat	0	0	0	0
	Oats	87.5	Depends on manure elemental makeup		
	Barley	0	0	0	0
	Hay	0	0	0	0
	Hay	87.5	Depends on manure elemental makeup		
CF	N/A	16	9	0	15
CG	N/A	90	22	46	20
8Y**	Barley	208	22	46	20
	Barley	35	22	46	20
	Faba beans	4	22	46	20
	Barley	89	22	46	20
	Barley	18	22	46	20
	Hay	0	22	46	20
	Hay	0	22	46	20
	Hay	28	22	46	20

* post-harvest application and incorporation (i.e., in fall after oats and after the second year of hay).

** averages for 1980 - 2019; N is applied as manure and is determined as 70% of the forage N uptake over one rotation cycle. Sum up averages for all phases and divide by 8 to get an average annual rate of 48 kg N ha^{-1}. Rotation phases were updated in 2000 (see Table S3 for more details and historical rotation patterns).

*** S rates in the Classical Plots and Hendrigan Plots were 5.5 kg S ha^{-1} for 1980-2006; 20 kg S ha^{-1} for 2007 onward.

Table S3. Historical crop rotation patterns for the 8Y treatment from 1980-2019

Phase	**1980-1999**	**2000-2019**
1	Barley (grain)	Barley (grain)
2	Faba beans	Barley (grain)
3	Barley (grain)	Faba beans
4	Faba beans	Barley (grain)
5	Barley underseeded to red clover/brome	Barley underseeded to alfalfa/brome
6	Red clover/brome	Alfalfa/brome
7	Red clover/brome	Alfalfa/brome
8	Red clover/brome	Alfalfa/brome

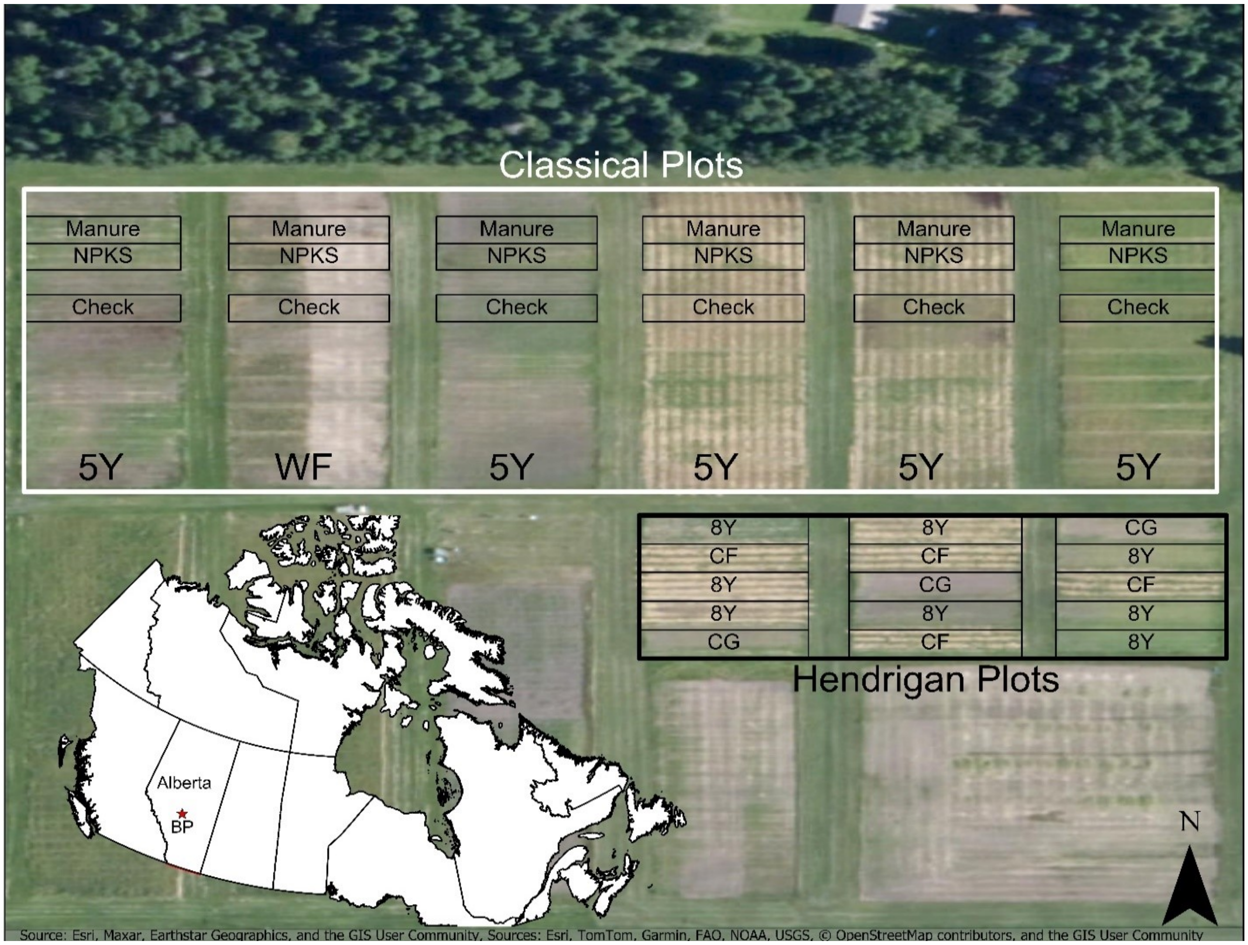

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Figure S1. Layout and location of the Breton Plots (BP) in Alberta, Canada. WF = 2-year wheat-fallow rotations; 5Y = 5-year, wheat-oats-barley-hay-hay rotation; CG = continuous grain system; 8Y = 8-year, barley-barley-barley-faba-barley-hay-hay-hay rotation. See Table S1 and S2 for details of rotations.