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Do UK grasslands have the ability to sequester more carbon? Assessment of stability and resilience to changing climate and management

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Declaration of Authorship

I declare that:

- the thesis has been composed by myself,
- the work submitted is my own, except where explicitly stated otherwise in the text,
- the work has not been submitted for any other degree or professional qualification,
- any included publications are my own work, with author contributions detailed below.

The work presented in **Chapter 2**, was published in Biogeosciences as: Kirsty C. Paterson, Joanna M. Cloy, Robert. M. Rees, Elizabeth M. Baggs, Hugh Martineau, Dario Fornara, Andrew J. Macdonald, and Sarah Buckingham (2021) Estimating maximum fine-fraction organic carbon in UK grasslands. With the following author contributions, KCP, SB, JMC, RMR and EMB formulated the research question and study design. KCP conducted the experimental work, data analysis, and prepared the manuscript draft. All authors contributed to editing and reviewing of the manuscript. I further acknowledge assistance from John Parker and Lydia Guo in the field and laboratory. Part of the data was used by Lydia Guo in fulfilment of her MSc dissertation.

Kirsty C. Paterson

Date: 03/01/2023

For Margaret,

Acknowledgements

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Finally, I wish to dedicate this thesis to my late grandmother, Mrs. Margaret Kilpatrick, for encouraging a love of learning and adventure in life - no fly cemetery will ever compare to yours.

Abstract

Soil organic matter (SOM) is the largest terrestrial pool of organic carbon (C). Carbon can persist in the soil due to several mechanisms, i) inherent chemical recalcitrance, ii) association with minerals in the soil and iii) limitations to microbial decomposition due to inaccessibility within aggregates, or conditions which limit microbial decomposition. However, C within mineral associated organic matter (MAOM-C), is often considered to be the most persistent, and consequently efforts to increase SOC largely focus on this long-lived SOC pool. The careful management of agricultural soils can contribute to climate mitigation by increasing C within MAOM, with co-benefits for ecosystem services and contributes to food security by increasing soil fertility. However, uncertainty remains with respect to the maximum sequestration potential, and the response of MAOM-C to changes in climate and management. This thesis aims to determine the carbon sequestration potential of agricultural grasslands, and to examine the effect of climate and management on MAOM-C formation within the United Kingdom.

Estimating the C sequestration potential of soils is essential in determining their potential contribution to climate mitigation policies. However, the myriad of processes that contribute to the formation, and persistence of soil C makes this difficult. Consequently, a balance must be struck between the accuracy of estimates and resource inputs. In this thesis a relatively simple, but widely used linear regression equation, developed by Hassink (1997), to estimate maximum C was examined in conjunction with boundary line and quantile regression analysis – both suggested as options to overcome the shortcomings of linear regression. The quantile regression estimate of maximum soil organic C was almost double that of the linear regression and boundary line analysis (0.89 ± 0.074, 0.43 ± 0.017 and 0.57 ± 0.052 g C per

kg soil, respectively). Additionally, the linear regression generated from the selected UK grasslands, was significantly different to that of Hassink (1997), demonstrating the importance of estimation methods which account for *in situ* context, such as management and climatic factors which play a role in C accrual.

To date, much of the focus on stability and resilience of persistent soil C has focused on its mineralisation and losses due to climatic and management changes. However relatively less focus has been paid to the influence of these factors on the formation of persistent soil C. This thesis examines the influence of management factors relevant to agricultural grasslands, and climatic change on the formation of MAOM-C. Nitrogen fertilisers are frequently used to enhance plant productivity, increasing plant C inputs to the soil. Additionally, nitrogen is thought to play an important role in the formation of organo-mineral associations. Therefore, the effect of the addition of ammonium nitrate and the quantity of C substrate on MAOM-C formation was examined. It was anticipated that greater C inputs would result in greater absolute retention of substrate within MAOM-C, but less proportionally, due to changes in microbial C use efficiency (the amount incorporated into biomass versus respired as carbon dioxide). It was found that absolute substrate C retention increased with higher C addition rates, with no apparent effect on proportional retention within MAOM-C. The results also suggest that the role of nitrogen in the retention of labile substrate C, such as glucose, may be a function of C addition rate.

Looking to the future it is necessary to understand how warming will influence the formation of MAOM-C. This was examined in conjunction with the effect of substrate type, to improve the understanding of the effects of substrate type and temperature on MAOM-C formation.

There was no effect of temperature or substrate type (glucose versus acetic acid) on substrate C recovered in MAOM-C. However, substrate C recovery was significantly affected by substrate and temperature in the bulk SOC. The substrate C recovered in bulk SOC was significantly higher for glucose than acetic acid at 10 and 15°C, but not at 20 and 25°C. Suggesting that MAOM-C formation was independent of substrate type and temperature, but that substrate type and temperature have an influence on retention of fresh labile C inputs within the bulk soil, possibly within dissolved organic C or microbial biomass.

An important aspect of agricultural land management is the maintenance of soil pH. Changes to soil pH can influence the retention of C within the soil by altering the bonds between organic matter and minerals in the soil. In acidic conditions C may be lost due to disruption of organo-mineral associations and reduced microbial activity. Whilst, in alkaline conditions additional C may be preserved due to cation bridging. Soils from a long-term pH trial were used to examine the effect of soil pH, grass ley duration and depth on MAOM-C. Ley duration had no effect on total SOC or MAOM-C, and as expected both declined with depth. In the topsoil, both SOC and MAOM-C increased with soil pH, possibly due to additional abiotic cation stabilisation, due to the presence of calcium, magnesium and manganese associated with lime application in pH management.

The findings of this thesis contribute to the understanding of the current C status of a selection of UK grasslands, and how typical management and climatic factors influence the formation and persistence of MAOM-C. There is likely to be potential for additional C sequestration within persistent C pools within agricultural grasslands in the UK. However, further work is required to improve the understanding of the formation and persistence of

MAOM-C in response to typical land managements. This would help to guide land management policies which enhance and limit losses of existing SOC.

Lay Summary

Carbon dioxide in the atmosphere can be reduced by increasing organic carbon (C) in the soil, so called soil carbon sequestration. The long-term retention of C in the soil is determined by processes which influence its persistence. Soil organic carbon (SOC) persistence arises due to several mechanisms; the inherent resistance to decomposition, by chemically binding to mineral surfaces in the soil, and due to conditions, which limit the decomposition of C by soil microbes such as physical separation. The C that binds with minerals (MAOM-C) is considered to have the longest lifetime within the soil. Therefore, increasing MAOM-C can play a role in mitigating the effects of climate change. To determine the potential benefit of increasing MAOM-C in soils, it is necessary to estimate the additional MAOM-C that can be stored, as MAOM-C is known to have a limited storage capacity. Additionally, there is a need to understand how agricultural land management and climate change, such as warming, can affect the accumulation and decomposition of MAOM-C. The overall aims of this thesis were to examine methods used to estimate the potential MAOM-C in UK grasslands and contribute to the current understanding of how land management and climatic warming can influence the formation and persistence of MAOM-C.

In Chapter 2, maximum MAOM-C was estimated by simple linear regression, boundary line analysis and quantile regression. The results demonstrated that the estimation method can result in significantly different estimates of maximum C. The quantile regression estimate of maximum soil organic C was almost double that of the linear regression and boundary line analysis. This demonstrates the importance of ensuring that estimation methods are accurate, so as not to mislead the sequestration potential of soils.

Chapters 3 and 4 examined the effects of climate and management on the formation of MAOM-C. Under warmer temperatures the amount of C entering the soil from plants may increase. Additionally, nitrogen (N) fertilisers are commonly used to enhance plant growth in agricultural land management. Therefore, in Chapter 3 the effect of C input rate and N fertilisers on MAOM-C formation was explored. At higher C input rates, more of the added C was recovered within MAOM-C. Whilst the effect of N varied with the amount of C input. In Chapter 4, the effect of the type of C input and soil warming on MAOM-C were investigated. There was no effect of temperature or C type on the amount of C recovered in MAOM-C. These results contribute to the understanding of the formation of MAOM-C under different land management and climatic scenarios. However further research is required to identify the mechanisms that led to the results observed.

To support plant growth, it is necessary to maintain soil pH at a favourable level. Changes to soil pH can also affect how C chemically binds to soil minerals. Additionally, soil disturbance associated with seeding new crops, alters its structure, and can break the chemical bonds between C and soil minerals. Therefore, in Chapter 5, the effects of soil pH and grass ley duration (time since soil disturbance) on MAOM-C were investigated at two different soil depths 0 – 20 cm and 20 – 40 cm. At 0 – 20 cm MAOM-C increased with soil pH, possibly due to the addition of lime which helps create bonds between C and soil minerals. There was no effect of grass ley duration on MAOM-C, suggesting that soil disturbance had a minimal impact on MAOM-C.

These results contribute to the understanding of the current C status of a selection of UK grasslands, and how typical management and climatic factors influence the formation and

persistence of MAOM-C. This is helpful in guiding future research and the design of land management policies which aim to increase and prevent losses of SOC.

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Abbreviations

Abbreviation	Definition
AIC	Akaike information criterion
Al	Aluminium
AN	Ammonium nitrate
ANOVA	Analysis of variance
BD	Bulk density
BL	Boundary line
Ca	Calcium
CO_2	Carbon dioxide
CUE	Carbon use efficiency
DCB	Dithionite – citrate – bicarbonate
DOC	Dissolved organic carbon
Fe	Iron
GHG	Greenhouse gas
HCl	Hydrochloric acid
HSD	Honest significant difference
KCL	Potassium chloride
K_2SO_4	Potassium sulphate
LMM	Linear mixed model
LMWOC	Low molecular weight organic carbon
MAOM	Mineral associated organic matter
MAOM-C	Mineral associated organic matter carbon
MBC	Microbial biomass carbon
MEMS	Microbial Efficiency-Matrix Stabilisation
Mg	Magnesium
Mn	Manganese
N	Nitrogen
NH_4^+	Ammonium
NO_{3-}	Nitrate

OC	Organic carbon
OM	Organic matter
P	Phosphorus
POM	Particulate organic matter
POM-C	Particulate organic matter carbon
QR	Quantile regression
SEM	Standard error of the mean
SOC	Soil organic carbon
SOM	Soil organic matter
TOC	Total organic carbon
UK	United Kingdom
WHC	Water holding capacity
WRB	World Reference Base

Chapter 1 Introduction

1.1 Introduction

The organic carbon (C) within soils is the largest terrestrial pool of C, and estimated to be twice the amount in global vegetation (Lehmann and Kleber, 2015; Smith, 2012). Soil organic carbon (SOC), is found within the heterogeneous mix of plant, microbe and animal organic matter (OM) residues at varying stages of decomposition, collectively known as the soil organic matter (SOM) (Baldock and Skjemstad, 2000). Therefore, SOC refers to the C found within the SOM. Fluxes of C within the soil are a function of the balance of photosynthesis and decomposition (Horwath, 2015). Plant photosynthesis removes carbon dioxide (CO_2) from the atmosphere, the C then cycles through as plant litter decomposing into the soil C stock (Ciais et al., 2013). Carbon is then released by autotrophic (plant) and heterotrophic (soil microbes and animal) respiration, and disturbance processes such as fires, land use change (LUC), and land management (Ciais et al., 2013).

SOC has various functions in soils and contributes to ecosystems services, such as (but not limited to); maintaining the cation exchange and acid buffering capacity, soil aggregation, nutrient provision, and water retention (Baldock and Skjemstad, 2000; Kimetu et al., 2009). The quantity and composition of SOC is determined by multiple factors including biotic (soil microbes and amount, composition and allocation of plant residues), abiotic (climate, elevation, aspect, parent material, clay content), and anthropogenic (fire, climate change, land management and use) factors. This is further influenced by the balance between inputs (primarily plant photosynthesis, root exudates and litter, and additional animal manure C) and outputs (respiration, decomposition, erosion, leaching, fire and biomass removal) (Jackson et al., 2017; Lorenz and Lal, 2018; Smith, 2012; Ward et al., 2016). Interventions in the

biological C cycle, to increase SOC are proposed as a means to mitigate climate change (Lorenz and Lal, 2018).

Carbon sequestration is the removal of CO_2 from the atmosphere into long lived C pools, which would not otherwise occur (Lal, 2004; Powlson et al., 2011). The ability of a soil to sequester C is determined by the balance of C inputs and C losses. The term is becoming synonymous with climate change mitigation, however this does not occur in every instance, and the term should not be confused with carbon storage. Carbon storage refers to an increase in SOC, but is not always associated with the removal of CO_2 from the atmosphere (Chenu et al., 2019). The potential of C sequestration in soils as a means to mitigate anthropogenic climate change has been widely discussed in the literature (e.g. Lorenz and Lal, 2018; Lugato et al., 2018; Smith, 2012; Soussana et al., 2004; Ussiri and Lal, 2017). Carbon sequestration is often presented as a win – win scenario to meet short term climate change goals (Lal, 2004; Smith, 2012), a proposal that has long existed as a measure to contribute towards decarbonisation of the economy (Dyson, 1977). Growing attention to the multifaceted benefits of C sequestration is acknowledged by the "4 per mille – Soils for food security and climate" Initiative, launched at Conference of Parties 21 alongside the Paris Agreement 2015 (Schiefer et al., 2018). The initiative recognises the need for C removal from the atmosphere in order to achieve the target of staying within a 1.5°C global warming limit. The 4 per mille Initiative aims to increase SOC globally by 4‰ per year over the next 25 years in the top 40 cm of soil (Schiefer et al., 2018). However criticisms regarding the practicality and methodology used in estimating additional C storage have been raised (Schlesinger and Amundson, 2019). The widespread implementation of C sequestration for climate change mitigation has been limited due to concerns of non-permanence, requirement of perpetual

management of the stabilised C and difficulties with verification (Horwath, 2015; Jones et al., 2017; Smith, 2012).

Efforts to increase SOC primarily focus on increasing the most persistent forms of SOC. Whilst much of the C entering the soil is mineralised and respired by soil microorganisms over short term scales (< 10 years), there is a portion which persists in the soils for centuries to millennia (Dungait et al., 2012; Sokol et al., 2018). Understanding the formation and persistence of this slower cycling pool of SOC is important in the context of incorporating land management focused on soil carbon sequestration within climate mitigation policies (Bossio et al., 2020). This review is composed of three main sections which provide the background for the following chapters within this thesis. Firstly, the formation of a persistent pool of SOC - mineral associated organic matter (MAOM), and several factors influencing its accumulation are considered, this is followed by an examination of typical grassland managements in the context of MAOM-C accumulation, and finally provides an overview of saturation, an important consideration in efforts to increase persistent SOC, and methods to estimate saturation point in soils.

1.2 Persistent SOC

Soil organic matter (SOM) is a heterogeneous mix of plant, microbe and animal organic matter residues at various stages of decomposition (Baldock and Skjemstad, 2000). Previously, the permanence of SOC within SOM was attributed to the chemical recalcitrance of the compound (Lehmann and Kleber, 2015). However, modern analysis indicates the presence of labile compounds (e.g. sugars, amino acids, and proteins) suggesting that OM persists due to other means, than inherent chemical recalcitrance alone (Lehmann and Kleber, 2015). The emerging view is that the most persistent SOC interacts with mineral surfaces in the soil, or is incorporated into aggregates (Dungait et al., 2012; Lehmann and Kleber, 2015). With mineral interactions conferring the longest persistence (Cotrufo et al., 2013).

Typically SOM is separated into functional pools to better understand the mechanisms which determine its formation and decomposition (Poeplau et al., 2018). Particulate organic matter (POM) and MAOM are fundamentally different components of SOM when considering the formation, persistence and functioning of SOM (Lavallee et al., 2020). MAOM is composed of low molecular weight compounds of plant and microbial origin (Geyer et al., 2020; Lavallee et al., 2020; Miltner et al., 2012; Schmidt et al., 2011) which are associated with minerals in the soil (Cotrufo et al., 2013; Kopittke et al., 2020; Lugato et al., 2021). In contrast, POM, represents the faster cycling pool of soil C and is composed of fragments of plant origin which persists due to physical protection within aggregates, or chemical recalcitrance (until conditions facilitating decomposition arise) (Lavallee et al., 2020), see **Figure 1.1**.

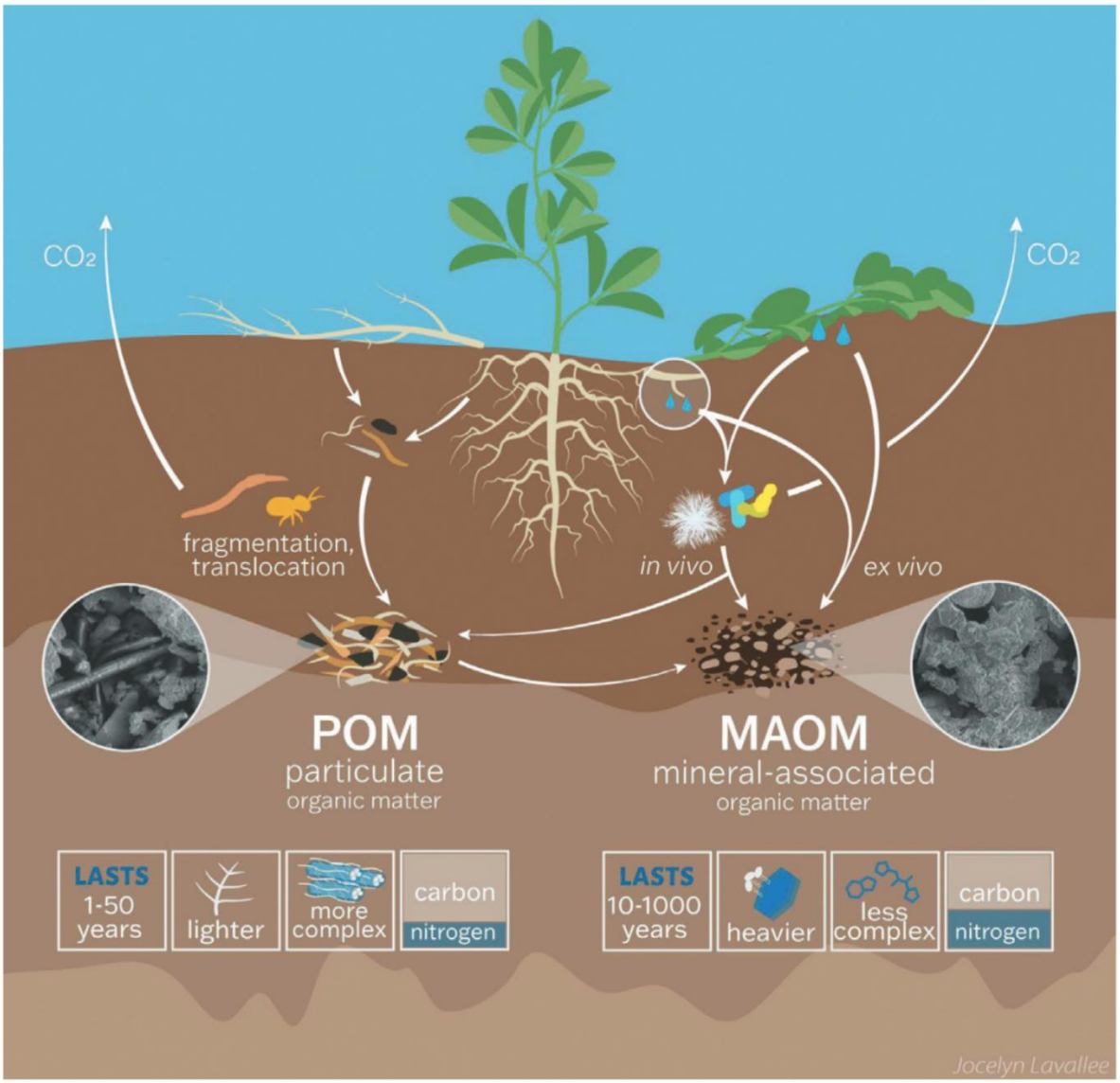

Figure 1.1. Overview of particulate organic matter (POM) and mineral-associated organic matter (MAOM). Key differences in formation pathways and composition are presented. Source: Cotrufo and Lavallee, 2022.

Organo-mineral associations may form by several different, and simultaneous mechanisms due to the heterogeneous nature of OM, diversity of minerals, and soil solution properties, such as pH (Kleber et al., 2021). These may include weaker interactions such as van der Waals forces and H-bonding, or stronger ligand exchange and cation bridging (von Lützow et al., 2006). OM may also be associated with minerals via occlusion in micropores and micro-

aggregates (Kleber et al., 2015; Kögel-Knabner et al., 2008) or bind to existing organo-mineral associations forming layered MAOM complexes (Kleber et al., 2007). MAOM complexes are conceptualised as multilayer structures with an inner mineral core, to which OM associates, in a disordered, heterogenous manner (as opposed to a uniform coating of mineral surfaces) (Possinger et al., 2020; Vogel et al., 2014). The C within MAOM complexes may persist due to i) the organo-mineral bond strength, ii) spatial inaccessibility due to occlusion within aggregates and, iii) limitations on microbial activity (spatial separation or abiotic constraints such as temperature or moisture which constrains enzyme diffusion for example) (Baldock and Skjemstad, 2000; Dungait et al., 2012; Kleber et al., 2015; Schmidt et al., 2011). However MAOM also contains a dynamic portion with a faster turnover time (sub-annual). This may be an important nutrient source for plants and soil microbes (Jilling et al., 2018) and susceptible to land management losses (Bischoff et al., 2016; Schrumpf et al., 2013; Torn et al., 2013).

MAOM can be isolated from the soil by physical fractionation (e.g. Bradford et al., 2008; Cotrufo et al., 2019; Sokol and Bradford, 2019), but may also include additional chemical or density-based separation stages to isolate additional light or heavy POM fractions, and coarse MAOM (e.g. Córdova et al., 2018; Samson et al., 2020; Witzgall et al., 2021; Wu et al., 2022; Ye et al., 2018). Despite predominantly isolating two pools of SOC, the distinct characteristics of MAOM and POM (summarised in **Table 1.1**), provides a suitable means to understand the formation and vulnerability of MAOM-C, and better inform land management practices focused on SOC accrual (Lavallee et al., 2020; Lugato et al., 2021). A caveat to this is that the fractionation requires total soil dispersion prior to separation. Lavallee et al., 2020, justify this on the basis of aggregates (greater than 50 – 63 μm), being composite mixes of MAOM and POM. From a resource and practicality perspective, excluding further separation

steps saves time and resources, providing a clear and simple method to compare SOM dynamics at scale (e.g. Cotrufo et al., 2019).

Table 1.1. Summary of MAOM characteristics, information from Lavallee et al., 2020.

	POM	**MAOM**
Dominant chemical constituents	Plant and fungal derived	Low molecular weight compounds of plant and microbial origin
Density (g cm^{-3})	< 1.6 to 1.85 for light or > 1.6 to 1.85 for heavy POM	> 1.6 to 1.85
Size (µm)[a]	> 50 to 63	< 50 to 63
C:N ratio	10 to 40	8 to 13
Residence time	< 10 years - decades	Decades – millennia

[a] The upper size limit of 50 – 63 µm varies with region (Lavallee et al., 2020; Totsche et al., 2018).

1.3 MAOM Formation

Empirical evidence of microbial by-products in MAOM supports the notion that OM undergoes microbial assimilation, biosynthesis and turnover prior to inclusion with MAOM; so called microbial mediated or *in vivo* MAOM formation (Bradford et al., 2013; Kallenbach et al., 2016; Liang et al., 2017; Miltner et al., 2012). However, Sokol et al., 2018, highlights the presence of low molecular weight organic carbon (LMWOC) compounds with plant signatures also found in MAOM complexes. This indicates the existence of an alternative formation pathway – a direct sorption or *ex vivo* MAOM formation pathway, see **Figure 1.2.**.

The understanding of the formation of MAOM builds on the "*Microbial Efficiency-Matrix Stabilisation"* (MEMS) framework presented by (Cotrufo et al., 2013), which synthesised emerging concepts recognising the role of soil microbial community, interactions with soil mineral matrix and input quality, in the formation and persistence of SOM. Central to this framework is the understanding that soil microbes act not only as agents of SOM decomposition, but also contribute to SOM formation (Bradford et al., 2013; Lehmann and Kleber, 2015; Schimel et al., 2012; Schrumpf et al., 2013). The high contribution of microbially derived compounds in MAOM (Miltner et al., 2012) and lower C:N ratio of MAOM (Giannetta et al., 2018), confirm the presence of a microbially mediated MAOM formation pathway. The close proximity between minerals and microbes, facilitates the formation of MAOM via the sorption of microbial cells, debris, exopolysaccharides and root exudates on mineral surfaces (Lehmann and Kleber, 2015; Schimel et al., 2012).

The presence of compounds with plant signatures in MAOM, suggests an alternative formation pathway (Sokol et al., 2018). The persistence of these compounds arises due to

direct sorption of partially metabolised compounds, or compounds mobilised by microbial enzymes to mineral surfaces, without microbial assimilation (Córdova et al., 2018; Sokol et al., 2018).

Whilst the MEMS framework focused on the first stage of organo-mineral association formation, once a compound is associated with minerals, it undergoes an iterative cycle of adsorption, desorption and exchange reactions (Lehmann and Kleber, 2015). Higher affinity compounds (e.g phenolic and proteinaceous compounds) from fresh OM entering the topsoil, displace older lower affinity compounds (e.g polysaccharides) within MAOM complexes (Mikutta et al., 2019). Resulting in compositional and age differences in MAOM with depth, with lower affinity and older compounds increasing down the soil profile (Mikutta et al., 2019). A more holistic understanding of the role of the two formation pathways throughout the whole soil profile, within various agri-ecological contexts (i.e considering microbial activity and composition, soil mineralogy, climate, and land management) is needed to better represent the mechanisms influencing MAOM-C persistence.

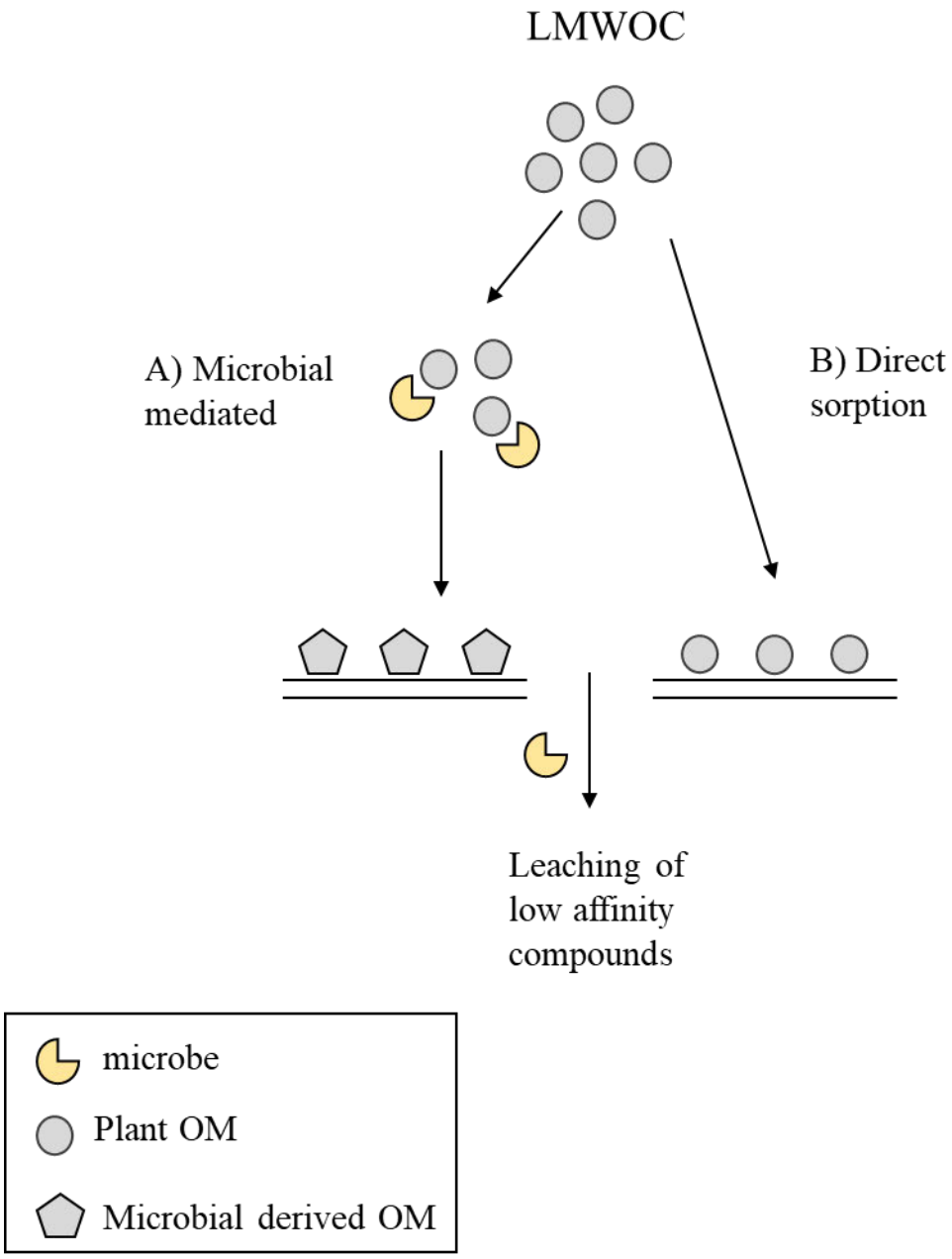

Figure 1.2. Pathways of mineral associated organic matter (MAOM) formation from low molecular weight organic carbon (LMWOC) compounds in the soil. Microbial mediated formation (A) and direct sorption (B), followed by remobilisation and downwards movement of lower affinity compounds. Diagram adapted from Sokol et al., 2018 and Mikutta et al., 2019.

1.3.1 Factors influencing MAOM-C formation

LMWOC compounds such as sugars, amino acids, organic acids and phenolics, account for the majority of the diversity of root exudates, (Bais et al., 2006). Although LMWOC compounds account for 4 to 25% of DOC in the soil (Van Hees et al., 2008), they are estimated to fuel 30-50% of heterotrophic soil respiration, and are a core substrate for microbial growth and turnover (Van Hees et al., 2005). LMWOC compounds from plants vary with species and environment, (Bais et al., 2006). Therefore, LMWOC compounds such as glucose, glycine, oxalic acid, alanine, alone or in combination are frequently used in studying the dynamics of MAOM-C (Kallenbach et al., 2016; Oldfield et al., 2018; Sokol and Bradford, 2019). Such work brought to light the influence of substrate quality, microbial physiology, community composition, and soil nutrient status, on the formation pathway and rate of MAOM-C accumulation.

Microbial uptake outcompetes mineral sorption for sugars (glucose), some amino and monocarboxylic acids (alanine and acetic acid). In contrast, compounds with higher sorptive affinity such as polyvalent carboxylic acid (citric, oxalic), phenolic acids, and lignin monomers (Jagadamma et al., 2012), are more likely to directly adsorb to soil mineral surfaces than compounds of lower affinity (Sokol et al., 2018). Consequently, at the initial stages of MAOM formation, the type of LMWOC compound influences the route with which it may be incorporated within MAOM complexes. The direct sorption pathway may also be more prominent in regions of the soil with lower microbial density such as the bulk soil, compared to the rhizosphere or with increasing depth (Sokol et al., 2018).

Once assimilated by the soil microbial community, the fate of C within LMWOC compounds depends on microbial physiology. The carbon use efficiency (CUE), the amount of C allocated to growth versus other processes such as respiration, of total C uptake (Geyer et al., 2019; Kallenbach et al., 2016), has a direct impact on the quantity of microbial residue production, and subsequent MAOM formation (Cotrufo et al., 2013). Factors influencing CUE include inherent differences between specific microbial species (e.g different growth and turnover rates) and external environmental influences, such as substrate availability and temperature (Geyer et al., 2019). Current theory suggests that a higher CUE leads to greater microbial mediated MAOM formation, due to relatively greater biomass and microbial residue synthesis available for organo-mineral associations (Cotrufo et al., 2013; Frey et al., 2013; Kallenbach et al., 2015; Liang et al., 2017; Malik et al., 2018). However, in instances where higher CUE coincides with reduced gross C uptake, lower respiration and no change in biomass production, it is of no benefit to C accumulation via the microbial pathway (Kallenbach et al., 2019). Further to this, Córdova et al., 2018, found that the addition of high quality plant residues (oat and alfalfa, with high C:N ratios), led to greater absolute, but less efficient, MAOM accumulation, than low quality litters (maize and soybean with low C:N ratios). This was possibly due to greater recycling of microbial biomass and by-products initially derived from the litter (Córdova et al., 2018).

It is important to consider the substrate induced changes in microbial community composition, and the consequences for CUE (Kallenbach et al., 2016). Additions may favour emergence of copiotrophic communities, with an inherently lower CUE (Fierer et al., 2007; Fischer et al., 2010). Indeed, Oldfield et al., 2018, found that higher substrate addition rates resulted in greater absolute substrate C retention in MAOM, but less per unit C added. This

was attributed to microbial community composition changes (Oldfield et al., 2018). Such changes are important to consider in circumstances with varying substrate availability, and how that may impact MAOM-C formation.

Evidence suggests that the nutrient status of the soil influences the retention of substrates of differing qualities within MAOM. Despite suggestions that higher quality litter (with low C:N and fast decomposition rate) results in faster, more efficient formation of MAOM-C (Cotrufo et al., 2013), a review by Castellano et al., 2015, did not find this to be the case. The effect of litter quality, may be a function of SOC content and is more evident in soils with more MAOM-C (closer to saturation) than those with less (Castellano et al., 2015). In agreement with this Wu et al., 2022, found substrate C retention to be determined by soil C saturation level. Soil nutrient status also influences the pathway by which compounds are incorporated in MAOM. In nutrient poor soils, MAOM formation via direct sorption may be the primary formation pathway, as microbes mine OM for nutrients, the by-products of which directly sorb to mineral surfaces (Córdova et al., 2018). Highlighting the role of soil nutrient status influencing the formation pathway, and rate of MAOM-C accrual.

Increasing C within MAOM can provide a means to mitigate the effects of climate change (Bossio et al., 2020). However, the response of plant and microbial productivity to climate change may alter SOC allocation within the soil fractions (Jandl et al., 2014). Much of the focus of the effects of soil warming on SOC has been on its decomposition. A recent meta-analysis found no clear effect of soil warming on MAOM-C (Rocci et al., 2021), however much less attention has been paid to the effects of warming on the formation of persistent SOC (Oldfield et al., 2018). With increases, decreases or no change in microbial CUE and

turnover being observed in the literature (Creamer et al., 2015; Frey et al., 2013; Li et al., 2018), the subsequent implications for MAOM-C are rarely considered. Oldfield et al., 2018, found a negligible effect of temperature (20 versus 25^{o}C) on SOM formation. Interestingly they found a small increase in substrate C within microbial biomass at higher temperatures (Oldfield et al., 2018). This may result in more substrate derived C within MAOM, but any losses of native MAOM-C, as a consequence of greater microbial activity, needs to be accounted for to determine the net effect on total MAOM-C. More work is required to determine effects of increasing temperature on MAOM-C formation, accrual, and persistence. Overall the theoretical link between higher CUE and greater microbial mediated MAOM accumulation is lacking empirical evidence (Sokol et al., 2022).

1.4 Grassland Management

In a European wide study, it was found that C in grasslands is predominantly stored within the MAOM fraction (Cotrufo et al., 2019). Consequently, it is necessary to consider the effects of agricultural grassland management on MAOM-C, not just total SOC, in the context of increasing persistent SOC through suitable land management. This section focuses on the effects of i) soil disturbance due to ploughing associated with reseeding events, ii) introduction of legumes, iii) addition of N fertilisers and iv) pH management by liming, as these are the most relevant in the context of this thesis. It is acknowledged that additional grassland managements such as grazing regime (Hewins et al., 2018; Mosier et al., 2021), mowing events (Gilmullina et al., 2020), the addition of organic amendments, such as manures (Fornara et al., 2020; Maillard et al., 2015; Samson et al., 2020) and management intensity (Grayston et al., 2004), are all likely to influence MAOM-C dynamics as well.

1.4.1 Soil disturbance due to ploughing events

Reseeding of grassland swards, is an essential part of maintaining sward productivity (Carolan and Fornara, 2016; Reinsch et al., 2018). However, reseeding events are associated with high levels of disturbance due to ploughing, causing changes to soil structure, with consequent impacts on microbial activity, nutrient cycling, and the formation and persistence of SOM (Carolan and Fornara, 2016; Drewer et al., 2017; Necpálová et al., 2014; Soussana et al., 2004). Studies assessing the impacts of reseeding events primarily focus on total SOC stocks and gaseous losses (Necpálová et al., 2014; Reinsch et al., 2018; Willems et al., 2011), with relatively few examining impacts on SOC fractions (Carolan and Fornara, 2016; Fornara et al., 2020; Linsler et al., 2013). Changes in MAOM-C accounted for 65% of the difference in C between chisel and no till in an arable system (Plaza et al., 2013). However, in grasslands systems it appears that reseeding has little impact on MAOM-C irrespective of time since, and frequency of reseeding events, all with traditional mouldboard ploughing to a depth of 20 to 25 cm (Carolan and Fornara, 2016; Fornara et al., 2020; Linsler et al., 2013). A decline in MAOM-C as a result of reseeding, may be offset, by microbial activity, and sward regrowth. The rise in CO_2 after reseeding events has been attributed to increased microbial activity due to the creation of favourable conditions associated with soil tillage; increased soil aeration and temperature, and greater access to OM due to aggregate destruction (Necpálová et al., 2014; Reinsch et al., 2018; Willems et al., 2011). Recovery of dense root biomass may also facilitate MAOM formation due to C inputs and promotion of aggregates (O'Brien and Jastrow, 2013; Rasse et al., 2005; Sokol and Bradford, 2019).

1.4.2 Legumes

The incorporation of N_2 fixing legumes, can be used to reduce reliance on N fertilisers, and generally has a positive impact on soil physical, chemical and biological properties (Rumpel et al., 2015). Fornara and Tilman, 2008, found that diverse grass-legume mixes increase total SOC, but it is necessary to identify which fractions C is accumulating. Canarini et al., 2018, observed a positive relationship between legume biomass and MAOM-C. This may be due to high plant residue quality stimulating microbial growth and activity (Canarini et al., 2018; Cong and Eriksen, 2018). Changes to the quantity and variety of compounds from rhizodeposition, and plant residue senescence may alter microbial community composition (Grayston et al., 2004; Lange et al., 2015), with potential implications for microbially mediated MAOM-C accrual. Grayston et al., 2004, found a positive correlation between gram -ve bacteria and *Lolium perenne* and *Trifolium repens*, across swards with differing sward compositions and agricultural management intensity. Gram -ve bacteria are known to utilise root exudate C, which may increase MAOM-C due to increased activity and subsequent by-product formation (Fornara et al., 2011; Holland et al., 2017; Treonis et al., 2004). Canarini et al., 2018, also linked legumes to microbial biomass N content, which is important in the formation of organo-mineral associations (Kopittke et al., 2020). In contrast, Prommer et al., 2019, found the inclusion of legumes reduced grass root biomass (due to lower N requirement), and subsequently reduced microbial growth, biomass and turnover, reducing SOC concentrations.

1.4.3 Nitrogen fertiliser

N availability impacts SOM processing due to C and N stoichiometry from inputs, and whether this meets microbial demand (Lavallee et al., 2020). Empirical evidence supports the

important role of N in the formation of organo-mineral and organo-organic associations (Kleber et al., 2015; Kopittke et al., 2020; Possinger et al., 2020). Cotrufo et al., 2019, attribute greater C in MAOM in grasslands to higher labile C inputs from grassland species, and N from fertiliser application which alleviates the high N requirements of MAOM formation. However, the effects of N fertilisers on MAOM-C accumulation remain unclear, with increases (Cenini et al., 2015; Neff et al., 2002), decreases (Chen et al., 2020) and no effects observed (Riggs et al., 2015).

Cenini et al., 2015, observed an increase in MAOM-C after 19 years of ammonium nitrate addition in a UK grassland. This coincided with an increase in the glucose degrading enzyme, β-1,4-glucosidase. They proposed that the lower C:N ratio of plant material, increased microbial C demand, and subsequent enzyme production increased the formation of microbial metabolites, increasing MAOM-C (Cenini et al., 2015). Similarly, Neff et al., 2002, found that 10 years of N addition increased POM decomposition but also increased C within MAOM, in an alpine tundra meadow. Additionally microbial mediated formation of MAOM-C may be increased by N-induced increases in plant OM, which serves as an important hotspot of microbial activity (Witzgall et al., 2021), and preventing C losses from MAOM due to mining of N within MAOM, in N limited systems (Jilling et al., 2018).

Soil acidification due to N addition, may increase MAOM-C due to changes in soil pH, increasing the solubility of aluminium (Al) and iron (Fe) cations and increased mineral surface reactivity (Riggs et al., 2015). However, iron oxides can inhibit microbial activity, reducing microbial derived C accrual (Wang et al., 2022). However, Riggs et al., 2015, found no effect of N enrichment on MAOM-C despite decreased pH, possibly due to low clay

content of soils, and short duration (5-3 years) of the experiment. Conversely, in a subtropical forest, Chen et al., 2020, attributed the decline in MAOM-C to supressed microbial activity and reduced direct sorption due to leaching of cations (specifically Ca^{2+}) as a result of N-induced soil acidification. Additionally, inorganic nutrients may block mineral sorption sites hindering the formation of new organo- mineral associations (Zhao et al., 2020).

1.4.4 Liming

Liming, the application of magnesium and calcium rich materials, to ameliorate acidity and enhance productivity is a global practice (Fornara et al., 2011; Goulding, 2016; Holland et al., 2017). The alteration of the soil environment due to liming may affect MAOM-C dynamics, by both the microbial mediated formation and direct sorption pathways. Direct sorption of OM to minerals surfaces may be increased due to the addition of polyvalent cations, forming organo-mineral associations via cation bridging, or direct sorption to Mn-oxides (Kleber et al., 2015; Rasmussen et al., 2018; Rowley et al., 2018). Liming induced changes in the abundance, composition and activity of the microbial community (Grover et al., 2017; Holland et al., 2017), alters the existing balance between microbial mediated MAOM-C formation and losses of MAOM-C. However, there is little data available linking liming practice and SOC stocks, making it hard to derive definitive conclusions on the relationship (Paradelo et al., 2015). Of four grassland papers included in the review by Paradelo et al., 2015, only one isolated a MAOM fraction. Fornara et al., 2011 reported a ~137% increase in MAOM-C in ungrazed limed plots, compared to unlimed plots, despite reduced CUE. They suggest higher total microbial biomass and activity and changes in community composition (increase gram- bacteria) contributed to an increase in MAOM-C (Fornara et al., 2011).

1.5 Soil Carbon Saturation

A limitation of SOC sequestration within MAOM, arises due to soil C saturation The concept of saturation or a limited protective capacity of organo-mineral associated C is generally well established (Castellano et al., 2015; Hassink, 1997; Kimetu et al., 2009; Smith, 2014; Stewart et al., 2009). The basis of saturation theory is that an upper threshold of C accumulation within MAOM complexes exists due to limited number of available binding sites on mineral surfaces (Hassink, 1997; Six et al., 2002) , and thus is dependent on a soils physiochemical characteristics (Kimetu et al., 2009). When a soil is at saturation, any further increases in steady state C input, do not result in a higher level of SOC, in either the whole soil, or a soil fraction, **Figure 1.3** (Castellano et al., 2015; Orgill et al., 2017).

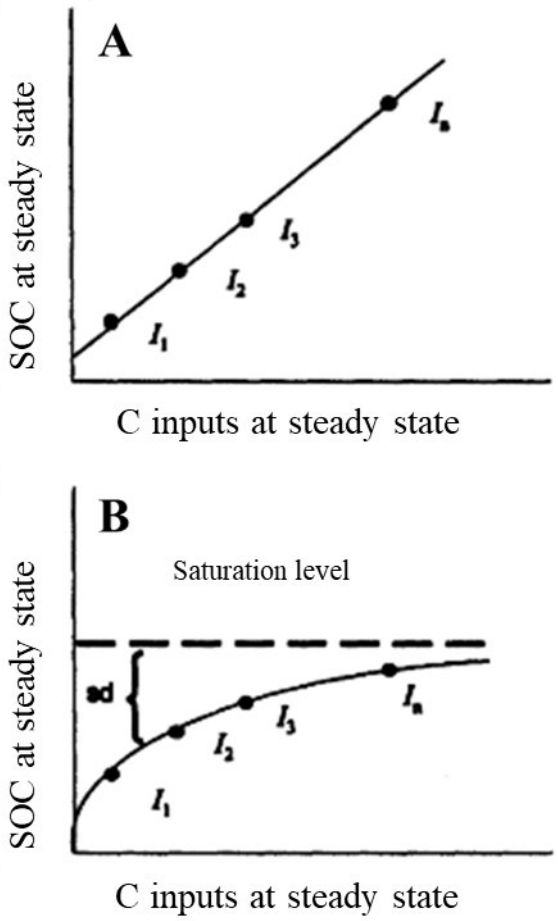

Figure 1.3. Relationship between C input and SOC without (**A**) and with saturation behaviour (**B**). Figure adapted from Stewart et al., 2007.

Hassink, 1997, and Six et al., 2002, found positive correlations between fine soil fraction and C within these fractions. In a comparison of paired Dutch arable and grassland soils, Hassink, 1997, found that whole soil SOC was significantly different, but fine-fraction C (< 20 µm) was not. Consequently, Hassink, 1997, proposed that the saturation point of the fine soil fraction could be estimated by linear regression; $y = 0.37x + 4.07$, where y is the C content of the fine fraction (g C kg^{-1} soil), and x is the mass proportion of the fine fraction in a soil sample (g fraction 100 g^{-1} soil). Six et al., 2002, expanded this work further incorporating two

size cut-offs 0 – 20 µm and 0 – 50 µm, and considered differences between mineral types in arable, grassland and forest sites. Six et al., 2002, reported different slopes and intercepts to Hassink, 1997, but found the same linear relationship between the fine soil fraction and C within these fractions.

Further developments to improve estimates of maximum mineral associated C include consideration of mineral type and different mathematical estimation methods (Beare et al., 2014; Feng et al., 2013). Feng et al., 2013, highlighted the potential for linear regression to predict mean C rather than maximal C and suggested the use of boundary line (BL) regression. BL analysis uses a predetermined subset of data to estimate the boundary line, consequently when one uses the upper 90th percentile of a data set, this prevents maximum estimates being more indicative of mean C when using linear regression (Feng et al., 2013; Shatar and Mcbratney, 2004). Beare et al., 2014, found mineral surface area to be more related to SOC than mass proportion of the fine fraction, and in a multi-variate model inclusion of Al and soil pH provided best fit to predict maximum SOC. Building on the work of Feng et al., 2013, Beare et al., 2014, examined the use of quantile regression (QR) to more accurately predict maximum C. QR is more robust than BL as it doesn't reduce sample size, and makes no assumptions regarding homogeneity of variance (Beare et al., 2014; Cade and Noon, 2003). However, the relative ease, in terms of resources needed to compute the estimate, means that the simple linear regression as presented by Hassink, 1997, is still used (see Angers et al., 2011; Guillaume et al., 2022; Liang et al., 2009; Wiesmeier et al., 2014).

The overestimation of the mitigation potential of C sequestration, may mislead policy makers seeking measures for reducing greenhouse gas (GHG) emissions (Powlson et al., 2011;

Schlesinger and Amundson, 2019). Attention must also be paid to the interactions with other biogeochemical cycles, particularly the nitrogen (N) cycle, which may offset C sequestration benefits (Lugato et al., 2018; Powlson et al., 2011).The widespread implementation of C sequestration for climate change mitigation has been limited due to concerns of non-permanence, CO_2 displacement (increased soil C in one area leading to decreases elsewhere), requirement of perpetual management of the stabilised C and difficulties with verification (Horwath, 2015; Jones et al., 2017; Smith, 2012). The concerns and uncertainties mentioned makes understanding the true potential, and dynamics of additional C storage within soils imperative to realise the full potential of C sequestration for climate change mitigation.

1.6 Thesis structure

Including SOC sequestration within climate change policy frameworks requires a good understanding of the potential C sequestration capabilities. However, estimation methods are nuanced by resource inputs versus accuracy. Whether C within SOM is incorporated within MAOM complexes, and the efficiency of this incorporation is dependent on interactions between plants (OM input quality and quantity), microbes (activity and composition) and edaphic factors (mineral type, pH, and soil structure). To increase, or prevent losses from this persistent pool of SOC, it is necessary to improve the understanding of these interactions in the context of land management. However, empirical evidence for the effects of typical grassland management on MAOM-C are limited. The main aims of this thesis were to

- examine the sequestration potential across a representative sample of agricultural grasslands within the UK, and
- contribute to the understanding of MAOM-C formation in the context of land management and climate change.

The following four research chapters (Chapter 2, Chapter 3, Chapter 4 and Chapter 5) were designed to achieve these aims. Specifically, Chapter 2, achieves the first aim, and had the following objectives

- to assess the suitability of the Hassink (1997) equation to estimate maximum fine-fraction OC in UK grassland soils;
- to evaluate the linear regression, boundary line and quantile regression methods to estimate maximum fine-fraction OC; and
- to explore the relationship between sward age (time since the last reseeding event) and current and predicted maximum fine-fraction OC.

Chapters 3, 4 and 5 focus on the second aim with the following objectives:

- to determine the effect of substrate quantity and ammonium nitrate addition on the retention of substrate C within MAOM (Chapter 3),
- to determine the effect of substrate type and temperature on the retention of substrate C within MAOM (Chapter 4), and
- to examine the effect of soil pH manipulation, grass ley duration and soil depth on MAOM-C (Chapter 5).

Finally, Chapter 6 contains a general discussion of the results of the thesis, and some opportunities for future work.

Chapter 2 Estimating maximum fine-fraction organic carbon in UK grasslands

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Author contributions: KCP, SB, JMC, RMR and EMB formulated the research question and study design. KCP conducted the experimental work, data analysis and prepared the draft of the paper. All authors contributed to editing and reviewing of the paper.

The following text includes an additional chapter introduction **(2.1**) and conclusion (**2.8**). The text in between is final as published, with minor formatting changes to form a coherent thesis chapter. **Appendix A** contains supplementary information, as published (**A.1**) and a map with locations of each of the sites (**A.2**).

2.1 Chapter introduction

Increasing persistent SOC has the potential to offset rising atmospheric CO_2 (Chenu et al., 2019). Accurately estimating the potential additional C that can be retained in agricultural soils is important for the incorporation of SOC sequestration in climate change mitigation policies. However multiple concurrent processes contribute to the accumulation of SOC (Lehmann and Kleber, 2015). Therefore, a balance must be struck between resources required to make predictions, and prediction accuracy. This chapter examines the use of a relatively simple, but widely used method to estimate C sequestration potential of grassland sites across the UK. Soil used in this chapter was sampled from a combination of experimental and non-experimental farms, across differing pedo-climatic conditions in the UK.

The impact of soil disturbance, from ploughing events associated with the reseeding of swards, on fine fraction C is also considered. This generates insights into the legacy effects of typical agricultural grassland management on SOC persistence and resilience, and whether there are any implications for sequestration potential. This knowledge provides important empirical evidence which can help to guide land management which is not detrimental to SOC.

Estimating maximum fine-fraction organic carbon in UK grasslands

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2.2 Abstract

Soil organic carbon (SOC) sequestration across agroecosystems worldwide can contribute to mitigate the effects of climate change by reducing levels of atmospheric CO_2. Stabilisation of organic carbon (OC) in the fine soil fraction (< 20 μm) is considered an important long-term store of SOC, and the saturation deficit (difference between measured OC and estimated maximum OC in the fine fraction) is frequently used to assess SOC sequestration potential following the linear regression equation developed by Hassink (1997). However, this approach is often taken without any assessment of the fit of the equation to the soils being studied. The statistical limitations of linear regression have previously been noted, giving rise to the proposed use of boundary line (BL) analysis and quantile regression (QR) to provide more robust estimates of maximum SOC stabilisation. The objectives of this work were to assess the suitability of the Hassink (1997) equation to estimate maximum fine-fraction OC in UK grassland soils of varying sward ages and to evaluate the linear regression, boundary line and quantile regression methods to estimate maximum fine-fraction OC. A chronosequence of 10 grasslands was sampled, in order to assess the relationship between

sward age (time since the last reseeding event) and the measured and predicted maximum fine-fraction OC. Significantly different regression equations show that the Hassink (1997) equation does not accurately reflect maximum fine-fraction OC in UK grasslands when determined using the proportion of the fine soil fraction (< 20 μm, %) and measured fine-fraction OC (g C per kg soil). The QR estimate of maximum SOC stabilisation was almost double that of the linear regression and BL analysis (0.89 ± 0.074, 0.43 ± 0.017 and 0.57 ± 0.052 g C per kg soil, respectively). Sward age had an inconsistent effect on the measured variables and potential maximum fine-fraction OC. Fine-fraction OC across the grasslands made up 4.5% to 55.9% of total SOC, implying that there may be either high potential for additional C sequestration in the fine-fraction of these soils or that protection in aggregates is predominant in these grassland soils. This work highlights the need to ensure that methods used to predict maximum fine-fraction OC reflect the soil in situ, resulting in more accurate assessments of carbon sequestration potential.

2.3 Introduction

Carbon (C) sequestration in soils offers a significant opportunity to remove CO_2 from the atmosphere and store it in long-lived C pools (Lal, 2004; Powlson et al., 2011), with co-benefits for soil structure and functioning (Lorenz and Lal, 2018; Smith, 2012; Soussana et al., 2004). To utilise soils as a CO_2 drawdown mechanism, accurate estimates of their storage capability are required. With respect to soil organic carbon (SOC) sequestration, organic carbon (OC) stabilised via adsorption to mineral surfaces in the fine soil fraction (< 20 μm) is often regarded as the most important due to its longer residence time (Baldock and Skjemstad, 2000; Six et al., 2002). There is empirical evidence that there is an upper protective capacity limit or saturation point of the mineral-stabilised OC pool (Six et al., 2002; Stewart et al., 2007). Potential SOC sequestration (or saturation deficit) can be estimated by subtracting the current fine-fraction OC from the estimated maximum fine-fraction OC (Angers et al., 2011).

Hassink (1997) compared pairs of Dutch arable and grassland soils and found that while bulk SOC contents significantly differed among soils, fine-fraction OC did not. These findings led to the idea that the saturation point of the fine soil fraction could be estimated by linear regression using the mass proportion of the fine fraction in a soil sample (%) and the current fine-fraction OC (g C per kg soil). Several iterations of the concept have been proposed to overcome the limitations of linear regression. For example, boundary line analysis uses a defined upper or lower subset of a data set to estimate the boundary line, when a limiting response to one or more independent variables along a boundary is supported (Lark and Milne, 2016; Schmidt et al., 2000). Using the upper 90th percentile of a data set, boundary line analysis overcomes the limitation of linear regression, depicting the mean response to the

independent variable (Feng et al., 2013; Shatar and Mcbratney, 2004), which is thought to cause an underestimation of sequestration potential. Quantile regression estimates the response of a specific quartile using the entire data set. It also makes no assumptions regarding homogeneity of variance, thus increasing the robustness of the estimated maximum fine-fraction OC. In quantile regression sample size is not reduced as in boundary line analysis (Beare et al., 2014; Cade and Noon, 2003). Using a forced zero intercept overcomes the contradiction of a positive intercept, indicating the presence of fine-fraction OC without any fine soil fraction (Beare et al., 2014; Feng et al., 2013; Liang et al., 2009). To our knowledge no comparisons between the equation developed by Hassink (1997) and one generated in the same way with a different data set have been done within the UK. This type of assessment would help to determine the suitability of the Hassink (1997) linear regression equation to predict maximum fine-fraction OC in UK soils. Without this, carbon sequestration potentials may be both over- and underestimated.

In the UK, human-managed grasslands are the dominant land use, covering 36% of the land area (Ward et al., 2016). Managed grasslands are planted and maintained to increase agricultural productivity through fertiliser and liming applications and the reseeding of swards. The high levels of disturbance associated with reseeding events by mould board ploughing and harrowing in particular, result in changes in soil structure, nutrient cycling and SOC mineralisation (Carolan and Fornara, 2016; Drewer et al., 2017; Soussana et al., 2004). Organo-mineral associations form the basis of microaggregates (Baldock and Skjemstad, 2000). The destruction of aggregates makes the organic carbon protected within the aggregates more accessible for mineralisation by the soil microbial community. This may result in the increased mineralisation of existing SOC, known as the priming effect

(Kuzyakov et al., 2000). The long-term effect of such a reseeding event on SOC dynamics is understudied; it is therefore important to understand how disturbance might affect OC in the fine fraction and the SOC sequestration ability of managed grasslands.

The objectives of this study were (i) to assess the suitability of the Hassink (1997) equation to estimate maximum fine-fraction OC in UK grassland soils; (ii) to evaluate the linear regression, boundary line and quantile regression methods to estimate maximum fine-fraction OC; and (iii) to explore the relationship between sward age (time since the last reseeding event) and current and predicted maximum fine-fraction OC. We hypothesised that (i) the linear regression equation developed using UK grassland soils would be significantly different to that of Hassink (1997) and that (ii) grasslands with an older sward age would have a greater proportion of total SOC stabilised in the fine fraction (< 20 μm) and a lower sequestration potential.

2.4 Materials and methods

2.4.1 Site description and sampling

Ten grassland chronosequences covering a wide range of soil types, land use and climatic conditions were identified across the UK in 2016. The sites included the range of agricultural activity associated with UK grasslands (upland grazing, dairy, and mixed grazing), variations in soil type (organo-mineral, mineral and chalk) and the majority of UK climatic zones (**Table 2.1**). At each location, five to eight individual fields of different sward age (represented by years since a ploughing and reseeding event), ranging from 1 to 179 years, were identified for sampling. In each field, areas were avoided which had different

applications of manure, soil types or topography, headlands, areas near gates; where lime or manure had previously been dumped; or where livestock congregate. Two replicate soil cores were collected to a depth of 30 cm using a soil auger with a 2.5 cm diameter steel core and bulked to give a single composite sample. This was repeated 10 times in each field at regular intervals in a "W" shape across the field totalling 10 replicate samples per field per site. Intact soil cores for determining bulk densities were collected at three locations in each field at two depths (10 to 15 cm and 20 to 25 cm) using intact rings (7.5 cm diameter and 5 cm height). Replicate samples were sieved to 2 mm, and fresh subsamples were used to determine soil pH in water. Remaining sieved soils were dried at 40^{o}C and ball-milled prior to determination of total C and N contents (% by mass) using a Flash 2000 Elemental Analyser. Intact soils were dried at 107^{o}C and weighed to calculate dry bulk densities. Any stones were removed.

Table 2.1 Summary of UK grassland site characteristics.

Site	Age range (years)	Land Use[a]	Mean Annual Temperature (°C)[b]	Mean Annual Rainfall (mm)[b]	Elevation (m.a.s.l)	WRB Soil Type[c]	Soil Texture[c]
Aberystwyth (52°25'N 04°02'W)	2 to 33	UpG	9.5 to 11	1000	20 to 65	ST, CM	Clay to sandy loam
Crichton (55°02'N 03°35'W)	1 to 20	DP	9.5 to 9.9	1100	5 to 50	CM	Clay loam to sandy loam
Easter Bush (55°51'N 03°52'W)	3 to 6	MG	6 to 9	< 700	215 to 265	GL	Clay loam to sandy loam
Harpenden (51°48'N 00°22'W)	22 to 179	UnG	9.5 to 10.5	700	120 to 130	LV	Silty clay loam
Hillsborough (54°27' N 6°04' W)	1 to 37	DP	8.5 to 10	900	120	CM	Clay loam
Kirkton (56°25'N 04°39'W)	1 to 35	UpG	8 to 9.4	2528	163 to 170	PZ	Clay loam to sandy loam
Llangorse (51°55'N 03°16'W)	2.5 to 25	MG	8 to 10	1000		CM	Loam-Clay to Silty loam
Myerscough (53°51'N 02°46'W)	2 to 48.4	MG	9 to 10.5	1000	8 to 15	GL	Clay to sandy loam
Overton (51°48'N 02°08'W)	3 to 50	MGO	9 to 11	800	240 to 276	LP	Clay loam to silty loam
Plumpton (50°54'N 00°04'W)	1 to 20	MG	9.5 to 11	800	49 to 85 and 160 to 215	ST	Clay to clay loam, Chalky clay to chalky loam

[a] Land Use; DP; Dairy pasture, MG; Mixed grazing; MGO; Mixed grazing organic, UpG; Upland grazing, UnG; Ungrazed.
[b] Mean annual temperature and rainfall estimated from Met Office climatic region summaries, averaged over 1981 to 2010.
[c] World Reference Base (WRB) Soil Type: ST; Stagnosols, CM; Cambisols, GL; Gleysol, LV; Luvisols; PZ; Podzol; LP; Leptosol. Soil type and texture determined from GPS locations and UK Soil Observatory Map viewer.

2.4.2 Soil fractionation

The fine fraction (< 20 μm) of the soil was separated using a combined ultrasonic dispersion and sedimentation method adapted from Hassink (1997). Briefly, 20 g of dried sieved soil was soaked in 100 mL of deionised water for 24 h. The suspension was then sonicated with a Microson XL2000 Ultrasonic Processor for 20 min at 20 W in 50 mL centrifuge tubes, surrounded by ice to prevent overheating. The separated samples were recombined in 150 mL tubes and shaken end over end to disperse the soil water suspension. Sedimentation times were determined using a table applying Stokes' law for 20 μm particles, a particle density of 2.65 g cm^{-3} and sedimentation depth of 5 cm at temperatures between 20 and 35^{o}C (Jackson, 2005). After the appropriate sedimentation time, the fine fraction was siphoned off the soil suspension. The fine fraction was dried for 24 h at 107^{o}C and ball-milled prior to total C and N analysis (% by mass) using a Flash 2000 Elemental Analyser to determine the current OC content of the fine fraction. At each site, a minimum of three fields varying in age (young, intermediate and old at that location) were selected, and 3 of the 10 replicate field samples were selected at random for fractionation. Hydrochloric acid (HCl) fumigation was used to remove carbonates from the Plumpton samples. Ball-milled samples, in silver capsules, were moistened with deionised water (1: 4, sample: water ratio) to aid the efficiency of carbonate removal by HCl fumes (Dhillon et al., 2015). The samples were placed in a vacuum desiccator with a beaker of 100 mL of 12 M HCl for 24 h and subsequently dried in a ventilated oven at 60^{o}C for 16 h to remove excess moisture and HCl (Dhillon et al., 2015). Total C and N contents were determined as outlined above.

2.4.3 Statistical analyses

All statistical analyses were carried out using R software version 3.5.3 (Team, 2019). Significant differences were determined by ANOVA's and post hoc Tukey's tests ($\alpha = 0.05$). Where assumptions of normality and variance were not satisfied by testing (Shapiro–Wilk test and Levene's test), significant differences were identified using the Kruskal test and post hoc Dunn's test. A Kendall's tau (τ) correlation matrix was produced using the "corrplot" package (Wei and Simko, 2017).

2.4.3.1 Regression analyses

Linear regression was used to predict maximum fine-fraction OC, with the mass proportion of the fine fraction (< 20 μm, %) in a sample and the measured OC of the fine fraction (g C per kg soil) as the independent and dependent variables, respectively. Regression equations were developed for the combined UK data set and the individual sites. Linear regression with a forced zero intercept was used with data from this study and the data published in Hassink (1997). Boundary line analyses were performed as an alternative to linear regression, both with and without a forced zero intercept to predict maximum fine-fraction OC for all UK sites. The data were organised by mass proportion of the fine fraction (%) and divided into subgroups at 5 %, 10% and 15% intervals. The 10% interval reflects the method of Feng et al. (2013), whilst the 5% and 15% intervals were used to assess the effect of the interval on estimation of maximum fine-fraction OC. The groups were then ordered by measured fine-fraction OC (g C per kg soil), and the values in the 90th percentile were used to plot the boundary line. Boundary line analysis was not used for individual sites, as it resulted in too few data points. Quantile regression analysis was performed in RStudio using the "quantreg" package (Koenker, 2019), for the 90th and median percentiles ($\tau = 0.90$ and $\tau = 0.50$).

Forcing the intercept to zero overcomes the paradox of having C stabilised as mineral-associated organic carbon (MAOC) without any fine fraction in the soil. Significant differences between slopes were identified using the "lsmeans" package (Lenth, 2016), followed by post hoc Tukey's tests ($\alpha = 0.05$).

2.4.4 Carbon saturation ratio

The carbon saturation ratio was determined to identify the degree of saturation across the sites. The carbon saturation ratio was calculated by dividing the current fine-fraction OC by the estimated maximum fine-fraction OC content. Values < 1 were deemed undersaturated; 1 is at saturation; and values > 1 were deemed oversaturated.

2.5 Results

2.5.1 Current C concentrations

The measured total SOC and fine-fraction OC concentrations exhibited variation within the grassland sites (**Figure 2.1**). Total SOC varied from 8.2 to 85.8 g C per kg soil, with a median of 32.7 g C per kg soil. Hillsborough, Overton and Plumpton had significantly higher total SOC, whilst Harpenden and Llangorse had the lowest total SOC ($p < 0.05$) (**Figure 2.1**). The measured fine-fraction OC ranged from 1.4 to 20.9 g C per kg soil, with a median of 6.2 g C per kg soil. Overton had the highest total fine-fraction OC ($p < 0.05$) and was the only organically managed site (**Figure 2.1**). The proportion of OC stabilised in the fine-fraction (< 20 μm) had high variability across the UK sites accounting for 4.5 to 50.1% of total SOC with a median of 17.5%. The proportion of total SOC stabilised in the fine-fraction (< 20 μm), and proportion of fine-fraction in a sample did not significantly differ in Harpenden and

Overton, however they have significantly different measured fine-fraction OC contents (g C per kg soil) ($p < 0.05$), indicating different saturation potentials (**Figure 2.1**). Soil C: N ratio was positively correlated with fine-fraction C: N (0.30, $p < 0.0001$; **Table 2.2**); however there was no relationship between bulk soil C: N ratio and proportion of fine-fraction (data not shown). The fine-fraction and bulk soil C: N ratios were significantly different between the sites, (**Figure 2.1**). The mean value of fine fraction showed little deviation, 9.84 ± 1.00 (mean ± standard deviation). Full details of all the measured properties of bulk and fine fraction, per field are presented in **Table A.1** and **Table A.2**.

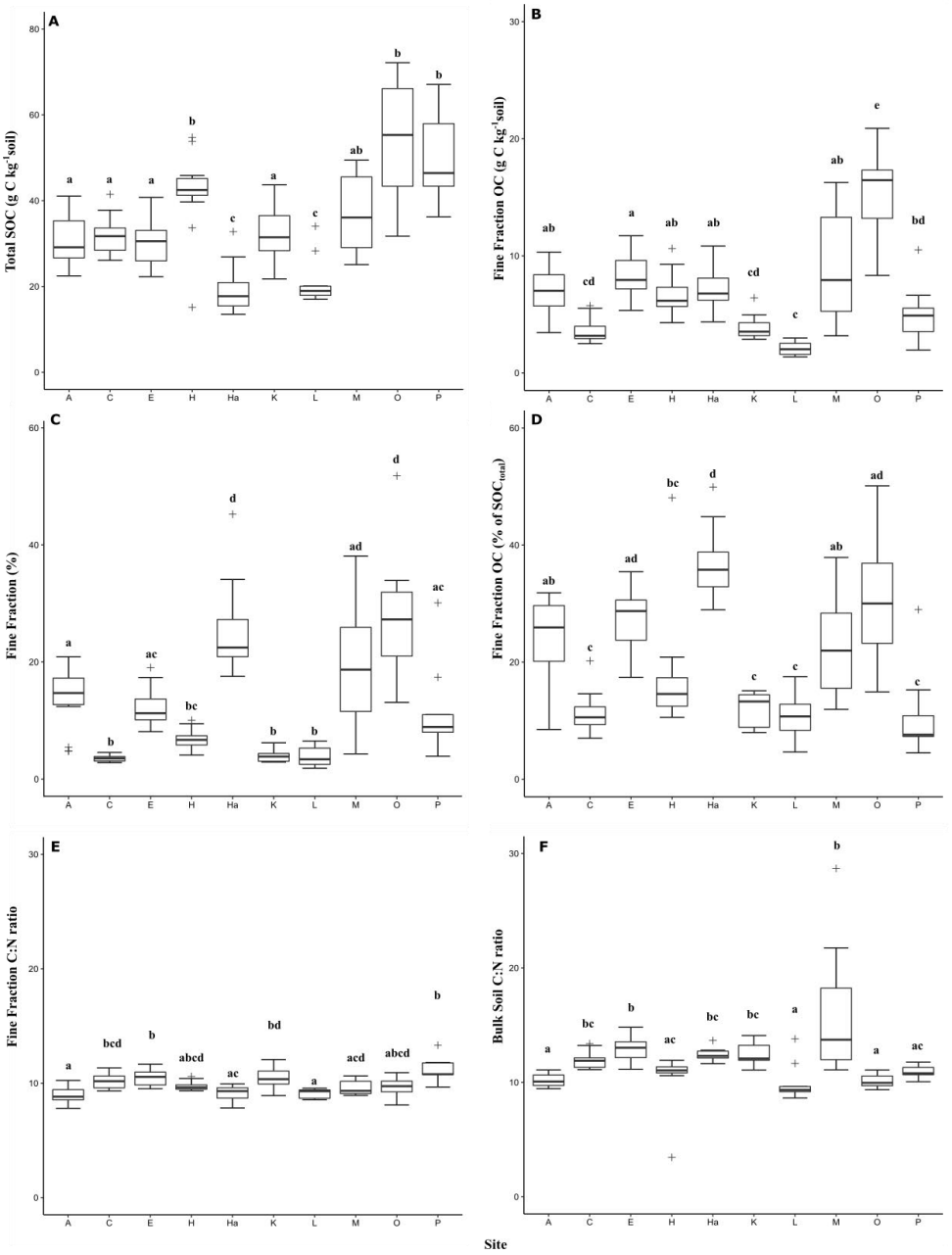

Figure 2.1. Measured total SOC (g C kg^{-1}soil) (**A**) total fine-fraction organic carbon (g C kg^{-1}soil) (**B**), mass proportion of fine-fraction (< 20 μm, %) (**C**), relative proportion of measured fine-fraction organic carbon of the total SOC content of the bulk soil (**D**), fine-fraction C:N ratio (**E**) and bulk soil C:N ratio (**F**), for each of the grassland sites; Aberystwyth (A), Crichton (C), Easter Bush (E), Hillsborough (H), Harpenden (Ha), Kirkton (K), Llangorse (L), Myerscough (M), Overton (O) and Plumpton (P). Boxes represent the 25th and 75th percentile, with lines showing the median value. Whiskers show the lowest and highest values with outliers indicates as crosses (> 1.5 times the interquartile range). Lettering indicates significant differences between soils ($p < 0.05$).

The significance of correlations between the measured soil properties, time since reseeding and known environmental factors were analysed. The matrix of Kendall tau (τ) correlation coefficients in **Table 2.2**, revealed that measured fine-fraction OC was positively correlated with median annual temperature ($\tau = 0.13$, $p < 0.05$), %N ($\tau = 0.26$, $p < 0.0001$) and %C ($\tau = 0.27$, $p < 0.0001$) in the bulk soil, and negatively correlated with mean annual rainfall ($\tau = -0.36$, $p < 0.0001$), and %N ($\tau = -0.15$, $p < 0.05$) in the fine-fraction. The mass proportion of the fine fraction and measured fine-fraction OC (g C per kg soil) were positively correlated in Cambisols ($R^2 = 0.61$, $p < 0.05$), Gleysols ($R^2 = 0.76$, $p < 0.05$), Podzols ($R^2 = 0.93$, $p < 0.05$), and Stagnosols ($R^2 = 0.88$, $p < 0.05$) (**Figure 2.2**). However, the proportion of total SOC stabilised in the fine fraction (< 20 μm), was greatest in Luvisols ($p < 0.05$) (**Figure 2.3**).

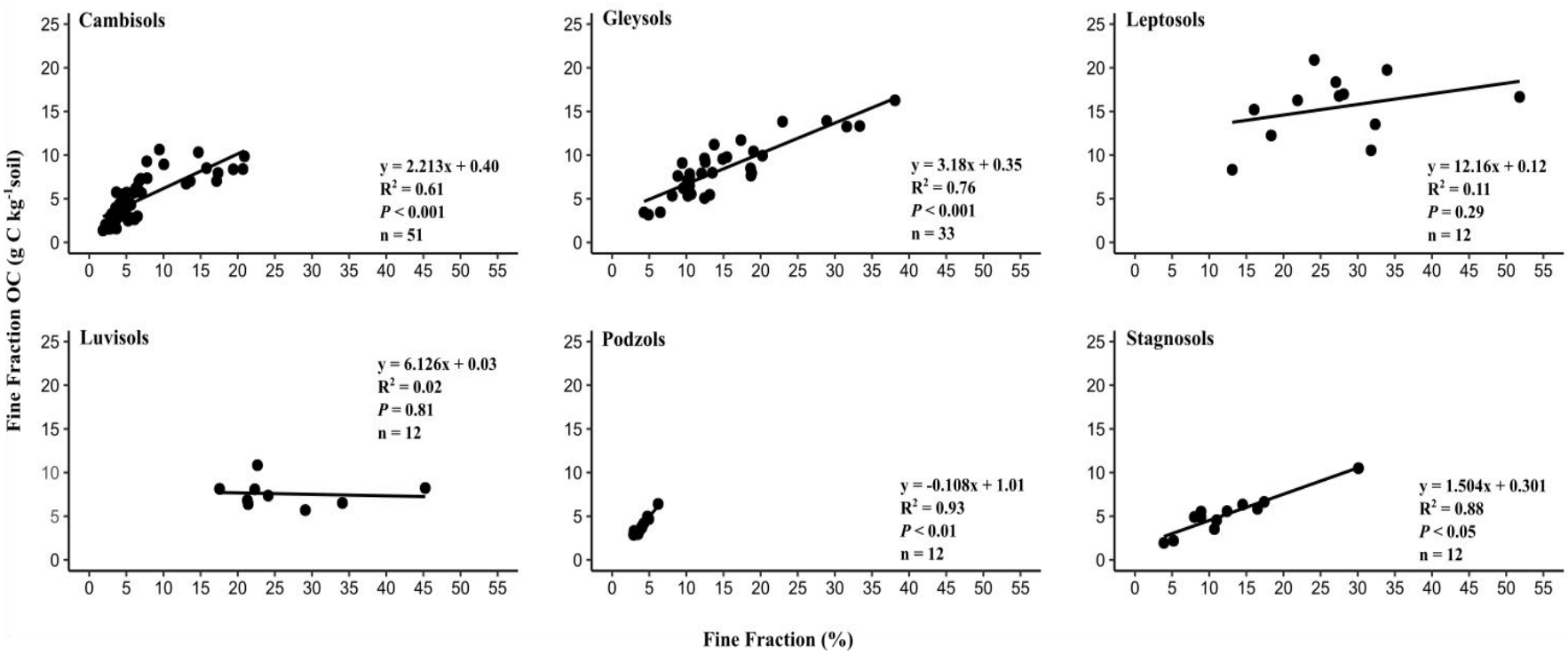

Figure 2.2. Relationships between mass proportion of the fine-fraction (%) and fine-fraction organic carbon (g C per kg soil) in the soil types used in this study.

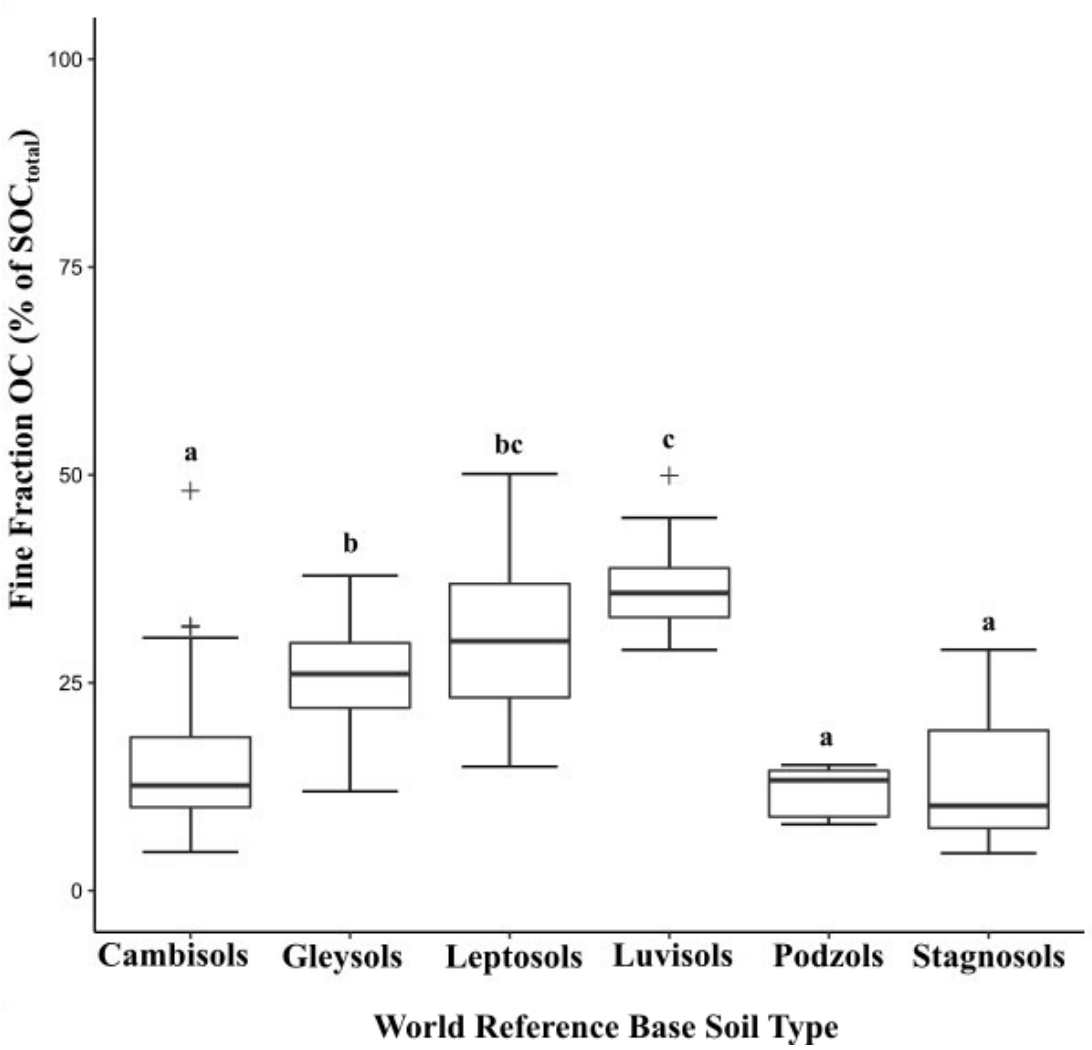

Figure 2.3. Relative proportion of measured fine-fraction organic carbon of the total SOC content of the bulk soil for the different soil types used in this study. Lettering indicates significant differences at $p < 0.05$.

Table 2.2. Correlation matrix of Kendal tau (τ) coefficients for bulk and fine-fraction (< 20 μm) soil properties, sward age and known environmental parameters.

				Bulk Soil							Fine-fraction		
		Temp.	Prec.	Age	%N	%C	C:N	pH	%SC	Fine-fraction OC	%N	%C	C:N
	Temp.	1											
	Prec.	-0.05	1										
	Age	0.15	-0.11	1									
	%N	0.23***	-0.07	0	1								
	%C	0.16*	0.06	0.04	0.73***	1							
Bulk soil	C:N	-0.25***	-0.05	0.02	-[a]	-[a]	1						
	pH	0.07	-0.30***	-0.07	-0.04	0.03	0.02	1					
	%SC	0.26***	-0.43***	0.14*	0.12	0.12	-0.01	0.21***	1				
	Fine-fraction OC	0.13*	-0.36***	0.1	0.26***	0.27***	0.01	0.12	-[a]	1			
	%N	-0.32***	0.28***	-0.07	0.17**	0.14*	-0.02	-0.27***	-0.47***	-0.15**	1		
Fine-fraction	%C	-0.33***	0.25***	-0.09	0.18**	0.17**	0.06	-0.25***	-0.47***	-[a]	0.87***	1	
	C:N	-0.21***	-0.08	-0.16	0.11**	0.21***	0.30***	-0.05	-0.15*	-0.02	-[a]	-[a]	1

[a] No correlation calculated as one variable used to calculate the other. Age; years since last reseeding event, Temp; median value from the mean annual temperature range (°C), Prec.; mean annual rainfall (mm), %SC; mass proportion of fine-fraction in a sample (%), Fine-fraction OC; measured fine-fraction OC (g C kg^{-1} bulk soil). Level of significance: * $p < 0.05$, ** $p < 0.01$, ***$p < 0.0001$

2.5.2 Estimated maximum fine-fraction organic carbon

The slope generated from the UK data used to estimate maximum fine-fraction OC (**Table 2.3**) was significantly different ($p < 0.05$) to the slope reported in Hassink (1997). There was no significant difference between the slopes generated from the UK data and the data from Hassink (1997) when estimated by linear regression with a forced zero intercept. Significantly, different ($p < 0.05$) slopes were found between the individual UK sites, owing to the range in the proportion of the fine fraction within each sample, from 1.85% to 51.8% (**Table A.3** and **Table A.4**).

Coefficients from boundary line analysis are presented in **Table 2.3**. There was no significant difference in slopes between the 5 %, 10% and 15% fine-fraction intervals used. The median-percentile quantile regression analysis had a similar slope to the boundary line and linear regression with a forced zero intercept. Quantile regression using the 90th percentile resulted in the steepest slope of all estimation methods (**Table 2.3**). The C saturation ratios revealed the difference in the number of samples with the potential to sequester more C (**Table 2.4**). The Hassink (1997) linear regression equation, without a forced zero intercept, predicted the greatest number of unsaturated sites, followed by the 90th percentile quantile regression, with a forced zero intercept. There was no clear relationship between oversaturated sites and the proportion of silt and clay contents, as oversaturation occurred across all proportions, indicated by points above the lines in **Figure 2.2**.

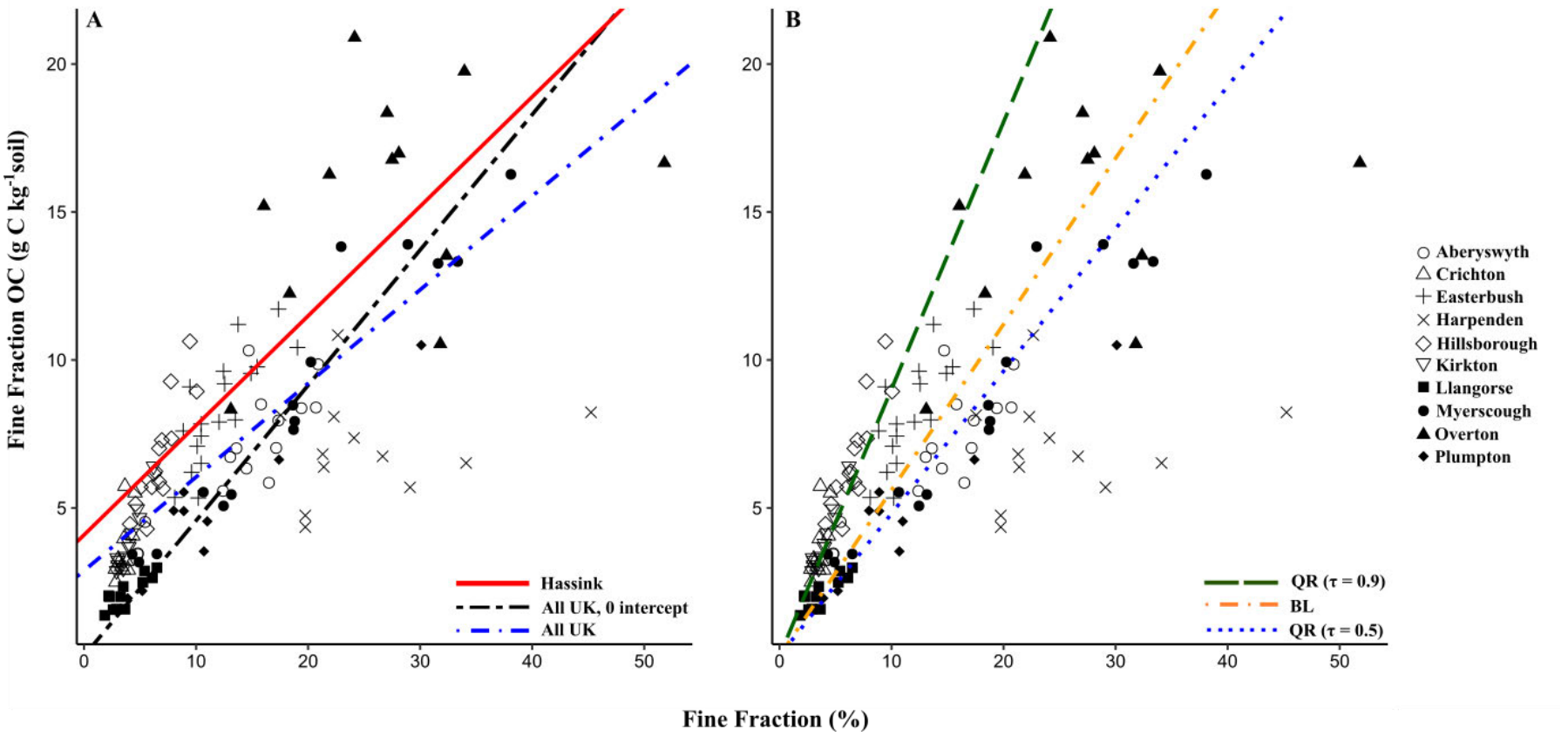

Figure 2.4. Measured fine-fraction organic carbon (g C per kg soil) in relation to mass proportion of fine-fraction of a soil sample (%). Line of best fit represent (A) linear regression method of Hassink, (1997) and data from this study, and (B) boundary line (BL) using 15% intervals, and quantile regression analysis (QR) at 90th and 50th percentiles.

Table 2.3. Analyses coefficients for the estimation of maximum fine-fraction organic carbon by linear regression (LR), linear regression with forced zero intercept (LR_0), boundary line (BL) and quantile regression (QR). Lettering indicates slopes that were significantly different within a method ($p < 0.05$).

Method		**Slope (± 1 SEM)**	***p***	**Intercept (± 1 SEM)**	***p***	**RMSE**	**n**	R^2
LR	Hassink, (1997)	0.37[a]		4.09			40	
	All UK	0.32 ± 0.023[b]	***	2.86 ± 0.368	***	2.58	129	0.61
LR_0	Hassink, (1997)[a]	0.45 ± 0.02	***			4.97	40	0.94
	All UK	0.47 ± 0.017	***			3.13	129	0.85
BL	5% intervals	0.48 ± 0.058	***			5.89	19	0.79
	10% intervals	0.48 ± 0.070	***			6.36	15	0.77
	15% intervals	0.56 ± 0.056	***			4.77	14	0.89
QR	QR (τ = 0.90)	0.92 ± 0.071	***			7.90	129	0.90
	QR (τ = 0.50)	0.49 ± 0.032	***			3.15	129	0.66

SEM: standard error of the mean. RMSE, root mean square error. Level of significance: *** $p < 0.001$.

[a] Data extracted from Hassink, (1997) used to generate slope value with forced zero intercept.

Table 2.4. Carbon saturation ratios calculated from the estimated maximum fine-fraction organic carbon by linear regression (LR), linear regression with forced zero intercept (LR_0), boundary line (BL) and quantile regression (QR). Values < 1 indicate unsaturated, = 1 at saturation and > 1 are oversaturated samples.

Method		**No. of unsaturated samples (*n* = 129)**	**Mean ratio**	**Median**
LR	Hassink, (1997)	105	0.77	0.73
	UK	75	0.98	0.94
	UK site specific	71	1	0.99
Forced 0 intercept				
LR_0	Hassink (1997)	30	1.52	1.44
	UK	34	1.47	1.39
	UK site specific	57	1.09	1.04
BL	5%	38	1.42	1.34
	10%	36	1.43	1.35
	15%	50	1.22	1.15
QR	50^{th}	38	1.4	1.32
	90^{th}	99	0.74	0.7

2.5.3 Effect of sward age on current C concentrations and estimated maximum fine-fraction organic carbon

Sward age (years since the last reseeding event) had a weak positive correlation with the mass proportion of the fine fraction (%) (**Table 2.2**). When grouped in 5-year intervals, significant differences were found between the age group and the mass proportion of the fine fraction (%), measured fine-fraction OC (< 20 μm) (g C per kg soil) and the C: N ratio of the fine fraction (**Table 2.5**); however there was no consistent increase or decrease with sward age. At each site, significant differences were observed between fields, for some properties, but again there was no consistent effect of sward age (**Table A.3** and **Table A.4**).

Table 2.5. Effect of sward age grouped at five year intervals on selected soil properties. Values are means ± standard error of the mean, and different letters indicate age groups which are significantly different ($P < 0.05$), by columns.

Age	***n***	**C:N**	**% SC**	**Fine-fraction organic carbon (g C per kg soil)**	**Fine-fraction organic carbon (% of SOC_{total})**
0 to 5	48	10.18 ± 0.15^{a}	10.00 ± 1.41^{a}	5.68 ± 0.49^{a}	18.32 ± 1.5^{ab}
6 to 10	18	9.79 ± 0.26^{ab}	14.47 ± 1.69^{b}	8.58 ± 0.59^{b}	24.94 ± 1.35^{c}
11 to 15	15	9.33 ± 0.11^{b}	15.27 ± 2.98^{ab}	9.17 ± 1.66^{ab}	20.66 ± 2.55^{abc}
16 to 20	9	10.41 ± 0.31^{a}	6.10 ± 0.77^{a}	4.68 ± 0.44^{a}	11.54 ± 1.12^{a}
21+	39	9.50 ± 0.11^{b}	15.19 ± 1.69^{b}	7.44 ± 0.69^{ab}	23.21 ± 1.94^{bc}

Age; years since last reseeding event, C:N ratio of the fine-fraction, %SC; proportion of fine-fraction in a sample (%) by mass, Fine-fraction organic carbon (% of SOC_{total}); relative proportion of measured fine-fraction OC of the total SOC content of the bulk soil.

2.6 Discussion

2.6.1 Estimation of maximum fine-fraction organic carbon

Determining the potential C sequestration capacity of soils is essential to predict the influence of land management for climate change mitigation. The determination of the saturation deficit using the mass proportion of the fine fraction and current fine-fraction OC content is an established method with a strong grounding in correlation between the variables. As mentioned earlier, previous studies have examined methods to improve estimates of maximum fine-fraction OC. However so far, no comparison has been made between the Hassink (1997) linear regression equation and one developed using grassland soils in the UK.

The significantly different slopes for the linear regression equations (**Table 2.3**) shows that the Hassink (1997) regression equation is not suitable for estimating maximum fine-fraction OC in UK grasslands. Previous concerns have focused on the potential for the equation developed by Hassink (1997) to underestimate maximum fine-fraction OC, as least-squares linear regression represents the mean response of the independent variable, rather than the maximum. For the UK grasslands in this study, estimating maximum fine-fraction OC using the Hassink (1997) regression approach resulted in a significant overestimation of fine-fraction OC sequestration potential. Future work using maximum fine-fraction OC prediction equations reported in the literature (e.g. Beare et al., 2014; Feng et al., 2014; Hassink, 1997; Six et al., 2002) should first conduct a validity test and determine if the regression equations match the soils in question or a subset of the data to ensure results are not significantly over- or underestimated.

To overcome the contradiction of an intercept greater than zero, indicating that C is stabilised in the fine fraction without any fine fraction, a forced zero intercept was used. The linear regression slopes with a forced zero intercept were not significantly different and were similar to that of Feng et al. (2013) at 0.42 ± 0.002. Liang et al. (2009) reported a lower slope of 0.36 in Chinese black soils, whilst Beare et al. (2014) reported a slope of 0.70 ± 0.03 in long-term New Zealand pastures. The range of reported values and differences across the UK sites (**Table A.3** and **Table A.4**) suggest that the effect of the proportion of the fine fraction of a sample on fine-fraction OC is not consistent and likely reflects differences in pedogenic and environmental conditions, land management and possibly the fine-fraction OC isolation method. It may be that the use of the mass proportion of the fine fraction to predict maximum fine-fraction OC is only suited for larger scales, rather than smaller, site-specific scales, as indicated by the variability in this study.

Boundary line analysis and quantile regression have been suggested as alternatives to overcome the limitations of linear regression. The estimation of maximum fine-fraction OC was greatest when using quantile regression ($\tau = 0.90$), whereas boundary line estimates at 5% and 10% intervals were similar to quantile regression ($\tau = 0.50$) and those estimated from linear regression (**Table 2.3**). The use of the median percentile quantile regression highlights the closeness of linear regression predictions being more indicative of mean values, thus underestimating SOC sequestration potential. The boundary line estimate of Feng et al. (2013), 0.89 ± 0.05, was nearly double their linear regression; this was not the case in our study. Boundary line analysis uses a subset of data to estimate, in this case, an upper limit; the data set used by Feng et al. (2013) had a wider spread of measured fine-fraction OC of 0.9 to 71.7 g C per kg soil, compared to 1.72 to 18.29 g C per kg soil in our UK soils. Therefore,

the upper subset of data was composed of higher values giving a steeper slope and demonstrates the range of data biases the C sequestration estimate generated by boundary line analysis.

The strength of using quantile regression analysis is that it makes no assumptions of homogeneity of variance and uses the entire data set to estimate the upper limit of a response. The measured fine-fraction OC in the UK sites lacks homogeneity of variance (**Figure 2.4**) where the variation in the measured fine-fraction OC increases with the proportion of the fine fraction. Standard deviation of the proportion of the fine fraction in the 10th percentile is 0.4 compared to 6.9 in the 90th percentile. Of the methods explored in this study for our grassland soils, we consider the quantile regression at the 90th percentile estimate of maximum fine-fraction OC to be the most robust. This method results in the greatest number of unsaturated samples (**Table 2.4**), suggesting great potential for additional sequestration.

When examining the estimated OC input versus existing fine-fraction OC using estimates generated by quantile regression at the 90th percentile, a positive correlation between current fine-fraction OC and estimated C input (Kendall's tau(τ); 0.323, $p < 0.001$) was observed for the entire data set. This was not the case at the site level (**Figure A.1**). In some instances, increasing fine-fraction OC (g C per kg soil) was associated with increased estimated C input until saturation, such as at Aberystwyth, Myerscough and Plumpton. Despite a higher fine-fraction OC content, these samples are furthest from saturation. In contrast the opposite was true for Crichton and Hillsborough (and Harpenden, Kirkton and Overton, although not statistically significant), implying that for these sites, samples with a higher fine-fraction OC are closer to or over saturation. It is unclear why this is the case particularly as in all sites; bar

Harpenden, there is a positive regression between mass proportion of the fine fraction and fine-fraction OC (**Table A.3**), meaning that higher fine-fraction OC is also associated with a higher mass proportion of the fine soil fraction. It is likely that the organic matter (OM) input to the soils with the higher mass proportion of the fine fraction is insufficient to bridge the gap between current and estimated maximum fine-fraction OC, as it is not possible to identify any other effect due to pedogenic or environmental conditions measured in this work. Further work investigating grasslands with similar soil types and textures and environmental conditions – but contrasting management in terms of fertiliser regimes, grazing densities, sward composition and management – may help to elucidate management factors that can be used to increase fine-fraction OC and explain the differences observed in this work.

Estimating maximum fine-fraction OC based on the mass proportion of the fine fraction is likely to be an oversimplification of the dynamics of fine-fraction OC accrual. Other parameters such as mineralogy, soil microbial community, environmental conditions (e.g. precipitation, **Table 2.2**) and land management can significantly influence fine-fraction OC stabilisation (Cotrufo et al., 2015; Kallenbach et al., 2016). This work has identified some soil and environmental properties that may play a role in fine-fraction OC stabilisation such as median annual temperature, %N and %C in the bulk soil, mean annual rainfall, and %N in the fine fraction (**Table 2.2**). Warmer median annual temperatures may enhance plant productivity and microbial processing, the byproducts of which are important precursors to fine-fraction OC (Cotrufo et al., 2013). It would be interesting to know at which point higher temperatures have a deleterious effect on fine-fraction OC accumulation. Mean annual rainfall and %N in the fine fraction were negatively correlated to fine-fraction OC. It was anticipated that fine-fraction OC would be positively correlated with fine-fraction N, as N-

rich microbial by-products have been found to form new organo-mineral associations onto which OC preferentially binds (Kopittke et al., 2018). These bonds may have been disturbed during the fractionation process, resulting in an N-rich fine fraction with less OC content. The influence of soil type on fine-fraction OC was also evident in our results, as all soil types had statistically significant positive correlations between the mass proportion of the fine fraction and measured fine-fraction OC, except for Leptosols and Luvisols (**Figure 2.2**). However, these soil types exhibited the greatest proportion of total SOC stabilised in the fine fraction (**Figure 2.3**). Luvisols have a high base saturation facilitating more fine-fraction OC stabilisation via complexation of organic ligands by free Ca^{2+} (Chen et al., 2020). Identifying soils where a greater proportion of total SOC is stored in the fine fraction is important for recognising not only where fine-fraction OC needs to be protected but also where it can be enhanced.

Whilst we consider the quantile regression at the 90th percentile method to provide the most robust estimate of maximum fine-fraction OC in the sites studied, further experimental work to test the saturation level of these soils would help to validate this. Incubation studies that force an unsaturated soil to its "saturation" level and the effect of influencing variables mentioned above will help to elucidate the factors controlling fine-fraction OC saturation. Further empirical evidence of practical methods to manipulate fine-fraction OC stabilisation processes to promote the formation of new organo-mineral associations and understand their stability is necessary to guide grassland management to enhance SOC sequestration.

2.6.2 Effect of sward age on fine-fraction OC

It was anticipated that for fields of an older sward age, a greater proportion of total SOC would be stabilised as fine-fraction OC, as tillage breaks up macroaggregates, making OC in the fine fraction available for mineralisation. Alternatively, fine-fraction OC is less sensitive to disturbance than particulate organic matter (POM), resulting in the accumulation of POM as the fine-fraction OC pool remains stable, if sufficiently saturated. The results seem to support neither hypothesis. The proportion of total SOC stabilised in the fine fraction was not consistently higher in the oldest field and, in some instances, was significantly less, such as at Aberystwyth (**Table A.2**). When grouped in 5-year intervals, significant differences in the C : N ratio of the fine fraction, the proportion of the fine fraction in a sample (%) by mass, measured fine-fraction OC (g C per kg soil) and the relative proportion of measured fine-fraction OC of the total SOC content of the bulk soil were found between age groups (**Table 2.5**). However, there was no consistent trend in the results. These data do not support the hypothesis that older swards will have a greater proportion of SOC stabilised in the fine soil fraction and a reduced potential for additional C sequestration. Equally, there was no negative correlation between sward age and the proportion of total SOC stabilised which would be supportive of the alternate hypothesis. From the data, fine-fraction OC makes up a greater proportion of SOC with increasing sward age when comparing the age groups of less than 5, 6 to 10 and 11 to 15 years. However, there is a significant decrease in the amount of SOC that is stabilised in the fine fraction in the group of 16 to 20 years; this is likely due to fields in this age range originating from Crichton, Hillsborough and Plumpton, which have some of the lowest mass proportion of the fine fraction (**Figure 2.1**, **C**). The sward age analysis may also be confounded by the variation of the proportion of the fine fraction, particularly on soil properties influenced by the mass proportion of the fine fraction such as %C and %N and current fine-fraction OC (g C per kg soil). However, it was not possible to conduct robust

ANOVA tests with a grouping variable with more than two levels. It may be possible to elucidate the relationship better from a wider study with more samples per age group, as our group of 16 to 20 years only has 9 values compared to 48 in the group of less than 5 years.

Fine-fraction OC only accounted for 4.5% to 50.12 %, indicating high OC storage in other soil pools such as POM or different aggregate fractions. The fine roots of grassland flora species promote aggregate formation (O'Brien and Jastrow, 2013; Rasse et al., 2005), which may be a dominant stabilisation process in grasslands. However previous work has found no effect of sward age or the frequency of grassland reseeding on the %C in differing aggregate fractions (>2000, 250–2000, 53–250 and <53 μm) (Carolan and Fornara, 2016; Fornara et al., 2020). The impact of reseeding disturbance may be offset due to the high density of roots in grasslands by facilitating aggregate reformation. Additionally, dissolved organic carbon from belowground inputs is more efficiently stabilised in organo-mineral associations than aboveground dissolved organic carbon (litter leachate) (Sokol and Bradford, 2019). The narrow rhizosphere to the bulk-soil ratio in grasslands may make the fine-fraction OC in grasslands more resilient to disturbance events.

2.7 Conclusions

Estimating the long-term sequestration of soil C in the fine fraction is difficult due to the lack of reliable methodologies that can be widely applied to all soils. Our study has demonstrated that the Hassink (1997) linear regression equation is not suitable to estimate maximum fine-fraction OC in a range of UK grassland soils. Therefore, caution should be applied to estimates of maximum fine-fraction OC obtained using the Hassink (1997) equation, in

instances where it may not accurately reflect fine-fraction OC of the soil in situ. After exploring various univariate estimation methods, we recommend the use of quantile regression at the 90th percentile to overcome the shortfalls of least-squares linear regression. However, such a simple estimate is unlikely to accurately reflect the dynamics of fine-fraction OC stabilisation. This work has helped to identify some key parameters that play a role in fine-fraction OC stabilisation, such as median annual temperature, mean annual precipitation, bulk-soil %C and %N and fine-fraction %N. Further work to understand how these parameters influence fine-fraction OC dynamics will help to accurately assess the feasibility of achieving soil carbon sequestration targets. Our results showed little evidence of the impact of time since the last reseeding event on the OC in the fine soil fraction. However, improving our understanding of SOC stabilisation processes and their resilience to grassland management is essential to ensure that current SOC is not only enhanced but also protected.

Author contribution

KCP, SB, JMC, RMR and EMB formulated the research question and study design. KCP conducted the experimental work, data analysis, and prepared the manuscript draft. All authors contributed to editing and reviewing of the manuscript.

Data Availability

All data resulting from this study are available from the authors upon request to Sarah Buckingham (sarah.buckingham@sruc.ac.uk)

Competing Interest Data Availability

The authors declare that they have no conflict of interest.

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2.8 Chapter conclusion

This chapter evaluated the use of a relatively simple method to estimate the maximum C content of the fine-fraction in 10 agricultural grasslands across the UK. Each of the estimation methods examined predicted different maximum C content within the fine-fraction. The QR estimate of maximum SOC stabilisation was almost double that of the linear regression and BL analysis. The differences in sequestration potential observed highlights the importance of estimation method when making predictions. Since the time of publication, Guillaume et al., 2022, reported an almost identical relationship between the proportion of fine-fraction in a soil and C within that fraction, in permanent grasslands to the one reported by Hassink (1997). However, this was not true for croplands, or sites with a land use change within the last 30 years. The significant differences between the Hassink (1997) and UK linear regression equations, demonstrates the context specificity of SOC accrual due to differences in the agri-ecological context of the grassland systems. Methods to predict maximum SOC need to account for such site-specific variance.

The exploration of the impacts of soil disturbance due to ploughing associated with reseeding events (analysed as sward age in this chapter) provided some novel insights which are beneficial for the guidance of land management. The lack of a clear effect on fine fraction C due to reseeding events suggests that these soils are resilient to these events and able to recover any C lost. Therefore, the impact of disturbance on persistent C within the fine fraction may be minimal. However further evidence at a finer temporal scale is required to accurately determine the impacts of ploughing on the mechanisms that lead to the formation and persistence of fine fraction C.

Chapter 3 The effect of glucose quantity and ammonium nitrate on mineral associated organic matter carbon formation

3.1 Introduction

The understanding of the persistence of soil organic carbon (SOC) has undergone a paradigm shift in recent years (Lehmann and Kleber, 2015). The persistence of C within soils is now thought to be primarily determined by interactions with mineral surfaces and reduced accessibility due to aggregation, rather than inherent biochemical recalcitrance (Dungait et al., 2012; von Lützow et al., 2007). The growing recognition of the importance of increasing and maintaining persistent SOC, as a means to mitigate climate change (Bossio et al., 2020), has increased focus on the processes which contribute to the formation of C within mineral associated organic matter (MAOM-C) (Córdova et al., 2018; Lavallee et al., 2018; Sokol and Bradford, 2019).

Soil organic matter inputs are broken down and transformed by soil microorganisms, and subsequently C may be lost via respiration. However, there is empirical evidence supporting the role of soil microbes in the formation of persistent SOC (Kallenbach et al., 2016; Liang et al., 2017; Miltner et al., 2012). Carbon entering the soil at microbial hotspots, such as the belowground rhizodeposition of dissolved organic carbon (DOC), is an important and efficient route of mineral associated organic matter carbon (MAOM-C) formation (Sokol and Bradford, 2019).

Carbohydrates are the most abundant compounds in root exudates, with glucose making up 40 – 50% of this (Gunina and Kuzyakov, 2015). Glucose is easily assimilated by multiple taxa (Yuan et al., 2020), and microbial uptake outcompetes direct sorption to mineral surfaces (Fischer et al., 2010). Subsequently, glucose additions to the soil provide a means to examine the microbial mediated accumulation of substrate C within MAOM, assuming consistency

with other rhizodeposited C compounds. However, uncertainty remains with regards to the effects of substrate quantity, and the role of nitrogen (N) in the accrual of fresh C within MAOM.

The carbon use efficiency (CUE), the amount of C allocated to growth versus other processes such as respiration, of total C uptake (Geyer et al., 2019; Kallenbach et al., 2016), has a direct impact on quantity of microbial residue production, and subsequently MAOM formation (Cotrufo et al., 2013). Increased C availability, may increase microbial turnover and growth, resulting in greater accumulation of microbial products for MAOM-C accrual. However, labile C addition can also alter the microbial community composition and activity (Derrien et al., 2014; Kallenbach et al., 2016). This may result in the emergence of a less efficient, faster growing community, resulting in proportionally less MAOM-C formation per unit increase in C input (Fierer et al., 2007). Subsequently higher input rates may result in greater absolute retention, but with lower efficiency (Oldfield et al., 2018). In an agricultural context additional C input may therefore result in an increase absolute MAOM-C but coincide with higher CO_2 emissions from soil respiration.

Nitrogen additions to agricultural soils from synthetic fertilisers, manure or through the introduction of N-fixing legumes, can affect the cycling of SOM (Gillespie et al., 2014). Additional N helps to alleviate N constraints on microbial activity, by narrowing the overall soil C:N ratio and increasing plant litter N content (Gillespie et al., 2014), facilitating OM decomposition and production of N-rich microbial by-products (Cotrufo et al., 2013). Imagining evidence reveals the importance of N in the formation of organo-mineral and organo-organo associations (Kopittke et al., 2020; Possinger et al., 2020). Córdova et al.,

2018, observed greater absolute MAOM-C accumulation, but with lower efficiency from plant litter with lower C:N. In a long term field trial Gillespie et al., 2014, found that N source (ammonium nitrate, manure or legume N) influenced the composition of MAOM. However N additions may also cause soil acidification, which has been associated with declines in MAOM-C due to reduced microbial activity and loss of C preserved by cation bridges (Chen et al., 2020; Ye et al., 2018).

However, less is known about the role of N addition on the fate of DOC additions, such as glucose in rhizodeposits. As glucose is a simple substrate, the subsequent incorporation in MAOM as a result of microbial transformation may differ to more complex plant matter, such as residues, that requires extracellular enzyme decomposition (Creamer et al., 2014). In a comparison of amino acid mix and glucose addition to soils, Wu et al., 2022, found that the initial soil nutrient availability had a greater effect on the retention of added substrate, than the N content of the substrate itself (Wu et al., 2022).

Recent interest in understanding the dynamics of MAOM-C has seen a growth in studies examining the fate of labile C substrates within the soil (Geyer et al., 2020; Oldfield et al., 2018; Sokol et al., 2018), rather than focusing on the uptake and mineralisation of inputs (Frey et al., 2013; Jones et al., 2019). However, to our knowledge only Wu et al., 2022, has considered the role of N in labile C retention within MAOM. The work in this chapter aims to better understand the role of substrate C quantity (in the form of glucose) and the addition of N, from ammonium nitrate, a commonly used agricultural fertiliser, in the retention of substrate C within MAOM. Specifically, we hypothesised that: i) with increasing C addition rate more absolute substrate-derived C would be recovered in MAOM, ii) the proportional

retention of substrate C within the MAOM fraction would decrease with increasing substrate C addition rate, and iii) N addition would increase absolute and proportional substrate C recovered in MAOM compared to treatments without N.

3.2 Methods

3.2.1 Soil Sampling and preparation

Soil was sampled in August 2020, to a depth of 30 cm from the Shepherd's cottage field located in the SRUC Bush Estate research farm in the southeast of Scotland (55°51'N 03°52'W). The mean annual temperature and rainfall in the area is from 6 to 9°C, and less than 700 mm respectively. The soil is a non-calcareous gley with a clay loam to sandy loam texture (Glenpark series, classified as Eutric Stagnosol according to WRB (FAO, 2006)). The field from which the soil was sampled is an agricultural grassland grazed by a mix of sheep and cattle. The soil was sieved to 4 mm and mixed to produce a homogenous sample. Large stones, roots and macro fauna were removed by hand. The sieved soil was packed into microcosms (pot height 10 cm, diameter of 5.3 cm) to a bulk density of 1.2 g cm^{-3}. The microcosms were incubated at 10°C, in the dark, for a 14-day microbial stabilisation period prior to treatment addition.

3.2.2 Experimental Design

Treatments consisted of 3 different glucose (uniformly labelled, 99 atom% ^{13}C) amendment levels; low (0.177 mg C g^{-1} soil), medium (4.425 mg C g^{-1} soil) and high (8.847 mg C g^{-1} soil), with or without ammonium nitrate (AN) addition, equivalent to 100 kg N ha^{-1}. Controls, with and without AN addition, received deionised water only, bringing all microcosms to

60% of water holding capacity. Treatments are referred to as follows: i) control, ii) control + AN, iii) low, iv) low + AN, v) medium, vi) medium + AN, vii) high and viii) high + AN. The C:N ratios of inputs to soil receiving both glucose and AN were as follows; 0.642, 16.043 and 32.085, for the low + AN, medium + AN and high + AN treatments respectively.

The microcosms were incubated in one incubator at 10°C, in the dark, in a randomised block design with three blocks. Each block contained one replicate of each glucose x AN treatment, per destructive sampling time point. Destructive sampling was done at 0.5, 8, 32 and 72 hours after treatment application, totalling 96 experimental microcosms. The deconstruction at 72 hours was chosen as previous studies have shown that microbial uptake and partitioning of low molecular weight C substrates is quasi-complete after 72 hours (Glanville et al., 2016; Jones et al., 2019).

Glucose was selected as a substrate in order to target the DOC microbial organo-mineral C stabilisation route, as its uptake outcompetes sorption with mineral phases (Gunina and Kuzyakov, 2015; Sokol and Bradford, 2019). Glucose addition rates were selected to not exceed theoretical maximum C within fine silt and clay fraction, estimated for this soil (Paterson et al., 2021, **Chapter 2**). The low addition rate is reflective of estimated root exudate inputs to the soil. The AN addition rate equivalent to 100 kg N ha^{-1} was selected as the midpoint of the typical N addition rates at the site (between 80 and 120 kg N ha^{-1}). Glucose was injected into the soil to the same depth (1 cm from the soil surface) at 5 locations in each pot, whilst AN and deionised water were applied to the soil surface. To achieve the time resolution for gas and destructive sampling, treatment additions were staggered within one day.

3.2.3 Destructive soil sampling and analysis

When the microcosms were removed from the incubator, the soil was gently homogenised and frozen immediately at -20°C as it was not possible to process the soil whilst continuing with the experimental measurements. However, at 0.5 and 72 hours, a subsample was taken for soil pH determination. Soil pH was measured in deionised water (1:2, soil:water ratio). For the remaining analysis soil was slowly defrosted and microbial biomass extractions were completed on the same day. Microbial biomass C (MBC) was estimated using the chloroform fumigation-extraction method (Vance et al., 1987). Fumigated and unfumigated samples were extracted with 0.5 M K_2SO_4 (1:4, soil to extract ratio). Fumigated samples were fumigated for 24 hours in a dark vacuum oven with chloroform. Dissolved organic carbon (DOC) concentrations were analysed using a Rosemount-Dohrmann DC-80 Total Organic Carbon (TOC) analyser. MBC was calculated as the difference between the DOC of the paired fumigated and non-fumigated samples, with a conversion factor k_{EC} of 0.45, (a predetermined factor indicating the fraction of microbial C that is extractable by fumigation) (Joergensen, 1996) and corrected for soil moisture. The remaining soil was dried at 60^{o}C until constant mass, and soil moisture was determined gravimetrically. Total soil C and N concentrations were determined using a Flash 2000 Elemental Analyser (% by mass).

3.2.4 Soil Fractionation

A subsample of dried soil was used to isolate MAOM by physical fractionation. Five grams of oven dried soil was shaken on an orbital shaker at 180 rpm for 18 hours in a dilute sodium hexametaphosphate solution (0.5%) at a soil to solution ratio of 1: 3 (Bradford et al., 2008; Córdova et al., 2018). The dispersed soil was then passed through a 53 μm sieve, and thoroughly rinsed with deionised water. Material < 53 μm was decanted into an aluminium

tray and dried at 60°C until constant mass and classified as MAOM. Material > 53 μm was backwashed into an aluminium tray and dried in the same way and classified as particulate organic matter (POM). This method may result in contamination of very fine particulate matter within the MAOM fraction, but this is expected to be minimal (Cotrufo et al., 2019). The isolated MAOM was ball milled and total C and N determined the same way as for the bulk soil.

3.2.5 Substrate derived C and N in MAOM and bulk soil

Bulk soil and MAOM fractions were analysed for ^{13}C enrichment on a 20/20 isotope ratio mass spectrometer (Sercon Ltd, Crewe, UK) after Dumas combustion of the sample in a Sercon GSL unit (Sercon Ltd, Crewe, UK). The contribution of substrate C to total SOC and MAOM-C was calculated using the following equation:

$$C_{substrate\text{-}derived} = C_{total} \times (atom\%^{13}C_{sample} - atom\%^{13}C_{control}) / (atom\%^{13}C_{glucose} - atom\%^{13}C_{control})$$

Where C_{total} is the total concentration of C in a pool, $atom\%^{13}C_{sample}$ is the $atom\%^{13}C$ value of the C pool at the sampling point, $atom\%^{13}C_{control}$ is the $atom\%^{13}C$ value of the C in the unlabelled control at the same sampling point, $atom\%^{13}C_{glucose}$ is the $atom\%^{13}C$ of the glucose added.

3.2.6 Gas analysis

Gas samples were taken at 1, 3, 5, 10, 24, 32, 52 and 72 hours after substrate addition, from the 72-hour microcosms. These sampling points where selected to provide a more detailed picture of respiration throughout the experimental period, than the destructive soil sampling

points. The microcosms used for gas sampling were fitted with airtight lids with three-way ports. Approximately 25 mL air was sampled from the headspace with a gas syringe connected to the port. This was injected into a sealed pre-evacuated glass vial and analysed on an Agilent 7890A gas chromatograph for concentrations of CO_2. Two microcosm replicates were used for gas analysis, due to time and lone working constraints within the experimental period. After sampling, the lids were removed, and a fan was used to re-equilibrate with laboratory air. Laboratory air was sampled and analysed in the same way and results were corrected for laboratory air.

3.2.7 Statistical analysis

All statistical analyses were carried out using R software version 3.6.3. Linear mixed models (LMM) were used, to determine effects of soil treatments and time on measured soil properties using the lme4 package (Bates et al., 2015). All models included carbon addition rate (control, low, medium, and high), AN addition (0 or equivalent to 100 kg N ha^{-1}) and time (0.5, 8, 32 and 72 hours) were included as fixed effects, and "block" as a random effect. The control treatment was excluded from models analysing the effect of experiment treatments on the absolute and proportional retention of ^{13}C retained, as no ^{13}C substrate was added to these treatments. Significance of effects was determined using 'anova' from the lmerTest package (Kuznetsova et al., 2017). Model fits were determined by ANOVA and lowest AIC. Interactions and terms were dropped from the models if not significant ($\alpha > 0.05$) until a minimally adequate model was reached. Differences between treatments were determined by multiple comparisons using Tukey's honest significant difference (HSD) test in the 'emmeans' package (Lenth, 2016). Residuals were visually checked using quantile - quantile and residuals versus fitted plots. Response variables that did not meet assumptions

were log-transformed. The proportional retention of substrate C within MAOM failed to meet assumptions, however, prior to log transformation the intercept was set to zero to overcome log transformation of negative values.

3.3 Results

3.3.1 Recovery of substrate ^{13}C in MAOM and whole soil

There was a significant interaction between glucose addition rate and AN on the absolute retention of substrate derived ^{13}C recovered in the MAOM fraction (**Figure 3.1**, **Table 3.1**, $P < 0.001$). Total substrate ^{13}C recovered in the medium + AN treatment (5.113 ± 0.32 µg ^{13}C g^{-1} soil, mean ± SEM, averaged over time) was significantly higher than medium treatment (2.082 ± 0.52 µg ^{13}C g^{-1} soil, mean ± SEM, averaged over time) ($P < 0.05$). There was no difference in total substrate ^{13}C recovered in MAOM between the low, and low + AN, or high, and high + AN treatments. Glucose addition rate had a significant effect on total substrate ^{13}C recovered in MAOM ($P < 0.05$). Total substrate ^{13}C recovered in MAOM increased with glucose addition rate, with 0.14 ± 0.20 µg ^{13}C g^{-1} soil, 3.60 ± 0.42 µg ^{13}C g^{-1} soil, and 4.60 ± 0.37 µg ^{13}C g^{-1} soil (mean ± SEM, averaged over time and AN), recovered in the low, medium, and high glucose treatments respectively. Although not significant at $P < 0.05$, the interaction between glucose addition rate and time was retained in the optimal model (**Table 3.1**, $P < 0.10$). At the low addition rate, substrate ^{13}C recovered in the MAOM fraction increased over time but remained constant at the medium addition rate. However, there was a decline in retention in the high addition rate treatment (**Figure 3.1**).

Similarly, there was a significant interaction between AN addition and time ($P < 0.001$), and a main effect of glucose addition rate ($P < 0.001$), on the recovery of substrate ^{13}C within the

bulk soil (**Table 3.1**, **Figure 3.2**). Substrate ^{13}C recovery was greater with AN addition 0.5 hours after addition, but declined thereafter, depending on glucose addition rate (**Figure 3.2**). Total substrate ^{13}C recovered in the bulk soil increased with glucose addition rate, with 0.51 ± 0.15 µg ^{13}C g^{-1} soil, 3.26 ± 0.30 µg ^{13}C g^{-1} soil, and 5.05 ± 0.33 µg ^{13}C g^{-1} soil (mean ± SEM, averaged over time and AN), recovered in the low, medium, and high glucose treatments respectively. There was a significant interactive effect between glucose addition rate and AN on the proportional retention of substrate ^{13}C in MAOM ($P < 0.05$, **Table 3.1**). This is likely driven by the negative values in the 0 kg AN treatment, at the low glucose addition rate (**Figure 3.3**). In the bulk soil there was a significant interaction between glucose addition rate and time ($P < 0.01$), and AN and time ($P < 0.001$), on the proportional retention of substrate ^{13}C (**Table 3.1**, **Figure 3.4**).

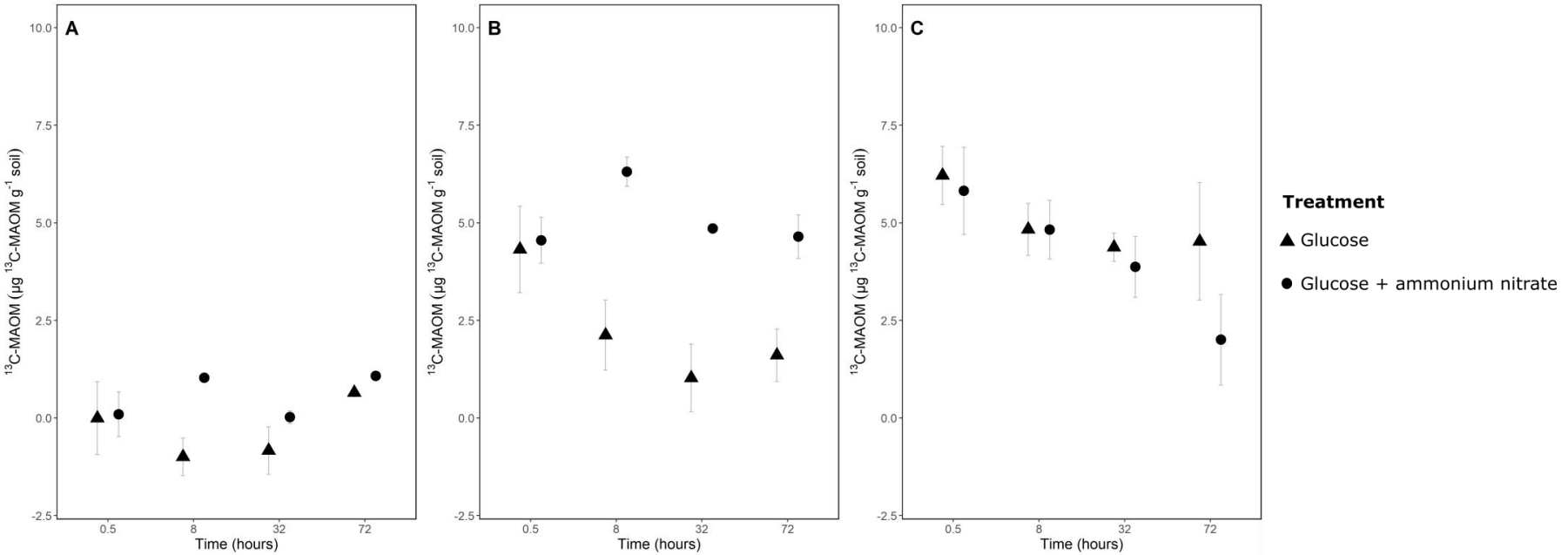

Figure 3.1. Absolute substrate derived ^{13}C recovered in the MAOM fraction (µg ^{13}C-MAOM g^{-1} soil) in the glucose (triangles) and glucose + ammonium nitrate (AN) (circles) treatments, for the low (0.177 mg C g^{-1} soil) (**A**), medium (4.425 mg C g^{-1} soil) (**B**) and high (8.847 mg C g^{-1} soil) (**C**) glucose addition rates, across time (hours since treatment addition). AN addition equivalent to 100 kg N ha^{-1}. Points are means (n = 3) and vertical bars indicate ± one standard error of the mean.

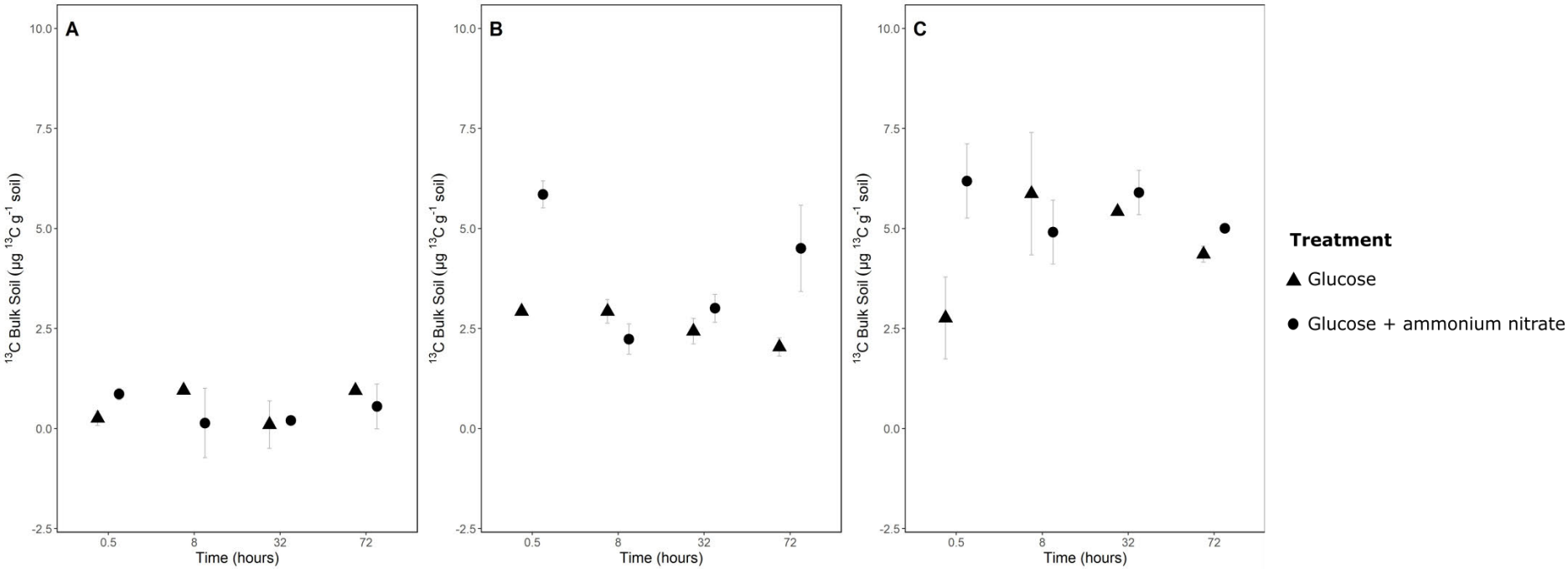

Figure 3.2. Absolute substrate derived ^{13}C recovered in the bulk soil (µg ^{13}C g^{-1} soil) in the glucose (triangles) and glucose + ammonium nitrate (AN) (circles) treatments, for the low (0.177 mg C g^{-1} soil) (**A**), medium (4.425 mg C g^{-1} soil) (**B**) and high (8.847 mg C g^{-1} soil) (**C**) glucose addition rates, across time (hours since treatment addition). AN addition equivalent to 100 kg N ha^{-1}. Points are means (n = 3) and vertical bars indicate ± one standard error of the mean.

Table 3.1. Results from ANOVA analysis examining the effect of glucose treatment (control, low (0.177 mg C g^{-1} soil), medium (4.425 mg C g^{-1} soil), and high (8.847 mg C g^{-1} soil)), ammonium nitrate addition (0 or equivalent to 100 kg N ha^{-1}) and time (0.5, 8, 32 and 72 hours after treatment addition), and their interactions on measured soil properties. Significant *P*-values in bold at $P < 0.05$.

Absolute ^{13}C retained in MAOM	***df***	***F***	**P-value**
Glucose treatment	2	77.385	**< 0.001**
Ammonium nitrate	1	8.995	**0.0041**
Time	3	3.224	**0.030**
Glucose treatment x Time	6	2.261	0.052
Glucose treatment x Ammonium nitrate	2	12.480	**< 0.001**
Absolute ^{13}C retained in bulk soil, log transformed			
Glucose treatment	2	93.590	**< 0.001**
Ammonium nitrate	1	3.191	0.080
Time	3	1.783	0.163
Glucose treatment x Time	6	3.5	0.006
Glucose treatment x Ammonium nitrate	2	0.323	0.725
Ammonium nitrate x Time	3	6.673	**< 0.001**
Proportion of ^{13}C retained in MAOM, log transformed			
Glucose treatment	2	1.514	0.228
Ammonium nitrate	1	4.519	**0.038**
Time	3	1.174	0.327
Glucose treatment x Ammonium nitrate	2	4.075	**0.022**
Proportion of ^{13}C retained in bulk, log transformed			
Glucose treatment	2	68.562	**< 0.001**
Ammonium nitrate	1	3.28	0.076
Time	3	2.001	0.126
Glucose treatment x Time	6	3.864	**0.003**
Ammonium nitrate x Time	3	6.794	**< 0.001**

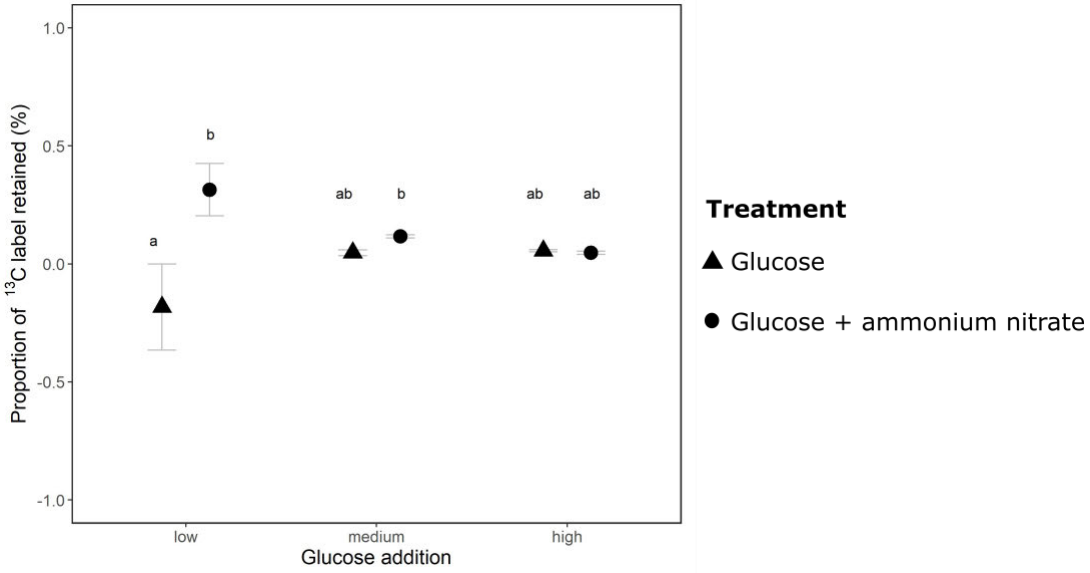

Figure 3.3. Proportion (%) of ^{13}C from glucose retained in the MAOM fraction in the glucose (triangles) and glucose + ammonium nitrate (AN) (circles) treatments, for the low (0.177 mg C g^{-1} soil), medium (4.425 mg C g^{-1} soil) and high (8.847 mg C g^{-1} soil) glucose addition rates. AN addition equivalent to 100 kg N ha^{-1}. Points are means and standard errors of treatments from each destructive sampling point (n = 12). Lowercase lettering indicates significant differences between the glucose addition rate x ammonium nitrate treatments, values which share a letter are not significantly different ($P < 0.05$).

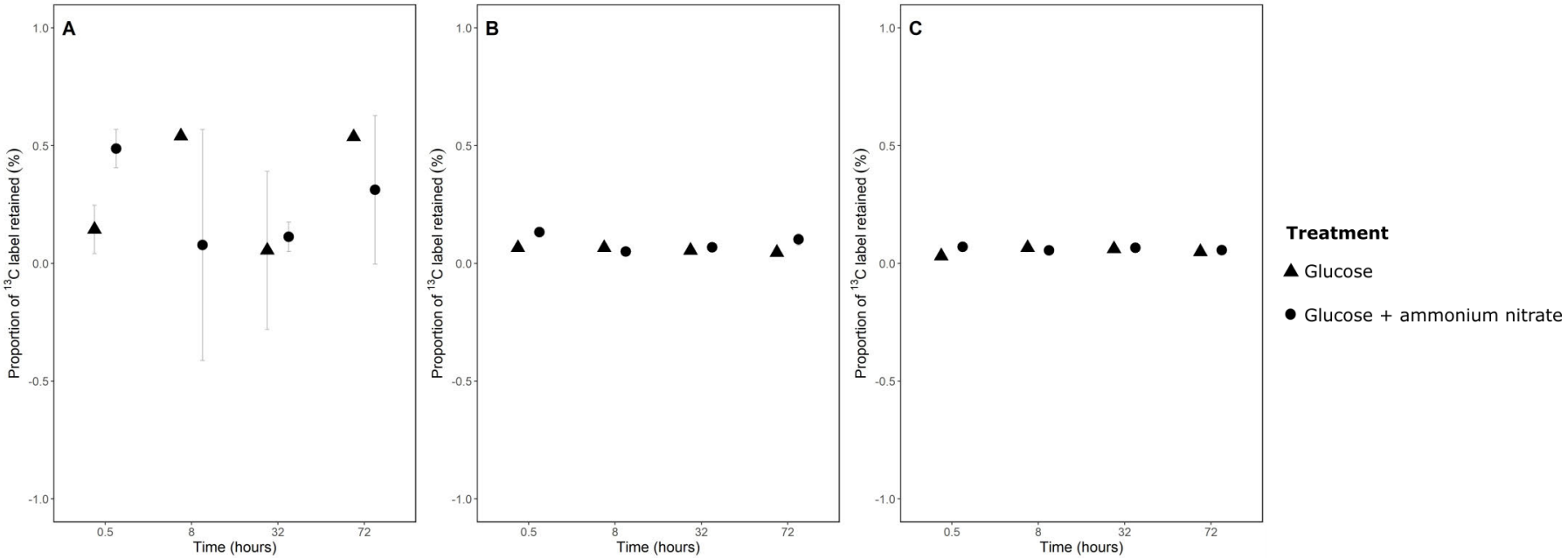

Figure 3.4. Proportion of ^{13}C from glucose retained (%) in the bulk soil in the glucose (triangles) and glucose + ammonium nitrate (AN) (circles) treatments, for the low (0.177 mg C g^{-1} soil) (**A**), medium (4.425 mg C g^{-1} soil) (**B**) and high (8.847 mg C g^{-1} soil) (**C**) glucose addition rates, across time (hours since treatment addition). AN addition equivalent to 100 kg N ha^{-1}. Points are means (n = 3) and vertical bars indicate ± one standard error of the mean.

3.3.2 Treatment effects on measured soil properties

There was no difference in MBC, DOC, bulk soil C, MAOM-C, whole soil N or MAOM-N concentrations between the glucose addition treatments (**Table 3.2**). MBC was significantly lower in treatments with AN addition than those without AN addition, 451.98 ± 20.72 and 496.11 ± 19.98 mg C g^{-1} soil (mean ± SEM, averaged over glucose addition and time), respectively ($P < 0.05$, **Table 3.2**). Concentrations of MBC also changed over time and were lowest at 8 hours ($P < 0.05$, **Figure 3.5**, **A**). There was a significant interactive effect of AN and time on DOC ($P < 0.01$, **Table 3.2**). DOC concentrations were significantly higher in the soils receiving AN compared to those to which no N was added, at 32 hours only ($P < 0.01$, **Figure 3.5**, **B**).

Ammonium nitrate addition and time were both significant main effects on N concentration within the bulk and MAOM, and bulk soil C:N ratio ($P < 0.05$, **Table 3.2**). In Bulk soil and MAOM N were highest at 32 hours, and in soils with AN addition (**Figure 3.5**, **C** and **D**). Bulk soil C:N was lowest at 32 hours, and significantly lower with AN addition ($P < 0.05$, **Table 3.2**). The C:N ratio of soils receiving AN was 10.59 ± 0.15 compared to 11.84 ± 0.22 in soils without AN addition (mean ± SEM, averaged over glucose addition and time). There was a significant interactive effect of AN and time on whole soil OC ($P < 0.05$, **Table 3.2**, **Figure 3.5**, **E**). There was no effect of glucose addition rate, AN or time on total MAOM-C concentrations (**Table 3.2**).

Glucose addition rate had no effect on soil pH. However, soil pH was significantly lowered due to AN addition ($P < 0.05$, **Figure 3.5**, **F**). Thirty minutes after treatment addition, the soil pH in treatments with AN addition was 5.87 ± 0.05, compared to 6.29 ± 0.05 in soils without

AN. After 72 hours the pH of soils treated with AN was 5.49 ± 0.07, compared to 6.12 ± 0.04 in soils without AN. There was no significant difference in soil pH over time in soils without AN addition. However, soil pH was lower at 72 hours than at 0.5 hours in treatments with AN, and both significantly lower than soils without AN ($P < 0.05$, **Figure 3.5**, **F**).

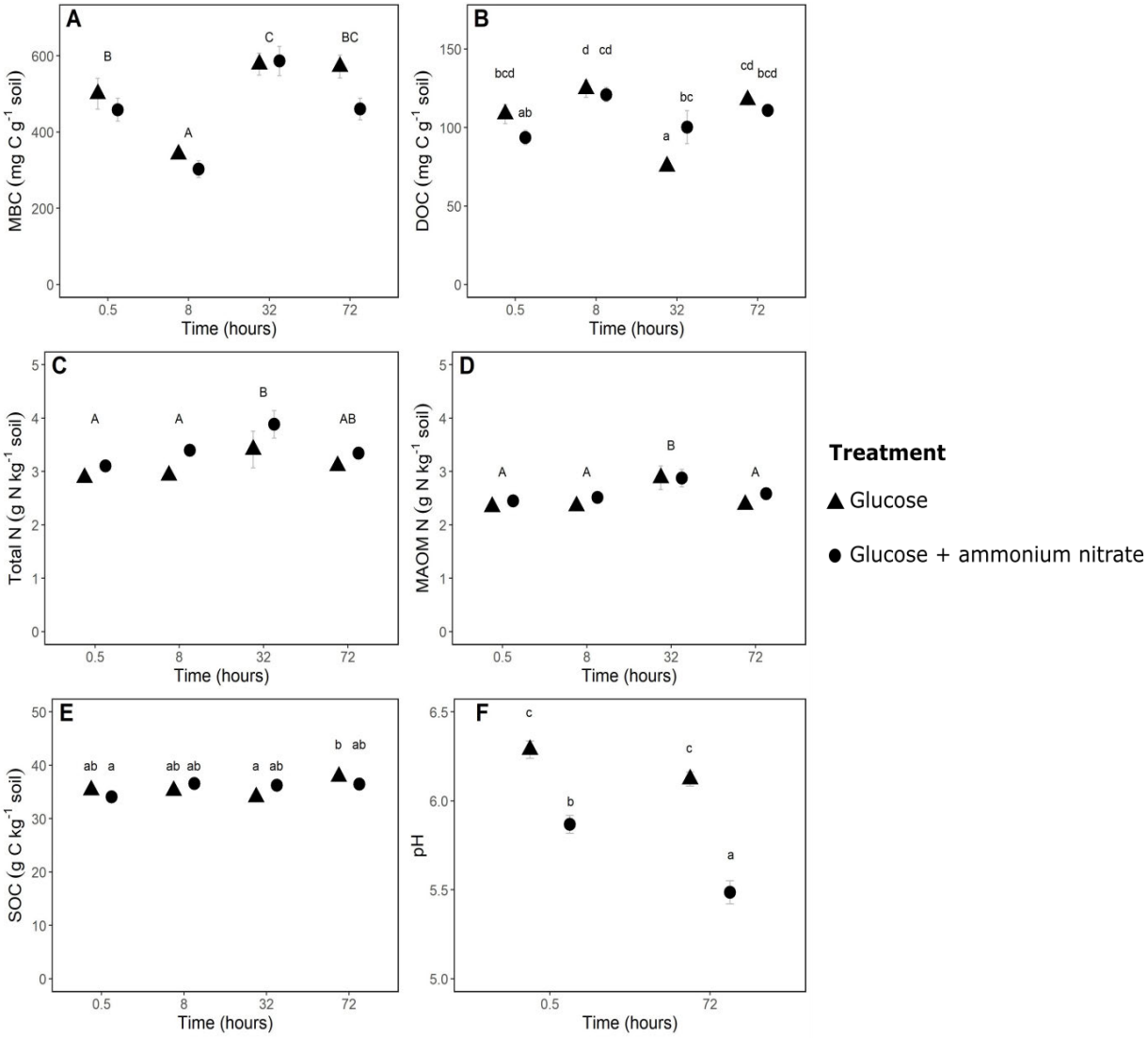

Figure 3.5. Microbial biomass carbon (mg C g^{-1} soil) (**A**), Dissolved organic carbon (mg C g^{-1} soil) (**B**), total soil N (g N kg^{-1} soil) (**C**), total MAOM-N (g N kg^{-1} soil) (**D**), total soil organic carbon (g C kg^{-1} soil) (**E**) and soil pH (**F**), at each time point (hours since substrate addition) in the glucose (triangles) and glucose + ammonium nitrate (AN) (circles) treatments. Points are means (n = 12), averaged across all glucose addition rates, as glucose treatment was insignificant in every instance (see Table 3.2). Vertical bars indicate ± one standard error of the mean. Uppercase letters indicate significant differences between time points ($P < 0.05$) and lowercase letters indicates significant differences between ammonium nitrate addition and time points ($P < 0.05$).

Table 3.2. Results from ANOVA analysis examining the effect of glucose treatment (control, low (0.177 mg C g^{-1} soil), medium (4.425 mg C g^{-1} soil), and high (8.847 mg C g^{-1} soil)), ammonium nitrate addition (0 or equivalent to 100 kg N ha^{-1}) and time (0.5, 8, 32 and 72 hours after treatment addition), and their interactions on measured soil properties. Significant *P*-values in bold at $P < 0.05$.

Microbial biomass carbon	***df***	***F***	**P-value**
Ammonium nitrate	1	6.348	**0.014**
Time	3	34.705	< **0.001**
Dissolved organic carbon			
Glucose treatment	3	2.322	0.081
Ammonium nitrate	1	0.001	0.982
Time	3	16.280	< **0.001**
Ammonium nitrate x Time	3	5.208	**0.002**
Total soil organic carbon			
Glucose treatment	3	0.287	0.835
Ammonium nitrate	1	0.179	0.673
Time	3	4.351	**0.007**
Glucose treatment x Time	9	1.734	0.096
Ammonium nitrate x Time	3	3.054	**0.033**
Total MAOM-C			
Glucose treatment	3	1.517	0.216
Ammonium nitrate	1	2.864	0.094
Time	3	0.864	0.463
Total soil nitrogen, log transformed			
Ammonium nitrate	1	14.985	< **0.001**
Time	3	5.642	**0.001**
Total MAOM-N, log transformed			
Ammonium nitrate	1	4.230	**0.043**
Time	3	9.886	< **0.001**
Bulk soil C:N ratio			
Glucose treatment	3	0.562	0.642
Ammonium nitrate	1	15.559	< **0.001**
Time	3	7.40	< **0.001**

Table 3.2. Continued.

CO_2 flux over the experimental period, log transformed	***df***	***F***	**P-value**
Glucose treatment	3	7.397	< **0.001**
Ammonium nitrate	1	5.061	**0.027**
Time	7	256.412	< **0.001**
Glucose treatment x Ammonium nitrate	3	2.685	0.052
Glucose treatment x Time	20	1.778	**0.037**
Ammonium nitrate x Time	7	1.846	0.089
Cumulative CO_2 at the end of the experimental period			
Glucose treatment	3	1.611	0.262
Ammonium nitrate	1	0.096	0.765
Glucose treatment x Ammonium nitrate	3	0.867	0.497

3.3.3 Respiration

Carbon dioxide flux (µg CO_2-C g^{-1} soil $hour^{-1}$) over the experimental period varied with glucose addition rate and time ($P < 0.05$, **Table 3.2**). The most variation occurred within the first 10 hours, after which soil CO_2-C efflux remained constant (**Figure 3.6**, **A**). There was a significant interaction between glucose addition rate and time ($P < 0.05$, **Table 3.2**). CO_2-C efflux was significantly higher 1 hour after treatment addition than at the sampling points after 10 hours. However post hoc testing revealed no difference between glucose addition and CO_2-C efflux at each time point. At the end of the experimental period there was no significant difference in cumulative CO_2 efflux (g CO_2-C g^{-1} soil) between the treatments (**Figure 3.6**, **B**, **Table 3.2**).

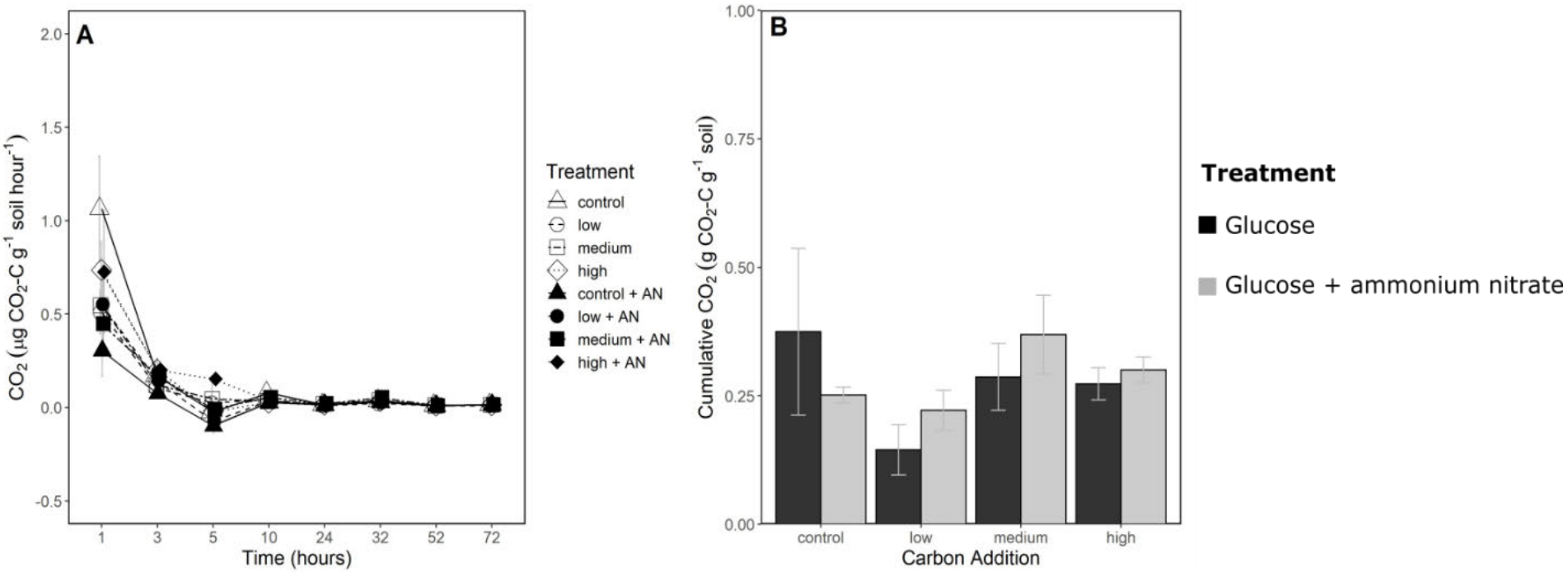

Figure 3.6. Soil CO_2-C efflux (µg CO_2-C g^{-1} soil) (**A**) and cumulative CO_2-C (g CO_2-C g^{-1} soil) at the end of the experimental period (72 hours) (**B**) for each of the soil treatments. In panel A, empty points indicate glucose only treatments, and filled indicate glucose + ammonium nitrate (AN). In panel B, glucose only treatments are black, and glucose + AN are grey (controls are deionised water or deionised water + AN). AN addition equivalent to 100 kg N ha^{-1}. Glucose addition rates were las follows, low (0.177 mg C g^{-1} soil), medium (4.425 mg C g^{-1} soil) and high (8.847 mg C g^{-1} soil). Points are means (n = 2) and vertical bars indicate ± one standard error of the mean.

3.4 Discussion

3.4.1 Substrate quantity

Greater plant C inputs may increase or decrease SOC, depending on microbial growth and turnover (Oldfield et al., 2018). In this study higher inputs of glucose were shown to lead to a greater total recovery of applied substrate ^{13}C within MAOM and the bulk SOC (**Figure 3.1** and **Figure 3.2**). This is similar to results of Oldfield et al., 2018, who observed greater substrate retention in the whole soil with higher addition rates, for glucose, glycine and oxalic acid, the majority of which was recovered in the MAOM fraction (Oldfield et al., 2018). Whilst there is potential for glucose to directly sorb with mineral surfaces within the soil, microbial uptake of glucose out competes direct sorption (Fischer et al., 2010). Therefore it is assumed that^{13}C recovered within the MAOM fraction has undergone microbial transformation, with evidence of microbially transformed C seen in MAOM after 24 hours (Wu et al., 2022).

However, there was no effect of glucose addition rate on total MAOM-C. This suggests that the additional retention of substrate ^{13}C at higher addition rates did not contribute to an increase in total MAOM-C. This suggests that the greater substrate recovery seen with higher addition rates is due to the fresh C displacing older lower affinity compounds within MAOM complexes as observed by Mikutta et al., 2019 (see **Figure 1.2**). Before drawing conclusions about the in-field implications of this, it would be necessary to carry out further investigations. Notably, plant derived LMWOC compound inputs to the soil typically contain a suite of compounds, some of which are known to destabilise organo-mineral associations (e.g. oxalic acid, Keiluweit et al., 2015). Examining the effect of grassland sward composition on the incorporation of fresh LMWOC compounds within MAOM provides an

important opportunity to better understand the mechanisms of MAOM-C accrual, and how interventions in land management interact with these.

There was no effect of glucose addition rate on microbial biomass C (MBC) (**Figure 3.5**, **A**). The increase in MBC after 8 hours may have been due to allocation of glucose to storage, biomass recycling, or growth from SOM released during priming (Reischke et al., 2014). Any substrate induced increase in microbial biomass may have been offset by increased biomass recycling. However, there was no difference in cumulative CO_2-C by the end of the experimental period between the treatments. (**Figure 3.6, B**). Unfortunately, it was not possible to determine the ^{13}C enrichment of CO_2 and MBC, which would be beneficial in identifying the source of CO_2, provide greater insight into microbial substrate use, recycling dynamics, and the subsequent effects of substrate addition rate and MAOM-C accrual. This would also provide empirical evidence to contribute to the theorised link between microbial CUE and MAOM-C accrual (Cotrufo et al., 2013; Sokol et al., 2022).

The proportional retention of substrate C is indicative of the formation efficiency of MAOM-C. It was anticipated that the proportional recovery would be higher in the lower C addition treatments, indicating less efficient formation with greater substrate additions (Oldfield et al., 2018). However, in this study there was no difference in proportional recovery of ^{13}C in MAOM between glucose addition rates (**Figure 3.3**), suggesting that at these addition rates, once substrate ^{13}C was taken up, its eventual incorporation into MAOM was not rate dependent.

3.4.2 Effect of ammonium nitrate addition

It was anticipated that AN addition would enhance both absolute and proportional substrate C recovery in MAOM, by alleviating N constraints on MAOM-C accrual (Cotrufo et al., 2019). There was a significant interaction between AN and glucose addition rate on substrate ^{13}C recovered in MAOM. This highlights the importance of nutrient stoichiometry in MAOM-C accrual. Absolute substrate ^{13}C incorporation into MAOM was only significantly different between the medium glucose addition rate and the medium + AN treatments (**Figure 3.1**). At the low and high glucose addition rates, AN addition had no effect on substrate ^{13}C incorporation into MAOM. The uptake and partitioning of glucose C is dependent on soil nutrient availability (Creamer et al., 2014), therefore the interactive effect of glucose and AN on substrate ^{13}C incorporation into MAOM, was likely due to nutrient imbalances as a result of the treatment additions. Wu et al., 2022, found that the addition of a N rich substrate (amino acid mix versus glucose), only increased recovery of substrate C within MAOM in soil with a lower initial C content. Twice as much amino acid mix was retained in the MAOM fraction than glucose, in soils with lower initial C and N content. However in soils with a high initial C and N content, there was no significant difference in substrate retention (amino acid mix versus glucose) in the MAOM fraction (Wu et al., 2022). Therefore initial SOM content had a larger effect on the retention of fresh inputs, than the N content of the substrate itself (Wu et al., 2022).

The C:N ratio of the high nutrient soils used by Wu et al., 2022, was 9.9, whereas the initial C:N ratio of soil used in this study was 11.6. Soils in this study were from the same site, however treatment additions may have induced nutrient imbalances. Glucose addition rate had no effect on bulk soil C:N, however AN did (**Table 3.2**), with AN addition resulting in a

narrower bulk soil C:N ratio of 10.59 from 11.83. In a long term field trial, ammonium nitrate addition increased MAOM-C (Cenini et al., 2015). A narrower plant C:N ratio increased microbial C demand, which was met by increased enzyme production, resulting in more MAOM-C due to the increased formation of microbial metabolites (Cenini et al., 2015). In this study the narrowest C:N ratio was in the low glucose + AN treatment. However, there was no difference in absolute recovery of substrate 13C in MAOM, between the low glucose and low glucose + AN. Further investigations are required to better understand the role of nutrient stoichiometry in the initial stages of MAOM-C accrual from DOC inputs.

The change in soil pH due to AN addition, may be important for MAOM-C accrual. Soils receiving AN had significantly decreased soil pH, with acidification being even greater by 72 hours (**Figure 3.5, F**). N-induced soil acidification has been linked to decreased microbial biomass and activity (Chen et al., 2020; Ye et al., 2018) and can cause changes in the type of organo-mineral associations (Rowley et al., 2018; Ye et al., 2018). In this study, the lower MBC concentration in treatments with AN addition are likely due to an acidification effect, with changes in pH potentially limiting bacterial growth and favouring fungal growth (Rousk et al., 2009). However, there were no differences in substrate ^{13}C recovered in MAOM between the soils without AN and with AN addition, expect for the medium C addition rate. Whilst substrate ^{13}C incorporation in microbial biomass was not determined, organisms present at higher C addition rates may have been better able to cope with nutrient imbalances, with more flexible stoichiometry (Reischke et al., 2014), and this may in part explain the similar absolute recovery of substrate ^{13}C in MAOM at the high addition rate, between treatments with and without AN addition. However there was no significant relationship between soil pH and cumulative respiration, substrate ^{13}C recovered in MAOM or the

proportion of substrate ^{13}C recovered in MAOM. Therefore the change in pH due to AN addition is unlikely to have influenced the processes leading to substrate ^{13}C incorporation into MAOM

Whilst there were differences in substrate ^{13}C recovered in MAOM in the medium glucose addition rate due to AN. This was not the same in the bulk soil (**Figure 3.2**). Creamer et al., 2014, found no difference in glucose derived C in total SOC with increasing N, phosphorus, and sulphur addition. Together this highlights the importance of N in MAOM formation. Córdova et al., 2018, observed greater absolute MAOM-C accumulation, but with lower efficiency from plant litter with lower C:N ratio. In this study, there was no difference in proportional retention of substrate ^{13}C between treatments receiving AN and those not for the medium and high glucose addition rates. There was a difference in proportional retention of substrate ^{13}C between the low glucose and low glucose + AN treatment (**Figure 3.3**). The glucose only addition had negative proportional retention of substrate ^{13}C, this is due to the ^{13}C atom % value of this treatment, being insignificantly lower than the control (**Table B.1**). At the medium and high glucose addition rates, there was no indication of AN addition impacting the efficiency of MAOM-C formation from added glucose.

The greater proportion of SOC that is within MAOM in grasslands, has been attributed to more labile C inputs from grass species and N additions from fertiliser applications which alleviates the high N requirements of MAOM formation (Cotrufo et al., 2019). From this work it is not possible to say that the addition of AN resulted in greater retention of labile substrate C within MAOM independent of C addition rate. Rather it highlights the need to consider the C:N ratio of inputs. In field this directly relates to application of different

sources of fertiliser (eg mineral fertilisers versus liquid slurry manure), which have different nutrient compositions. Further work which examines the effect of typical soil amendments with differing C:N ratios on eventual incorporation into MAOM, is essential for understanding how field level management influences the incorporation of C into MAOM.

3.4.3 Conclusions

Whilst this study considered a single substrate addition, which is less indicative of *in situ* processes of root inputs and decomposition (Wang et al., 2019) and does not consider the effect of substrate additions on associations with metal cations (Yuan et al., 2020), it was found that absolute substrate ^{13}C retention increased with higher addition rates, with no apparent effect on proportional retention within MAOM. Results obtained here highlight potentially different effects of N on MAOM-C accrual at different glucose addition rates, indicating need for further research on the role of C:N ratios (inputs and SOM-C concentrations) on labile substrate C retention within MAOM. In field examinations of the effect of fertiliser amendments with differing C:N ratios are essential to understand the influence of typical grassland management on the incorporation of fresh C within MAOM.

Chapter 4 Substrate incorporation into MAOM is independent of substrate type and temperature

4.1 Introduction

Soil organic matter (SOM) is the largest terrestrial pool of organic carbon (C), estimated to be between 3,500 and 4,800 petagrams (Pg) (Lehmann and Kleber, 2015). Whilst much of the C entering the soil is mineralised and respired by soil microorganisms over short term scales (< 10 years), there is a portion which persists in the soils for centuries to millennia (Dungait et al., 2012; Sokol et al., 2018). The organic carbon (OC) within the mineral associated organic matter (MAOM) pool is considered an important long-term store of soil OC (Baldock and Skjemstad, 2000). Mineral associations form after the processing of C by microbes or by direct sorption onto metals or soil minerals (Cotrufo et al., 2013), with microbial derived compounds making up a large proportion of MAOM (Bradford et al., 2013; Liang et al., 2017). Consequently, it is theorised that substrate carbon use efficiency (CUE), the proportion of substrate C used for growth versus respiration, is an important regulator of the rate of MAOM-C formed. A higher CUE results in a greater amount of microbial compounds potentially available for incorporation within organo-mineral associations (Cotrufo et al., 2013). Understanding the effects of future climate change, particularly temperature increase, on the formation of MAOM-C is important in the context of predicting future stocks of persistent SOC (Lavallee et al., 2020), and to direct land management focused on soil carbon sequestration within climate mitigation policies (Bossio et al., 2020).

Warming can stimulate SOM transformations by increasing microbial activity, however, the effect of warming on CUE, a key regulator of microbial mediated MAOM-C formation is unclear (Li et al., 2018). Laboratory studies suggest that CUE decreases with temperature (Tucker et al., 2013; Wen et al., 2019), but field studies have found weak or limited declines in CUE due to warming (Frey et al., 2013; Ye et al., 2019). More recently empirical (Takriti

et al., 2018), and modelling efforts (Ye et al., 2019), found that CUE increases with mean annual temperature. Creamer et al., 2015, observed an increase in priming (the loss of native SOC after the addition of fresh substrates) alongside an increase in microbial CUE. A greater microbial biomass per unit substrate may lead to higher SOM decomposition rates and losses of SOC (Wieder et al., 2013). Equally this may be offset by increased quantity of microbial derived compounds, which can potentially be incorporated within MAOM (Creamer et al., 2015; Geyer et al., 2020; Hagerty et al., 2014). There is an additional need to consider changes to microbial community composition and adaptation, and changes to the soil environment, such as moisture content, and plant C inputs that occur with warmer temperatures (Bradford, 2013; Ye et al., 2019). In the context of MAOM-C formation it is important to also understand the fate of assimilated C (Kallenbach et al., 2019) beyond microbial biomass, which is too infrequently determined in research focused on changes in microbial CUE with warming. Consequently the theoretical link between higher CUE and MAOM-C accrual is currently lacking empirical evidence (Sokol et al., 2022).

Belowground labile DOC inputs from root exudates are an important and efficient pathway of microbial MAOM-C formation (Sokol and Bradford, 2019). Root exudates are composed of various compounds, with the largest concentrations being of sugars, proteins and organic acids (Adeleke et al., 2017). This variety of compounds are likely to influence MAOM-C formation resulting from subsequent different microbial CUE and growth efficiencies (Cotrufo et al., 2013; Sokol et al., 2018). For example, microbial growth on sugars is more efficient than on organic acids (Geyer et al., 2016; Van Hees et al., 2005). In one of the few studies linking substrate compounds, the effects of warming and MAOM-C formation, Oldfield et al., 2018, found that substrate compound and quantity had a stronger effect on

SOC formation than temperature. Additionally, despite different proportional retention of the substrates within the soil organic matter (glucose > glycine > oxalic acid), once a substrate was metabolised the subsequent partitioning into MAOM was effectively equivalent (Oldfield et al., 2018). However, further work using different soil types and substrate compounds should build on the current understanding of the effects warming and substrate quality on MAOM-C formation.

The mechanisms leading to MAOM-C accrual are complex. The work in this chapter aims to improve understanding of substrate type and temperature on MAOM-C formation via the microbial DOC pathway. The hypotheses were i) substrate C retained in MAOM will be greater for glucose than acetic acid due to more efficient microbial growth efficiency on sugars than organic acids (Geyer et al., 2016; Van Hees et al., 2005), ii) whilst the effect of temperature on CUE remains uncertain – if temperature does lower microbial growth, it is anticipated that less substrate C will be retained in MAOM with increasing temperature, and iii) the reduction in substrate C retained in MAOM with increasing temperature, will be greater for acetic acid than glucose due to more ubiquitous use of glucose.

4.2 Methods

4.2.1 Soil Sampling and preparation

Soil was sampled in November 2021, to a depth of 30 cm from the Shepherd's cottage field located in the SRUC Bush Estate research farm in the southeast of Scotland (55°51'N 03°52'W). The mean annual temperature and rainfall in the area is from 6 to 9°C, and less than 700 mm respectively. The soil is a non-calcareous gley with a clay loam to sandy loam

texture (Glenpark series, classified as Eutric Stagnosol according to WRB (FAO, 2006)). The field from which the soil was sampled is an agricultural grassland grazed by a mix of sheep and cattle. In the laboratory, the soil was sieved to 2 mm, large stones, macrofauna and roots were removed, and the soil was homogenised into one composite sample.

4.2.2 Experimental Design

The sieved soil was packed into microcosms (pot height 10 cm, diameter 8 cm) to a bulk density of 1.14 g cm^{-3}. The microcosms were adjusted to 50% WHC and incubated at the specified treatment temperature of 10, 15, 20 or 25°C, in the dark, for a 14-day microbial stabilisation period prior to substrate addition. WHC was monitored throughout this period gravimetrically. Microcosms were arranged in a randomised block design with three blocks. Each block contained one replicate of each substrate treatment (glucose, acetic acid and a water only control) per destructive sampling time point (72 hours and 10 days after substrate addition).

Following the stabilisation period, uniformly labelled ^{13}C glucose or ^{13}C acetic acid were added (20 atom%) at a rate of 0.05 mg C per g fresh soil (2.5 mg C in 1 mL water, per microcosm). Deionised water was added to the controls and all microcosms were adjusted to 60% WHC. The substrate (glucose and acetic acid), or deionised water for the control, were injected 1 cm into the soil, at 5 locations in each pot. To achieve the time resolution for gas and destructive sampling, substrate additions at each incubation temperature were staggered within one day.

Incubation temperatures of 10, 15, 20 and 25°C, were chosen to reflect the current mean annual temperatures and potential future mean annual temperatures and summer highs experienced in the UK (Lowe et al., 2019). Glucose and acetic acid were chosen as they are reflective of common classes of root exudate compounds (carbohydrates and organic acids). The addition rate of 0.05 mg substrate C per g fresh soil was chosen to reflect typical rhizodeposition rates and avoid large increases in microbial biomass and changes in community composition (Brant et al., 2006; Frey et al., 2013). Two destructive harvests were selected as, at time of planning, it was unclear how quickly it would be possible to detect substrate ^{13}C within MAOM. The first deconstruction at 72 hours was chosen as previous studies have shown that microbial uptake and partitioning of low molecular weight C substrates is quasi complete after 72 hours (Glanville et al., 2016; Jones et al., 2019). The later 10 day deconstruction was chosen to allow for more time to detect substrate ^{13}C within MAOM, and if this was present at 72 hours, to track changes from 72 hours to 10 days. Recently, Wu et al., 2022, found substrate incorporation in fine silt and clay fraction after 24 hours, and attributed this to microbial processing.

4.2.3 Destructive soil sampling and analysis

When the microcosms were removed from the incubator, the soil was gently homogenised, and subsamples taken for soil pH and microbial biomass C (MBC) determination. Soil pH was measured in deionised water (1:2, soil:water ratio). Microbial biomass C (MBC) was estimated using the chloroform fumigation-extraction method (Vance et al., 1987). Fumigated and unfumigated samples were extracted with 0.5 M K_2SO_4 (1:4, soil to extract ratio). Fumigated samples were fumigated for 24 hours in a dark vacuum oven with chloroform. Dissolved organic carbon (DOC) concentrations were analysed using a

Shimadzu TOC-V analyser. MBC was calculated as the difference between the DOC of the paired fumigated and non-fumigated samples, with a conversion factor k_{EC} of 0.45, (a predetermined factor indicating the fraction of microbial C that is extractable by fumigation) (Joergensen, 1996) and corrected for soil moisture. The remaining soil was dried at 60°C until constant mass, and soil moisture was determined gravimetrically. Total soil C and N concentrations were determined using a Flash 2000 Elemental Analyser (% by mass).

4.2.4 Soil fractionation

A mineral associated organic matter (MAOM) fraction was isolated by dispersion and wet sieving (Cambardella and Elliott, 1993; Cotrufo et al., 2019). Briefly 15 g of air dried, 2 mm sieved, soil was shaken for 16 hours at 180 rpm in a 0.5% sodium hexametaphosphate solution (1:3 soil to solution ratio) (Bradford et al., 2008; Córdova et al., 2018). The suspension was poured over a 53 μm sieve, and thoroughly rinsed with deionised water. Material < 53 μm was decanted into an aluminium tray and dried at 60°C until constant mass. Material > 53 μm was backwashed into an aluminium tray and dried in the same way. The < 53 μm fraction was classified as mineral associated organic matter (MAOM). The mass recovery rate was 100.47% ± 0.06 (mean ± SEM). Multiple methods exist to isolate SOM pools (Poeplau et al., 2018), and fractions may be composite and contain C with varying turnover times (von Lützow et al., 2007). In this instance, very fine POM may contaminate the isolated MAOM, but we expect this to be negligible (Cotrufo et al., 2019; Lugato et al., 2021). The isolated MAOM was ball milled and total C and N contents were determined in the same way as the bulk samples.

4.2.5 Substrate derived C and N in MAOM and bulk soil

Bulk soil and MAOM fractions were analysed for ^{13}C enrichment on a 20/20 isotope ratio mass spectrometer (Sercon Ltd, Crewe, UK) after Dumas combustion of the sample in a Sercon GSL unit (Sercon Ltd, Crewe, UK). The contribution of substrate C to total SOC and MAOM-C was calculated using the following equation:

$$C_{substrate\text{-}derived} = C_{total} \times (atom\%^{13}C_{sample} - atom\%^{13}C_{control}) / (atom\%^{13}C_{substrate} - atom\%^{13}C_{control})$$

Where C_{total} is the total concentration of C in a pool, $atom\%^{13}C_{sample}$ is the $atom\%^{13}C$ value of the C pool at the sampling point, $atom\%^{13}C_{control}$ is the $atom\%^{13}C$ value of the C in the unlabelled control at the same sampling point, $atom\%^{13}C_{substrate}$ is the $atom\%^{13}C$ of the added substrate.

4.2.6 Gas analysis

Gas samples were taken at 1, 3, 5, 10, 24, 32, 52, and 72 hours after substrate addition from the 10-day microcosms. These microcosms were fitted with airtight lids with three-way ports. Approximately 20 mL of air was sampled from the headspace with a gas syringe connected to the port. The sample was split between two pre-evacuated vials one for CO_2 analysis (4 mL) and one for $^{13}CO_2$ analysis (15 mL). After sampling, microcosm lids were removed and microcosms were flushed with laboratory air, resealed, and placed back in the incubator. Laboratory air was sampled and analysed in the same way and results were corrected for laboratory air. Total CO_2 was determined on an Agilent 7890B gas chromatograph. Whilst samples were taken for isotope analysis, it was not possible to conduct this analysis prior to thesis submission. Additionally due to instrumental error, several samples taken at hour 3 were lost, and are not presented in the results. Therefore, results at hour 5 are only indicative

of CO_2 efflux in the 2 hours between hours 3 and 5, and not 4 hours between 1 and 5 hours after substrate addition.

4.2.7 Statistical analyses

All statistical analyses were carried out using R software version 3.6.3. Linear mixed models (LMM) were used to determine effects of substrate type, temperature and sampling time on measured soil properties using the lme4 package (Bates et al., 2015). All models included substrate type (control, acetic acid, and glucose), temperature (10, 15, 20 and 25°C) and sampling time (72 hours and 10 days after substrate addition) were included as fixed effects, and "block" as a random effect. The control treatment was excluded from models analysing the effects of experiment treatments on the absolute retention of substrate ^{13}C, as no ^{13}C substrate was added to these treatments. Significance of effects was determined using 'anova' from the lmerTest package (Kuznetsova et al., 2017). Model fits were determined by ANOVA and lowest AIC. Interactions and terms were dropped from the models if not significant ($\alpha > 0.05$) until a minimally adequate model was reached. Differences between treatments were determined by multiple comparisons using Tukey's HSD test in the 'emmeans' package (Lenth, 2016). Residuals were visually checked using quantile - quantile and residuals versus fitted plots. Response variables that did not meet assumptions were log-transformed. A correlation matrix of Pearson correlation coefficients of measured soil properties was achieved using the using the Hmisc and corrplot packages (Harrell and Dupont, 2021; Wei and Simko, 2017).

4.3 Results

4.3.1 Recovery of substrate ^{13}C in MAOM and the bulk soil

There was no significant difference in substrate derived ^{13}C recovered in the MAOM fraction, between the different temperatures, substrates (glucose or acetic acid), or deconstruction time points (**Figure 4.1**, **Table 4.1**). In the bulk soil there was a significant interaction between substrate and temperature ($P < 0.01$), on the recovery of total soil substrate ^{13}C (**Figure 4.2**, **Table 4.1**). There was no significant difference in substrate ^{13}C recovered at each temperature for acetic acid. For the glucose additions, substrate ^{13}C recovered was highest at 10 and 15°C, and then declined at 20 and 25°C. Substrate ^{13}C recovered in the bulk soil at 10 and 15°C, was significantly higher ($P < 0.05$) in soils receiving glucose than those with acetic acid additions. However, at 20 and 25°C there was no difference in substrate ^{13}C recovered between the two substrate treatments (**Figure 4.2**). For both the MAOM fraction and bulk soil, deconstruction time was not significant and removed from the final model (**Table 4.1**). There was no significant correlation between substrate ^{13}C recovered in MAOM or the bulk soil and total MBC (**Figure 4.3**).

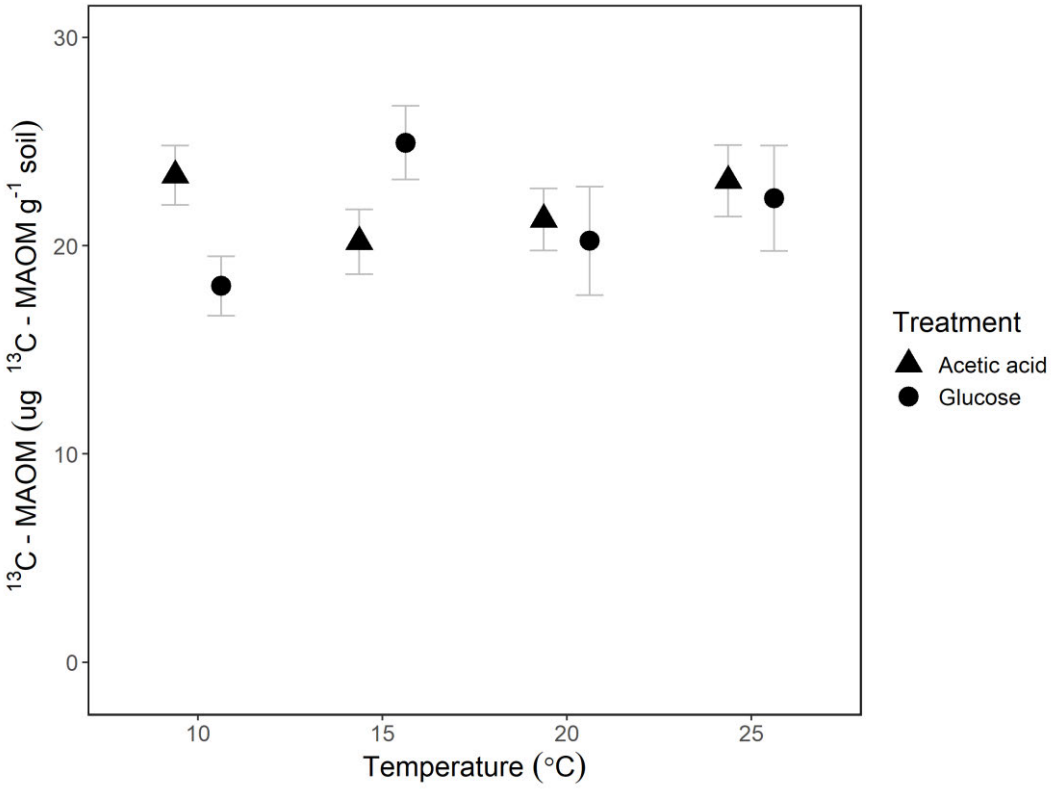

Figure 4.1. Absolute substrate derived ^{13}C recovered in the MAOM fraction (µg ^{13}C g^{-1} soil) in the acetic acid (triangles) and glucose (circles) treatments, at each temperature (10, 15, 20 and 25°C). Points are means (n = 6, averaged over deconstruction time) and vertical bars indicate ± one standard error of the mean.

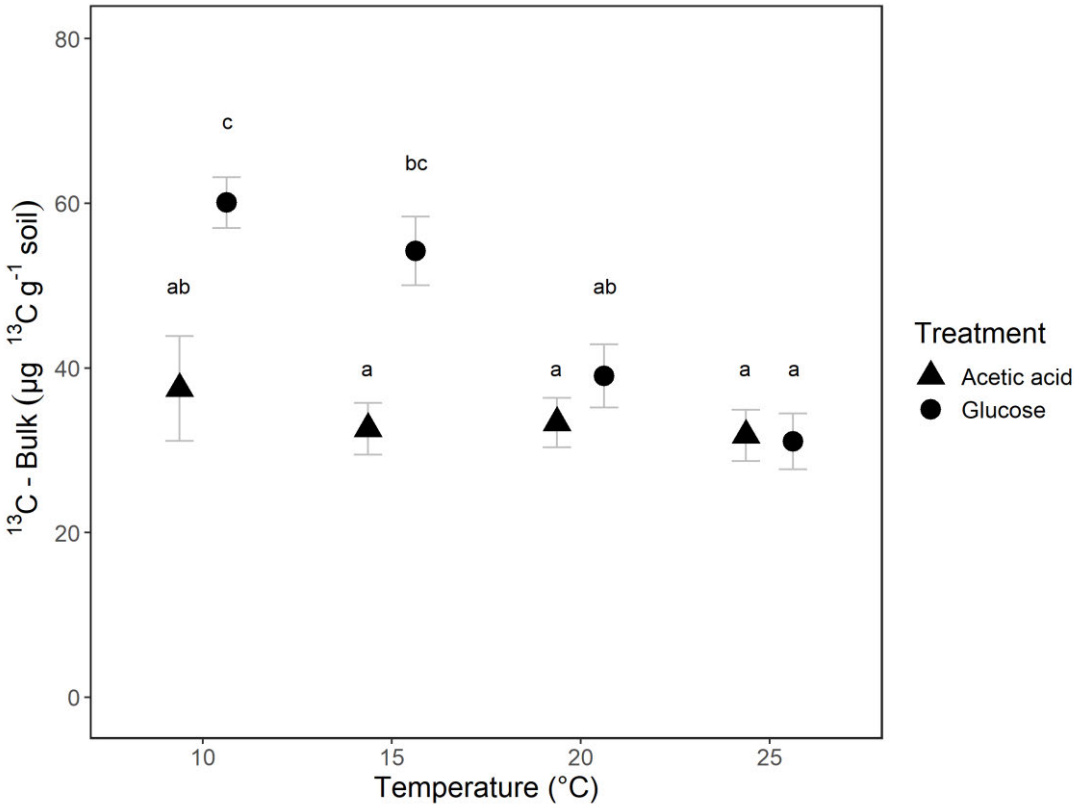

Figure 4.2. Absolute substrate derived ^{13}C recovered in the bulk soil (µg ^{13}C g^{-1} soil) in the acetic acid (triangles) and glucose (circles) treatments, at each temperature (10, 15, 20 and 25°C). Points are means (n = 6, averaged over deconstruction time) and vertical bars indicate ± one standard error of the mean. Different lowercase letters indicate significant differences between substrate and temperature treatments ($P < 0.05$).

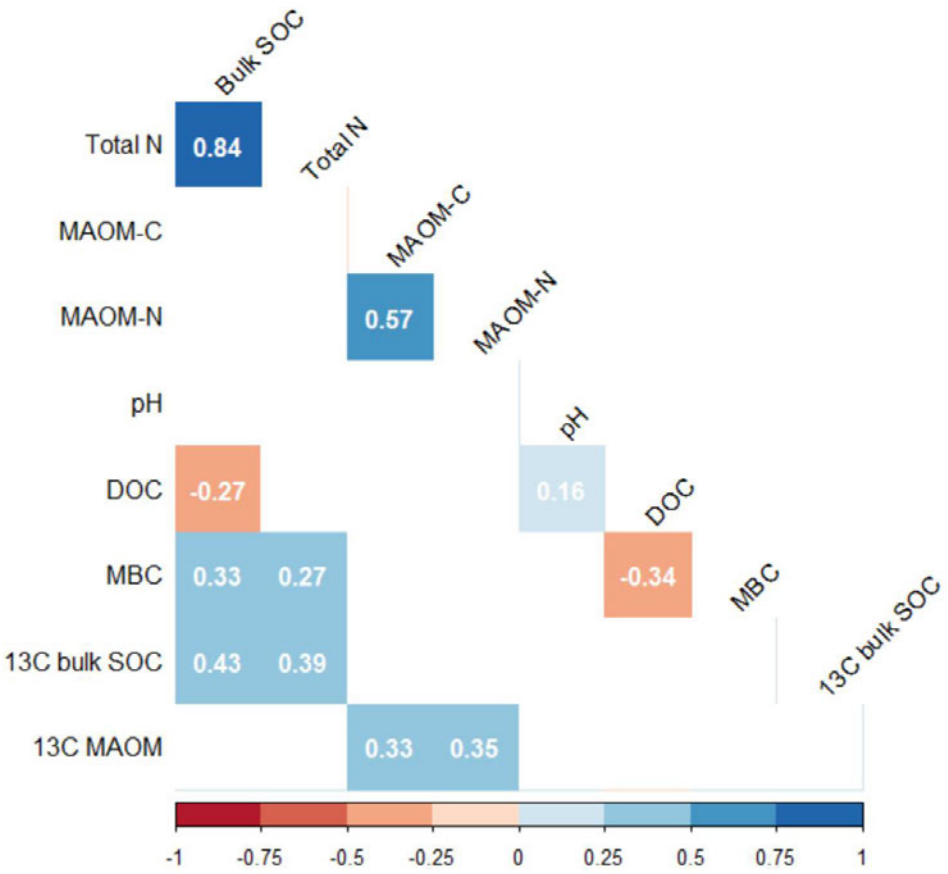

Figure 4.3. Correlation matrix of selected measured soil properties. Only correlation coefficients which are significant at $P < 0.05$ are displayed. The colour refers to the strength of the correlation, with red indicating negative and blue positive correlations. See **Figure C.1** for all correlation coefficients and their *P*-values.

Table 4.1. Results from ANOVA analysis examining the effect of substrate (glucose or acetic acid), temperature (10, 15, 20 and 25°C) and time (72 hours and 10 days after treatment addition) and their interactions on measured soil properties. Significant *P*-values in bold at $P < 0.05$.

^{13}C retained in MAOM	***df***	***F***	**P-value**
Substrate	1	0.078	0.781
Temperature	3	0.969	0.418
Time	1	1.059	0.310
Substrate x Temperature	3	2.326	0.091
Substrate x Time	1	3.243	0.080
^{13}C retained in bulk soil			
Substrate	1	19.749	**< 0.001**
Temperature	3	7.715	**< 0.001**
Substrate x Temperature	3	4.431	**0.009**
Microbial biomass C			
Temperature	3	2.670	0.055
Time	1	3.242	0.076
Dissolved organic C			
Substrate	2	0.962	0.388
Temperature	3	11.324	**< 0.001**
Time	1	15.152	**< 0.001**
Substrate x Temperature	6	2.833	**0.018**
Temperature x Time	3	16.340	**< 0.001**
Soil pH			
Substrate	2	1.157	0.322
Temperature	3	15.979	**< 0.001**
Time	1	0.801	0.375
Substrate x Temperature	6	4.376	**0.001**
Temperature x Time	3	2.988	**0.039**
Bulk SOC			
Temperature	3	12.046	**< 0.001**
Time	1	9.442	**0.003**
Temperature x Time	3	2.523	0.066

Table 4.1. Continued.

Total soil nitrogen, log transformed	***df***	***F***	**P-value**
Substrate	2	3.625	**0.033**
Temperature	3	10.208	< **0.001**
Time	1	2.142	0.149
Substrate x Temperature	6	2.113	0.066
Temperature x Time	3	2.927	**0.042**
MAOM-C			
Temperature	3	1.272	0.292
Time	1	0.653	0.422
Temperature x Time	3	3.709	**0.016**
MAOM-N, log transformed			
Substrate	2	5.992	**0.004**
Temperature	3	4.417	**0.007**
Time	1	0.565	0.455
Temperature x Time	3	5.504	**0.002**
Carbon Dioxide flux, log transformed			
Substrate	2	17.258	< **0.001**
Temperature	3	23.110	< **0.001**
Time	5	26.519	< **0.001**
Substrate x Temperature	6	2.801	**0.013**
Substrate x Time	10	1.801	0.064
Temperature x Time	15	2.126	**0.011**

4.3.2 Treatment effects on measured soil properties

There was no effect of substrate addition on bulk SOC, however bulk SOC was significantly affected by temperature ($P < 0.001$) and sampling time ($P < 0.01$, **Table 4.1**). Bulk SOC was significantly higher at 72 hours than 10 days (43.1 ± 0.49 and 41.6 ± 0.36, respectively; mean ± SEM, averaged over temperature and substrate). Bulk SOC declined with increasing temperature and was significantly higher at 10 and 15°C than at 25°C ($P < 0.05$, **Figure 4.4**, **A**). Whilst there was a significant main effect of substrate type on total soil nitrogen (N) ($P < 0.05$, **Table 4.1**), post hoc testing found no significant differences in total soil N between the substrate treatments. Total soil N was significantly affected by temperature and sampling time ($P < 0.05$). At each incubation temperature there was no significant difference in soil N between the 72 hour and 10-day sampling times. Averaged across substrate type, total soil N was higher at 10°C than in the 20 and 25°C treatments at 72 hours, but not by 10 days after substrate addition (**Figure 4.4**, **C**).

Substrate addition had no effect on total MAOM-C (**Table 4.1**). There was a significant interactive effect between temperature and sampling time on total MAOM-C ($P < 0.05$, **Table 4.1**). Seventy-two hours after substrate addition, MAOM-C in the 10°C treatment was 24.84 ± 0.14 g C kg^{-1} soil (mean ± SEM, averaged across all substrates) and significantly less than the 25°C treatment, 25.15 ± 0.23 g C kg^{-1} soil (mean ± SEM, averaged across all substrates). MAOM-C did not change between 72 hours and 10 days after substrate addition, in any of the temperature treatments (**Figure 4.4**, **B**).

MAOM-N content was significantly effected by both temperature and sampling time ($P < 0.01$, **Table 4.1**). MAOM-N was significantly lower at 72 hours compared to 10 days after

substrate addition at 10°C. At all other temperatures there was no difference in MAOM-N between the two sampling points, averaged across substrate (**Figure 4.4**, **D)**. There was a significant main effect of substrate on MAOM-N ($P < 0.01$, **Table 4.1**). MAOM-N was significantly lower in the soils receiving glucose (2.17 ± 0.01 g N kg^{-1} soil) than those receiving acetic acid (2.23 ± 0.01 g N kg^{-1} soil, mean ± SEM, averaged across temperature and sampling time). However, neither of these were significantly different to MAOM-N in the control treatment of 2.19 ± 0.01 g N kg^{-1} soil (mean ± SEM, averaged across temperature and sampling time).

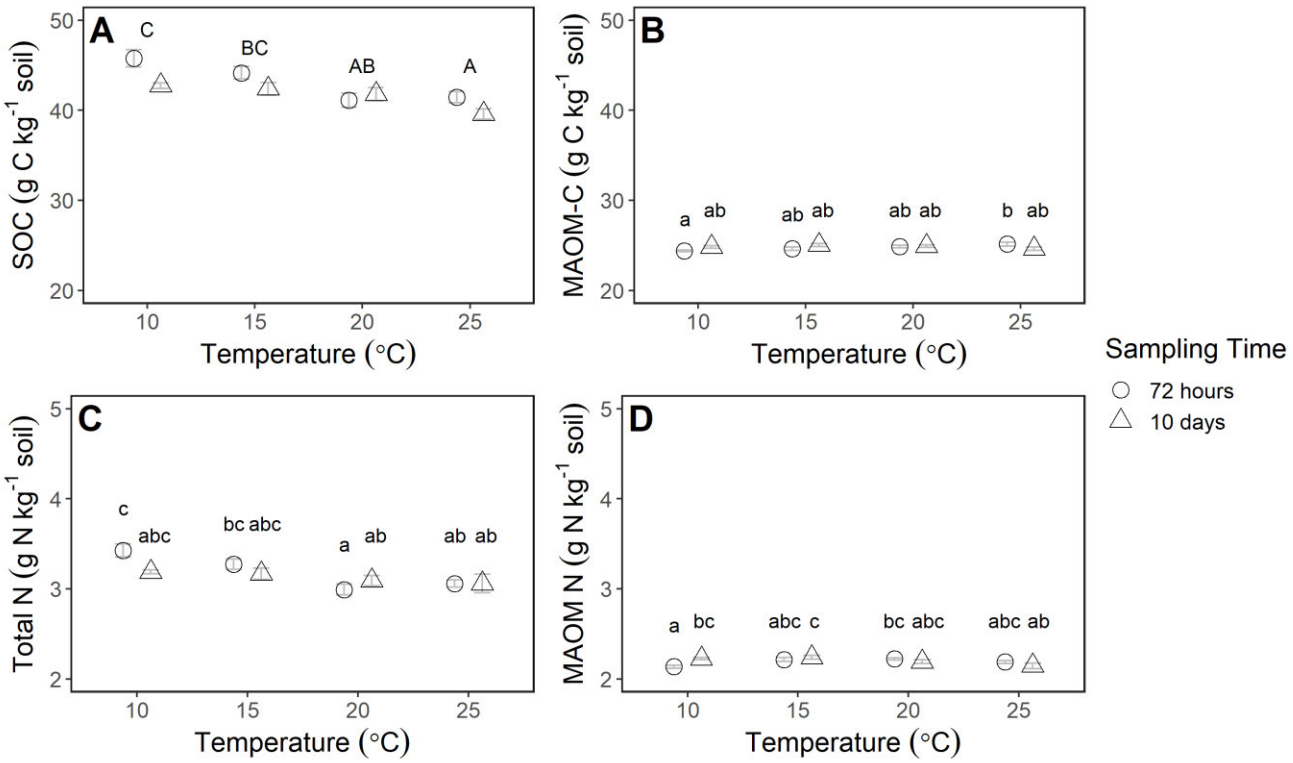

Figure 4.4. Bulk SOC (g C kg^{-1} soil) (**A**), MAOM-C (g C kg^{-1} soil) (**B**), total soil nitrogen (g N kg^{-1} soil) (**C**), and MAOM-nitrogen (g N kg^{-1} soil) (**D**) at 72 hours (circles) and 10 days (triangles) after substrate addition, at each temperature (10, 15, 20 and 25°C). Points are means (n = 9, averaged over substrate addition (acetic acid, glucose and control), due to no effect of substrate addition on the soil parameters in this panel. Vertical bars indicate ± one standard error of the mean. Different uppercase letters indicate significant differences between SOC at each temperature ($P < 0.05$). Different lowercase letters indicate significant differences at between sampling time and temperature treatments ($P < 0.05$).

There was no significant effect of substrate, temperature, or sampling time on MBC (**Table 4.1**). Both dissolved organic carbon (DOC) and soil pH had significant interactive effects of substrate and temperature, and temperature and sampling time ($P < 0.05$, **Table 4.1**). In both instances there was no difference in DOC or soil pH in soils receiving acetic acid, glucose or the water only control within each temperature treatment (**Figure 4.5**, **A** and **B**). DOC was lower at 72 hours than 10 days at 15 and 25°C but higher at 72 than 10 days at 20°C ($P < 0.05$, **Table C.1**). Within a temperature treatment there was no significant difference in soil pH, averaged across substrate type ($P < 0.05$, **Table C.1**).

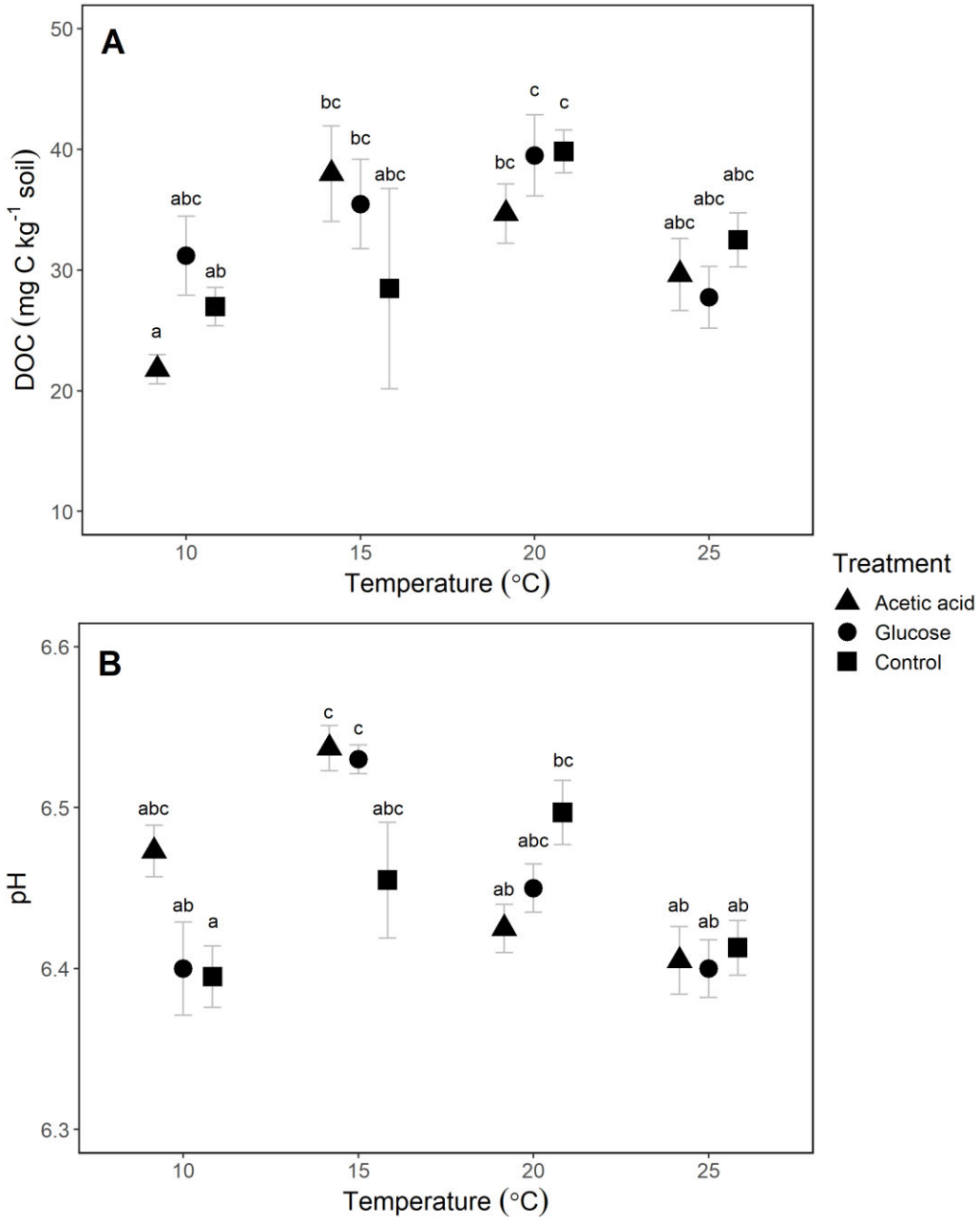

Figure 4.5. Soil dissolved organic carbon (DOC) (mg C kg^{-1} soil) (**A**) and soil pH (**B**) in the acetic acid (triangles) and glucose (circles) and control (squares) treatments, at each temperature (10, 15, 20 and 25°C). Points are means (n = 6, averaged over deconstruction time) and vertical bars indicate ± one standard error of the mean. Different lowercase letters indicate significant differences between substrate and temperature treatments ($P < 0.05$).

4.3.3 Respiration

Carbon dioxide flux (μg CO_2-C g^{-1} soil $hour^{-1}$) over the experimental period was affected by the interaction between substrate and temperature, and temperature and sampling time ($P < 0.05$, **Table 4.1**). There was no significant difference in CO_2 efflux between the acetic acid, glucose or water only controls within an incubation temperature, averaged over time, except at 15°C. At 15°C, the CO_2 efflux from the acetic acid and glucose substrate treatments was significantly higher than the water only control, 0.721 ± 0.14, 0.612 ± 0.14 and 0.161 ± 0.07 μg CO_2-C g^{-1} soil respectively, averaged over time (mean ± SEM) (**Figure 4.6**).

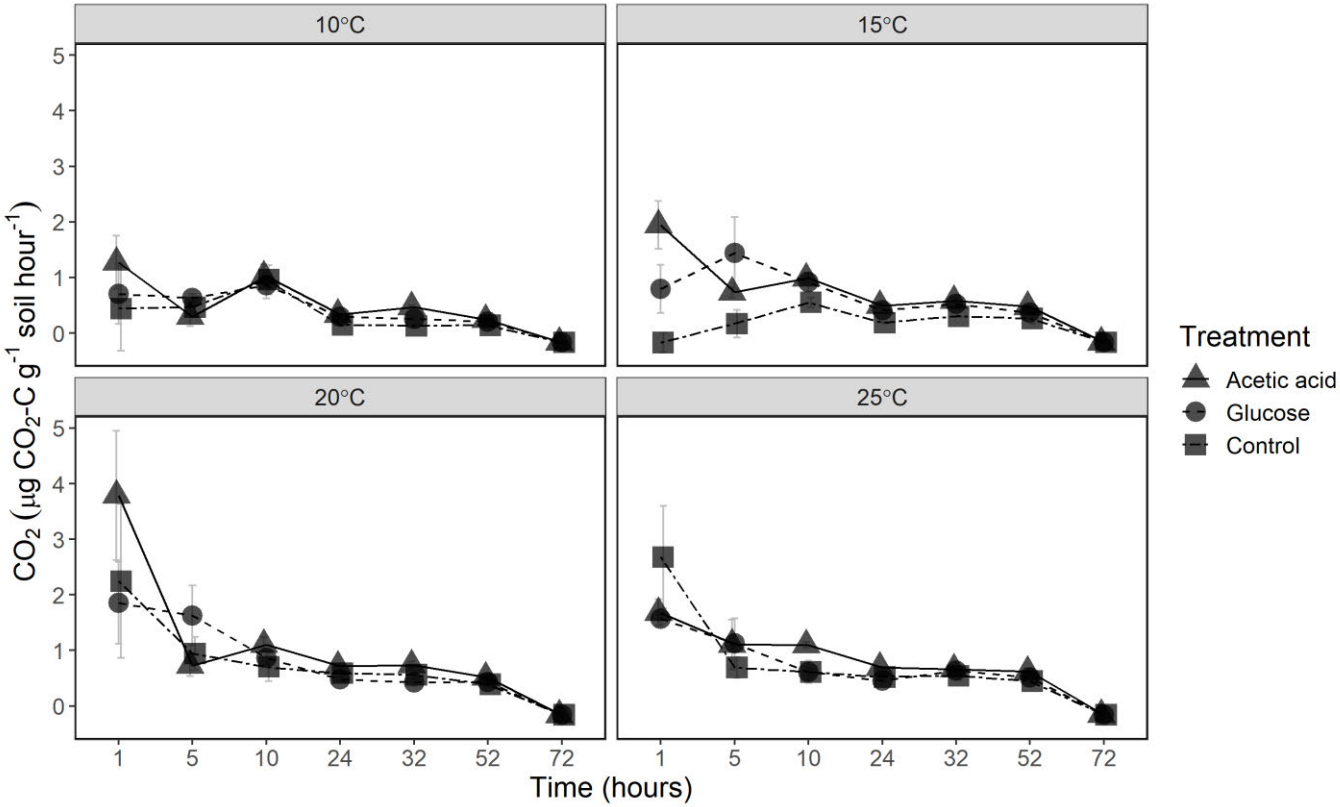

Figure 4.6. Soil CO_2-C efflux (µg CO_2-C g^{-1} soil) for each substrate treatment acetic acid (triangles) glucose (circles) and control (squares), at each temperature (10, 15, 20 and 25°C). Points are means (n = 3) and vertical bars indicate ± one standard error of the mean. Points at hour 5 represent flux over preceding two hours, due to analytical error when analysis gas sampling at 3 hours after substrate addition.

4.4 Discussion

4.4.1 Substrate type

There is growing evidence of the large contribution of microbial derived compounds contributing to the most persistent SOC within organo-mineral associations (Miltner et al., 2012). The microbial formation of MAOM-C from labile DOC inputs is an important and efficient pathway of MAOM-C accrual (Sokol and Bradford, 2019). This formation pathway is likely to be affected by the type of DOC compound due to differing microbial growth and use efficiencies (Geyer et al., 2016; Van Hees et al., 2005). It was hypothesised that substrate ^{13}C retained in MAOM will be greater for glucose than acetic acid due to these different growth efficiencies. However, there was no significant difference in substrate ^{13}C in the MAOM fraction between the glucose and acetic acid treatments (**Figure 4.1**). There was however, a significant effect of substrate and temperature on substrate ^{13}C recovered in the bulk SOC. The substrate ^{13}C recovered in bulk SOC was significantly higher for glucose than acetic acid at 10 and 15°C, but not at 20 and 25°C (**Figure 4.2**). At 10 and 15°C, the greater retention of glucose ^{13}C in the bulk soil, compared to acetic acid may be due to its rapid incorporation into microbial biomass. McFarland et al., 2022, reported 45% of applied glucose was retained in chloroform-labile pools (comparable to DOC and MBC here). The microbial community present in the soil may be more adept to operating at these temperatures with mean annual temperature of the region being between 6 to 9°C.

It was anticipated that more substrate ^{13}C would be recovered in MAOM fraction for glucose than acetic acid, however this was not evident in this study. However, more glucose substrate ^{13}C was retained in the bulk soil at 10 and 15°C than acetic acid, but this was not evident in the MAOM fraction. This is likely due to similar uptake of the two substrates, but different

subsequent partitioning into metabolic pathways determining the fate of C within the soil (Gunina et al., 2014). With sugars (glucose) being used more for anabolic processes and carboxylic acids (acetic acid) for energy production (Gunina et al., 2014). Whilst it is generally assumed that substrate ^{13}C recovered within the MAOM fraction has undergone microbial transformation, due to microbial uptake of LMWOC compounds outcompeting direct mineral sorption (Fischer et al., 2010). There was no correlation between MAOM-C or substrate ^{13}C within MAOM and MBC (**Figure 4.3**), the presence of such a relationship is often used to indicate the role of soil microbes in MAOM-C.

Further isotopic analysis of DOC, MBC and CO_2 would provide greater insights to the partitioning of the two substrates and establish a full substrate C budget. This would help to determine if the different retention of substrate ^{13}C within bulk SOC is due to differing rates of microbial incorporation. This would also shed light on microbial uptake and subsequent partitioning into MAOM-C and may reveal relationships between substrate derived ^{13}C in MBC and MAOM-C observed in other studies (e.g. Oldfield et al., 2018). It is anticipated that there would be greater glucose substrate ^{13}C within MBC than acetic acid, with more acetic acid ^{13}C lost as CO_2. This additional empirical evidence would contribute to the understanding of the dynamics of MAOM-C in the short term, which is important in the context of fresh substrate inputs such as in the rhizosphere.

There was no difference between substrate ^{13}C in the MAOM fraction or the bulk SOC at 72 hours versus 10 days after substrate addition (**Table 4.1**). This may be due to MAOM-C reaching saturation, or microbial recycling of substrate derived compounds contributed to the retention of substrate ^{13}C over time (McFarland et al., 2022). Given estimates of saturation

for the fine fraction (< 20 μm) of this soil (Paterson et al., 2021, see chapter 2), the addition rates in this study are unlikely to achieve saturation. Consequently, microbial recycling may be more likely, but further investigations at a finer temporal scale would be required to discern the mechanisms contributing to the constant substrate ^{13}C contribution to MAOM-C over the two sampling times in this study.

4.4.2 Temperature effect

Investigating the effects of warming on MAOM-C formation and persistence is necessary to understand the mechanisms of change and will help to better predict future stocks of persistent C with climate change (Lavallee et al., 2020). Typically warming is associated with a decline in CUE, however this relationship remains unclear (Bradford et al., 2013; Creamer et al., 2015; Frey et al., 2013; Ye et al., 2019). A decrease in CUE may result in a decrease in MAOM-C as less microbial derived compounds are available for incorporation in organo-mineral associations. In contrast, increases in CUE may lead to higher availability of microbial compounds potentially increasing MAOM-C (Creamer et al., 2015). This however depends on the balance between new microbial compounds and loss of SOC due to priming (Creamer et al., 2015). Given the role of soil microbes in MAOM-C formation, the effect of temperature on MAOM-C accrual is relatively understudied. This study found that temperature had no effect on substrate ^{13}C retained within MAOM, but did have a significant interactive effect with substrate on substrate-derived ^{13}C retained within the bulk soil (**Table 4.1**,**Figure 4.2**). Increasing temperature lowered the retention of substrate ^{13}C within the bulk soil for glucose, with retention at 10°C significantly higher than at 15, 20 and 25°C. However, after addition of acetic acid, there was no temperature effect on retention (**Figure 4.2**), suggesting the effect of temperature is substrate dependent. Oldfield et al., 2018, also

reported an interactive effect of substrate type and temperature but found that warming (20 to 25°C) generally increased substrate retention in bulk soil and was attributed to the greater total mass of substrate derived microbial biomass with warming (Oldfield et al., 2018). Here, although temperature was retained in the final model (**Table 4.1**), it had a non-significant effect on total MBC. Therefore, there was no change in total MBC between the temperatures. However, without additional isotope analysis it is not possible to say whether or not changes in substrate incorporation in MBC due to temperature led to the differences in substrate ^{13}C retention in this study.

The higher temperatures generally lowered concentrations of bulk SOC and resulted in lower total soil N at 72 hours compared to 10°C, but this difference was not observed 10 days after substrate addition (**Figure 4.4 A** and **C**). This may have contributed to differences in substrate ^{13}C retained in the bulk soil, due to nutrient limitations. However, substrate type has been found to have a stronger influence on microbial CUE than stoichiometry (Takriti et al., 2018).

Carbon dioxide flux (µg CO_2-C g^{-1} soil $hour^{-1}$) between the substrates was significantly different at 15°C. CO_2 efflux was highest for acetic acid, then glucose then the water only control. This may be due to a lower substrate CUE of acetic acid, or the dissolution of organo-mineral associations releasing C facilitating microbial activity (Ding et al., 2021; Jilling et al., 2018; Keiluweit et al., 2015). However as mentioned in the previous section, additional isotope analysis would reveal further insights into microbial CUE and its relationship with temperature and the implications for MAOM-C accrual. This would provide

empirical evidence for links between microbial CUE and MAOM-C, which are currently lacking (Sokol et al., 2022).

This chapter used single substrate compounds and one time addition, however, *in situ*, for example in the rhizosphere, microbes are exposed to a continuous flow of a mixture of C compounds (Kallenbach et al., 2015; Van Hees et al., 2005). The examination of the effect of warming under repeat additions of a mix of compounds indicative of rhizodeposits, would be more indicative of *in situ* conditions. Changes to the composition of exudates due to alterations of sward species or induced by climatic change may alter MAOM-C formation processes in the field. Further work to determine the effects of sward composition on MAOM-C accrual would help to develop guidance on sward composition which promotes the formation of persistent SOC.

4.4.3 Conclusions

The effects of climate change on SOC have primarily focused on losses due to mineralisation. However, it is also necessary to understand how warming may impact the formation of the most persistent forms of SOC. The results from this study found no effect of temperature or substrate on the retention of substrate ^{13}C within MAOM. However, substrate ^{13}C within the bulk SOC was significantly greater for glucose than acetic acid at 10 and 15°C, but not at 20 and 25°C. This highlights that the retention of a substrate in the bulk soil is temperature and substrate specific. The lack of a difference in retention within MAOM-C suggests that, despite differences in bulk soil retention, the portion retained within MAOM-C is equivalent. Additionally, there was no effect of sampling time (72 hours versus 10 days) on substrate ^{13}C recovered in the bulk SOC and MAOM fraction, the reasons for this however are unclear.

Further isotope analysis of DOC, MBC and CO_2 would provide greater insights into the effect of temperature and substrate type on MAOM-C formation; however, it was not possible to complete this prior to submission. Notably, such data would help to clarify links between microbial CUE and MAOM-C accrual. Despite the work in this chapter examining single substrate additions, rather than a continuous flow of a mixture of C compounds, more indicative of in field conditions. The results indicate that substrate type had a greater influence on retention in the bulk soil under conditions more aligned with mean annual temperatures in the UK, and that eventual incorporation into the MAOM fraction was independent of substrate type and incubation temperature. Future work examining the effect of changes in LMWOC compounds entering the soil in the field, would help to determine the effect of changes in sward species composition on MAOM-C formation in agricultural grasslands.

Chapter 5 Mineral associated organic matter carbon is differently affected by long-term soil pH amendment, grass ley duration and soil depth

5.1 Introduction

Globally soils are the largest terrestrial store of carbon (C) with an estimated 3,500 to 4,800 petagrams (Pg) of C within soils (Lehmann and Kleber, 2015). However, agricultural intensification has led to significant losses of soil organic carbon (SOC) (Bossio et al., 2020). Protecting and increasing existing SOC can be achieved by appropriate land management, and has multiple benefits such as improving soil fertility and resilience to climate change, and presents an opportunity to mitigate anthropogenic CO_2 in the atmosphere, by soil carbon sequestration (Bossio et al., 2020; Chenu et al., 2019). SOC sequestration focuses on the most persistent C pools, with turnovers of centuries to millennia (Chenu et al., 2019). Increasingly persistence is recognised as a function of soil structural mechanisms that determine the accessibility of substrates to microbial decomposition and to mineral surfaces, rather than inherent chemical recalcitrance (Dungait et al., 2012). Separation of SOC into pools with differing turnover times provides an opportunity to examine SOC dynamics, with recent focus on isolation of particulate organic matter carbon (POM-C) and mineral associated organic matter carbon (MAOM-C) (Cotrufo et al., 2019; Lugato et al., 2021; Sokol et al., 2018). POM is primarily composed of partially decomposed plant material, and persists due to its inaccessibility or microbial inhibition, and is relatively short lived (10 – 50 years) (Cotrufo and Lavallee, 2022). In contrast, MAOM is composed of microbial by-products and dissolved organic matter, which persists in the soil by organo-mineral associations and within micro aggregates (Jilling et al., 2018; Kleber et al., 2007). Organo-mineral associations form by sorption of SOC via multiple mechanisms including ligand exchange, cation bridging, and adsorption to minerals such as phyllosilicate clays and Al-, Fe-, and Mn-oxides (Kleber et al., 2021; Rowley et al., 2018), and due to the direct deposition of plant and microbial cells and by-products onto mineral surfaces (Cotrufo et al., 2013; Lünsdorf et al., 2000).

In agricultural systems, soil pH may be altered through the addition of nitrogen (N) fertilisers resulting in acidification, or application of lime (calcium and magnesium rich) to ameliorate soil acidity (Fornara et al., 2011). The changes in soil pH may influence the accrual of MAOM-C by both abiotic and biotic means. N-induced soil acidification decreases MAOM-C due to reduced microbial biomass, and leaching of Mg^{2+} and Ca^{2+} and the release of C adsorbed through cation bridging (Chen et al., 2020; Shen et al., 2018; von Lützow et al., 2006). However, the increased solubility of Al and Fe phases could increase C in MAOM in acidic conditions. In alkaline soils cation bridging with Mg^{2+} and Ca^{2+}, and adsorption with Mn-oxides plays a greater role in C preservation within MAOM complexes (Rowley et al., 2018). See **Figure D.1**, for an overview of the role of cations in SOC preservation at different pH's. A shift to more alkaline conditions may increase MAOM-C due to greater plant C inputs and higher microbial biomass (Fornara et al., 2011). Despite liming being a global practice, there is little data available linking liming rates and SOC stocks (Paradelo et al., 2015).

With increasing depth the relative proportion of SOC within MAOM complexes increases (Kögel-Knabner et al., 2008). Therefore, due to the concurrent increase in available mineral surface area and decrease in C concentration with depth, it is suggested that subsoils have a greater capacity for additional C sorption within MAOM complexes (Abramoff et al., 2021). However, as MAOM-C dynamics with depth are input limited, inherent soil properties exert a greater influence on C cycling (Cotrufo and Lavallee, 2022). Therefore, changes to soil pH, which alter the availability of cations deeper in the soil may have implications for MAOM-C.

However, the effect of soil surface applications to manipulate soil pH on deeper MAOM-C dynamics is unknown.

The inclusion of grass ley periods within arable rotations has been found to increase SOC (Börjesson et al., 2018; Crème et al., 2018) and plays a role in achieving SOC sequestration targets (Johnston et al., 2017). The increase in C is attributed to reduced soil disturbance and higher OM inputs (Zani et al., 2021), with SOC typically increasing with ley duration due to the development of an extensive root system, dependent on initial SOC status (Johnston et al., 2017). However the effects of grass leys on C within different soil fractions, particularly persistent SOC, is relatively understudied (Zani et al., 2021). The inclusion of legumes, such as clover has further benefits by increasing N availability (Rumpel et al., 2015; Zani et al., 2021). The presence of additional N may promote MAOM-C formation by facilitating the decomposition of POM (Bradford et al., 2008), and due to its role in organo-mineral association formation (Kopittke et al., 2020). However, Zani et al., 2021, found no change in MAOM-C with greater grass-clover ley durations in both conventional and organic systems. To our knowledge, no studies to date have assessed the effects of soil pH and grass-clover ley duration on MAOM-C.

The aim of this work was to examine changes in MAOM-C along a pH gradient under different grass ley durations at two depths, 0 – 20 cm and 20 – 40 cm. Two depths were selected to determine whether trends with pH and grass ley duration were apparent beyond the topsoil. Soil was sampled from a long-term pH manipulation trial. Using long-term trials such as this one are beneficial as it removes any confounding differences in historical land management, soil type or environmental conditions (Rousk et al., 2009). We hypothesised

that i) MAOM-C will increase along the pH gradient (from 4.5 to 7.5) due to more favourable biotic and abiotic retention conditions, ii) there will be no difference in MAOM-C between the grass ley durations, and iii) at 20 – 40 cm MAOM-C would make up a greater relative proportion of total SOC than at 0 - 20 cm.

5.2 Methods

5.2.1 Site and Sampling

Soil was sampled from three grass-clover ley rotations, which have been under ley grass for 1, 2 or 3 years, within a crop rotation, at the long-term pH trial at the SRUC Craibstone Estate, UK (57 11’ N, 2 12’ W). The trial (established in 1961), was divided into 7 subplots maintained at pH levels from 4.5 to 7.5, rising in 0.5 increments. All plots undergo the same 8-year crop rotation, which includes winter wheat, potatoes, spring barley, swedes, spring oat, and three years of ley grass (grass/white clover). In the first year of grass a nitrogen, phosphorus and potassium fertiliser is applied, with the following composition: ammonium nitrate (70 kg N ha^{-1}), triple superphosphate (TSP, 30 kg ha^{-1}) and murate of potash (MOP, 50 kg ha^{-1}). The soil pH is maintained regularly, based on soil testing, by liming (calcium carbonate) and ferric sulphate addition, to raise and lower pH respectively (historically aluminium sulphate was used instead of ferric sulphate) (Walker et al., 2015). Three replicate samples, per pH treatment (were collected in June 2021 from each of the ley grass phases (one, two and three years of ley grass), at depths 0 – 20 cm and 20 – 40 cm. Soil was sieved to 2 mm, and stored at 4°C until analysis, with a subsample used for fresh soil analysis and the remaining soil air dried.

5.2.2 Bulk soil measurements

Soil pH was determined in deionised H_2O. Soil water content was determined gravimetrically, by drying until constant mass at 107°C. Mineral N concentrations (ammonium (NH_4^+-N), and nitrate + nitrite (NO_3^-- N and NO_2-N)) was determined using a Skalar SAN^{plus} Segmented Flow analyser, after extraction of 10 g soil with 50 mL of a 2 M KCL solution.

Microbial biomass C (MBC) was determined by chloroform fumigation extraction (Vance et al., 1987). Fumigated and non-fumigated fresh soils (15 g) were extracted with 0.5 M K_2SO_4 and the organic C content of extracts was determined on a Rosemount-Dohrmann DC-80 TOC analyser. MBC was calculated as the difference between the fumigated and non-fumigated TOC contents, corrected by a K_{EC} factor of 0.45, (a predetermined factor indicating the fraction of microbial C that is extractable by fumigation) (Joergensen, 1996), and for soil moisture. TOC content of non-fumigated samples considered as dissolved organic C (DOC) (Canarini et al., 2018; Sokol and Bradford, 2019). Total C and N (% by mass) of ball-milled air-dried soil were determined using a Flash 2000 elemental analyser.

5.2.3 Soil fractionation

The MAOM fraction was isolated by dispersion and wet sieving. Briefly, 15 g of air dried, 2 mm sieved soil was shaken for 16 hours at 180 rpm in 45 mL dilute (0.5%) sodium hexametaphosphate solution to disperse the soil (Córdova et al., 2018; Cotrufo et al., 2019). The suspension was then poured over a 53 μm sieve, and thoroughly rinsed with deionised water. Material < 53 μm was decanted into an aluminium tray, dried at 60^{o}C until constant mass, and classified as MAOM. Material > 53 μm was backwashed into an aluminium tray

and dried in the same way. Multiple methods exist to isolate SOM pools (Poeplau et al., 2018), and fractions may be composite and contain C with varying turnover times (von Lützow et al., 2007). In this instance, very fine POM may have contaminated the isolated MAOM, but we expect this to be negligible (Cotrufo et al., 2019; Lugato et al., 2021). The isolated MAOM was ball-milled and total C and N were determined in the same way as the bulk samples. The mean mass recovery rate was 99.5% (minimum of 97.42% and maximum of 100.54%).

5.2.4 Extractable cations

Bulk soil and MAOM fractions were extracted using Dithionite – citrate – bicarbonate (DCB), according to (Cloy et al., 2014). Briefly, 2 g dried soil extracted with 30 mL, 0.3 M sodium citrate, 2.5 mL, 1 M sodium bicarbonate and 0.5 g of sodium dithionite, heated to 70^{o}C in a water bath for 15 minutes. The suspension was centrifuged at 4500 rpm for 10 minutes, and supernatant filtered (0.45 μm pore size). The aluminium (Al), calcium (Ca), iron (Fe), magnesium (Mg), manganese (Mn) and phosphorus (P) contents were determined by ICP-OES.

5.2.5 Statistical analysis

All statistical analyses were conducted in R (version 3.6.3). Differences in total soil properties (SOC, MAOM-C, MBC, DOC, total N and mineral N) between the ley duration (for all pHs combined) and soil depth was determined by two-way ANOVA. Followed by post hoc Tukey's HSD test, to identify differences where appropriate. Normality and homogeneity of variance was determined by Shapiro-Wilk test and Levene test (both $P > 0.05$), and visually using qqnorm and residuals versus fitted plots. Non-normal data was log

transformed to achieve normality. In instances where normality was not achieved, the Kruskal-Wallis test was used to determine differences between soil properties between ley duration and soil depths separately. Followed by post hoc Dunns test, to identify differences.

To examine the effect of soil pH on SOC, MAOM-C, MBC and DOC, measured soil pH was used instead of treatment pH, due to variation in mean pH levels within a pH treatment across years and soil depth. Linear regression analysis was undertaken to determine the effect of measured soil pH, ley duration (1, 2 and 3 years) and soil depth (0 – 20 cm and 20 – 40 cm) and their interactions on SOC, MAOM-C, DOC and MBC. Non-significant variables ($P > 0.05$) were dropped from the model. Model selection was based on the lowest AIC score. Normality and homogeneity of variance of residuals were checked. Factors were deemed significant at $P < 0.05$.

The relationships between measured soil properties, across all ley durations, at 0 – 20 cm and 20 – 40 cm were examined using correlation matrix of Pearson correlation coefficients, using the Hmisc and corrplot packages (Harrell and Dupont, 2021; Wei and Simko, 2017). This was primarily done to examine relationships with MAOM-C, as ley duration was insignificant in MAOM-C across the pH gradient (**Figure 5.1**), all ley durations were pooled together.

5.3 Results

5.3.1 Effect of soil pH on MAOM-C, SOC, MBC and DOC

There was an interactive effect between soil pH and soil depth on MAOM-C ($P < 0.05$), with no effect of ley duration on MAOM-C (**Figure 5.1**, **Table D.1**). MAOM-C increased with pH at 0 – 20 cm ($r^2 = 0.17$), but pH had no effect on MAOM-C at 20 – 40 cm depth (**Figure 5.1**). Across all ley years, MAOM-C was positively correlated with Ca ($r = 0.49$), Mg ($r = 0.45$) and Mn ($r = 0.31$) extracted from the MAOM fraction, and MAOM-N ($r = 0.75$) at 0 – 20 cm ($P < 0.05$, **Figure 5.2**). Whilst at 20 – 40 cm MAOM-C was positively correlated with soil moisture ($r = 0.32$), total N ($r = 0.33$) and C ($r = 0.48$), and Mn ($r = 0.26$) and P ($r = 0.38$) extracted from MAOM fraction ($P < 0.05$, **Figure 5.2**).

There was a significant interactive effect between soil pH and ley duration, and soil pH and soil depth on the relative contribution of MAOM-C to total SOC ($P < 0.05$, **Figure 5.3**, **Table D.2**). After 1 year of ley grass, the contribution of MAOM-C to total SOC increased with alkalinity at 0 – 20 cm. In all ley durations the contribution of MAOM-C to total SOC decreased with alkalinity at 20 – 30 cm ($P < 0.05$, **Figure 5.3**).

The effect of soil pH on total SOC differed between the years of grass and soil depth. There was no significant effect of soil pH on SOC in ley year 1 at either soil depth (**Figure 5.3**, **Table D.3**). After 2 years of ley grass, the effect of soil pH on SOC varied with soil depth ($P < 0.01$). SOC increased with alkalinity at 20 – 40 cm, with no effect of pH on SOC at 0 – 20 cm, after 2 years of ley grass (**Figure 5.3**). After 3 years of ley grass, there was a significant main effect of pH ($P < 0.01$), with SOC increasing with soil pH (**Figure 5.3**). The effect of soil pH on MBC varied with ley duration and soil depth (**Figure 5.3**, **Table D.4**). Soil pH had

a significant effect on MBC in ley year 1 and 3 at 0 – 20 cm. However, after 2 years of ley grass, MBC was consistent across the pH gradient, with no significant effect of soil pH (**Figure 5.3**).

There was an interactive effect of grass ley duration and soil depth, and soil pH and soil depth on DOC ($P < 0.05$, **Table D.5**). In all three ley durations, DOC was highest in acidic soils, declined in neutral, with a slight rise again in soils above pH of 7.0 (**Figure 5.3**). The relationship between soil pH and DOC was not significant in ley year 2 at 20 – 40 cm.

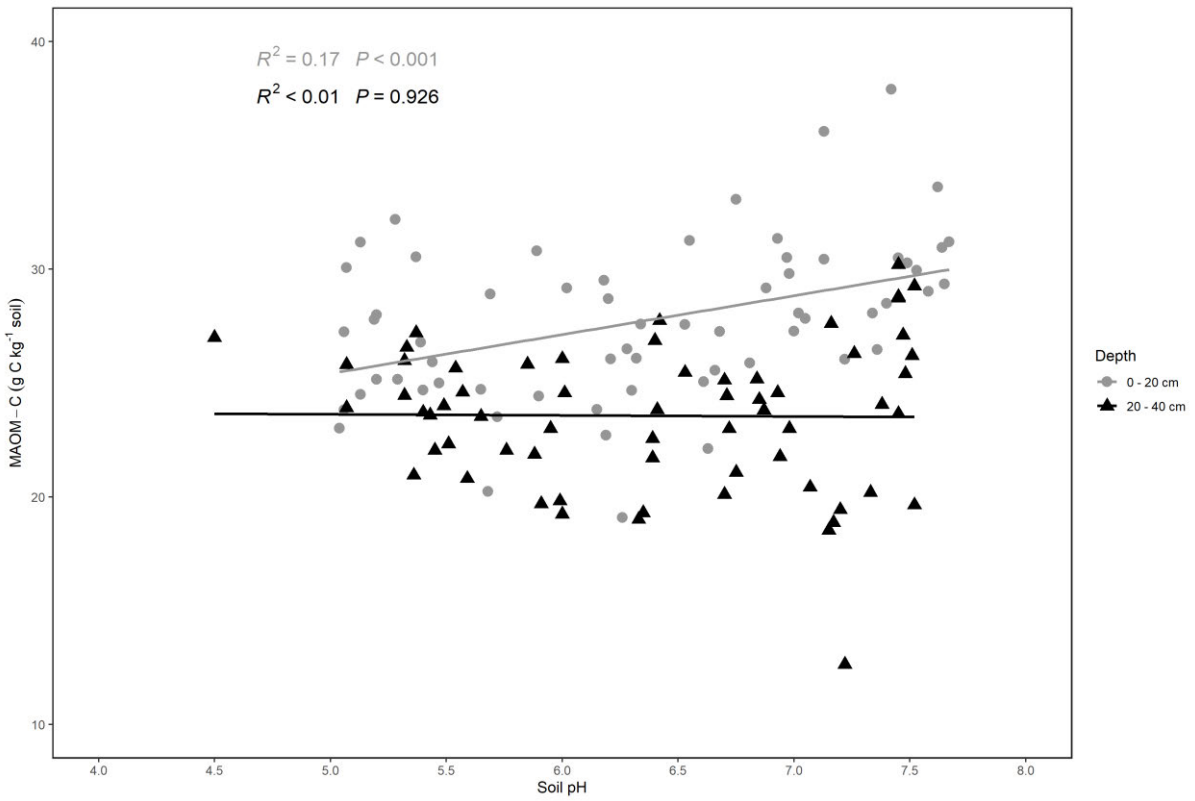

Figure 5.1. Relationship between measured soil pH and MAOM-C (g C kg^{-1} soil) at 0 – 20 cm (grey circles) and 20 – 40 cm (black triangles). Data from all three ley durations as ley duration was insignificant in MAOM-C across the pH gradient.

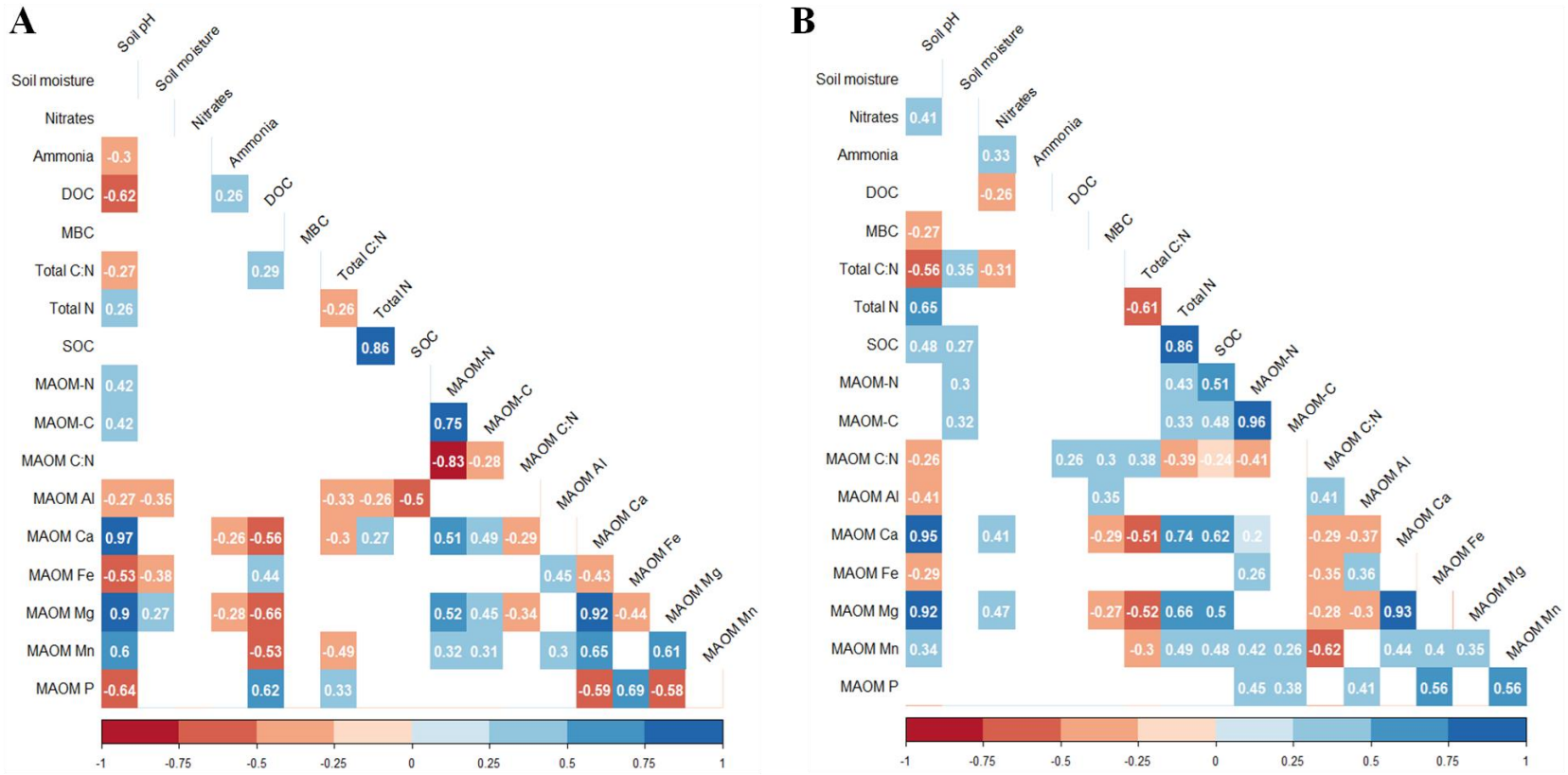

Figure 5.2. Correlation matrix of measured soil properties across all grass years at 0 – 20 cm (A) and at 20 – 40 cm (B). Only significant ($P < 0.05$) correlation coefficients are shown. Colours represent direction and strength of relationship, negative (red) and positive (blue). For individual P-values see **Figure D.2**.

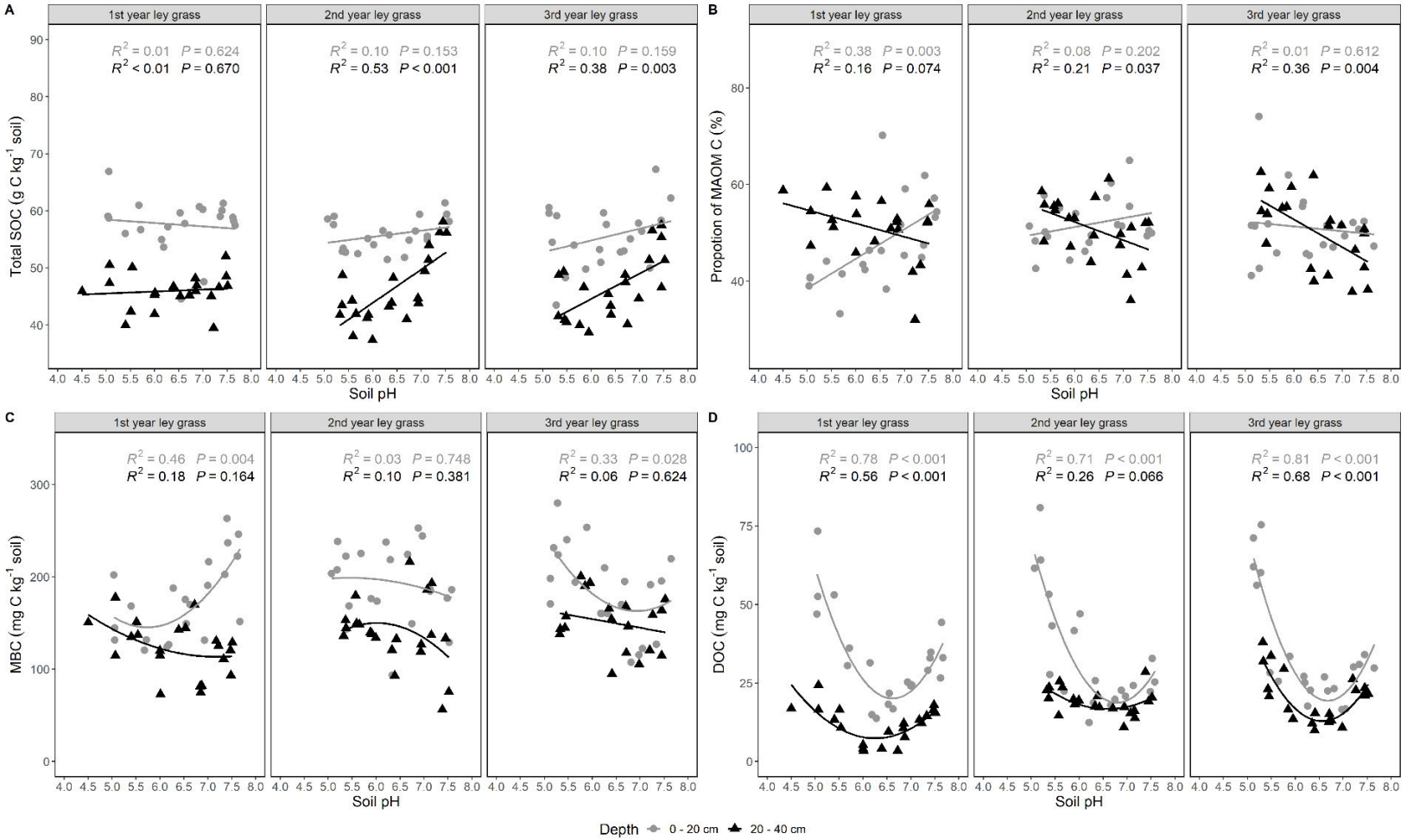

Figure 5.3. Relationship between measured soil properties and soil pH in each year of ley grass, total SOC (g C kg^{-1} soil) (**A**), the proportion of SOC that is MAOM-C (%) (**B**), microbial biomass carbon (mg C kg^{-1} soil, MBC) (**C**) and dissolved organic carbon (mg C kg^{-1} soil, DOC) (**D**).

5.3.2 The effect of grass ley duration

For all pH subplots together, there was no significant effect of ley duration on total SOC, MAOM-C, proportion of SOC that is MAOM-C, total soil N or nitrates (**Table D.6**). Total SOC, MAOM-C, total soil N and nitrates were all significantly lower at 20 – 40 cm than 0 – 20 cm ($P < 0.001$) (**Figure 5.4**, **Table D.6**). There was no significant difference in the relative contribution of MAOM-C to total SOC, at either depth, with MAOM-C accounting for 50.15 ± 0.93, and 50.67 ± 0.84 % of total SOC at 0 – 20 and 20 – 40 cm, respectively (mean ± SEM, averaged over all ley periods). There was a significant effect of ley duration on total MBC ($P < 0.05$), however post hoc testing revealed no significant differences (**Table D.6**). MBC at 20 - 40 cm was significantly lower than at 0 – 20 cm ($P < 0.001$) (**Figure 5.4**). Ammonium was significantly higher after 3 years of ley grass than after 1 ($P < 0.05$) and declined with soil depth ($P < 0.001$) (**Figure 5.4**, **Table D.6**). The effect of depth on DOC varied with ley duration ($P < 0.01$). DOC was lower at 20 – 40 cm, than at 0 – 20 cm in ley years 1 and 3 only (**Figure 5.4**, **Table D.6**).

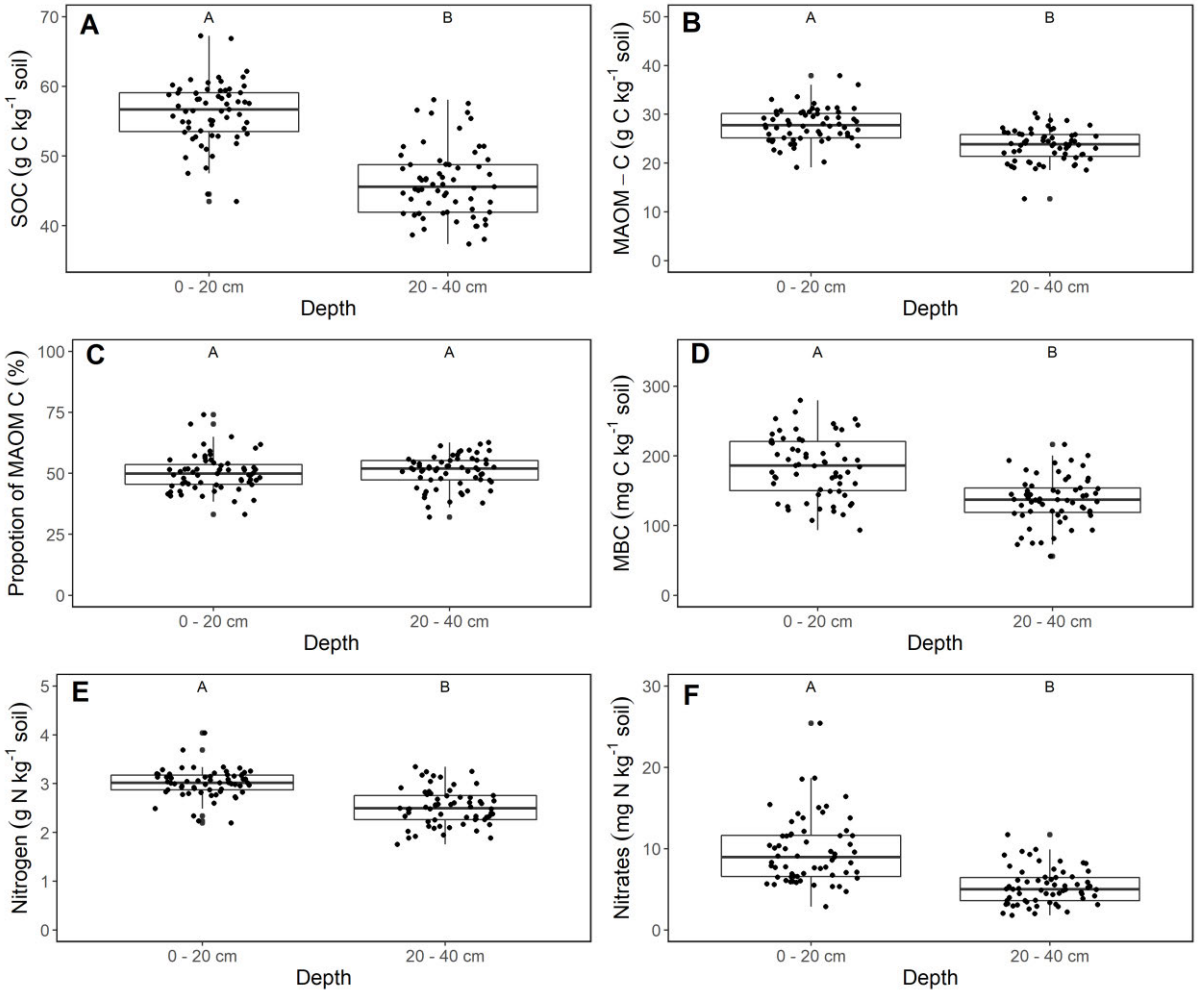

Figure 5.4. Effect of soil depth on total SOC (g C kg^{-1} soil) (**A**) MAOM-C (g C kg^{-1} soil) (**B**) proportion of total SOC that is MAOM-C (%) (**C),** MBC (mg C kg^{-1} soil) (**D**), total soil nitrogen (g N kg^{-1} soil) (**E**) and soil nitrates (mg N kg^{-1} soil) (**F**). Different uppercase letters indicate significant differences between means at 0 – 20 cm and 20 – 40 cm ($P < 0.05$). In each instance, ley duration was not significant, therefore boxplots represent samples from all 3 ley durations.

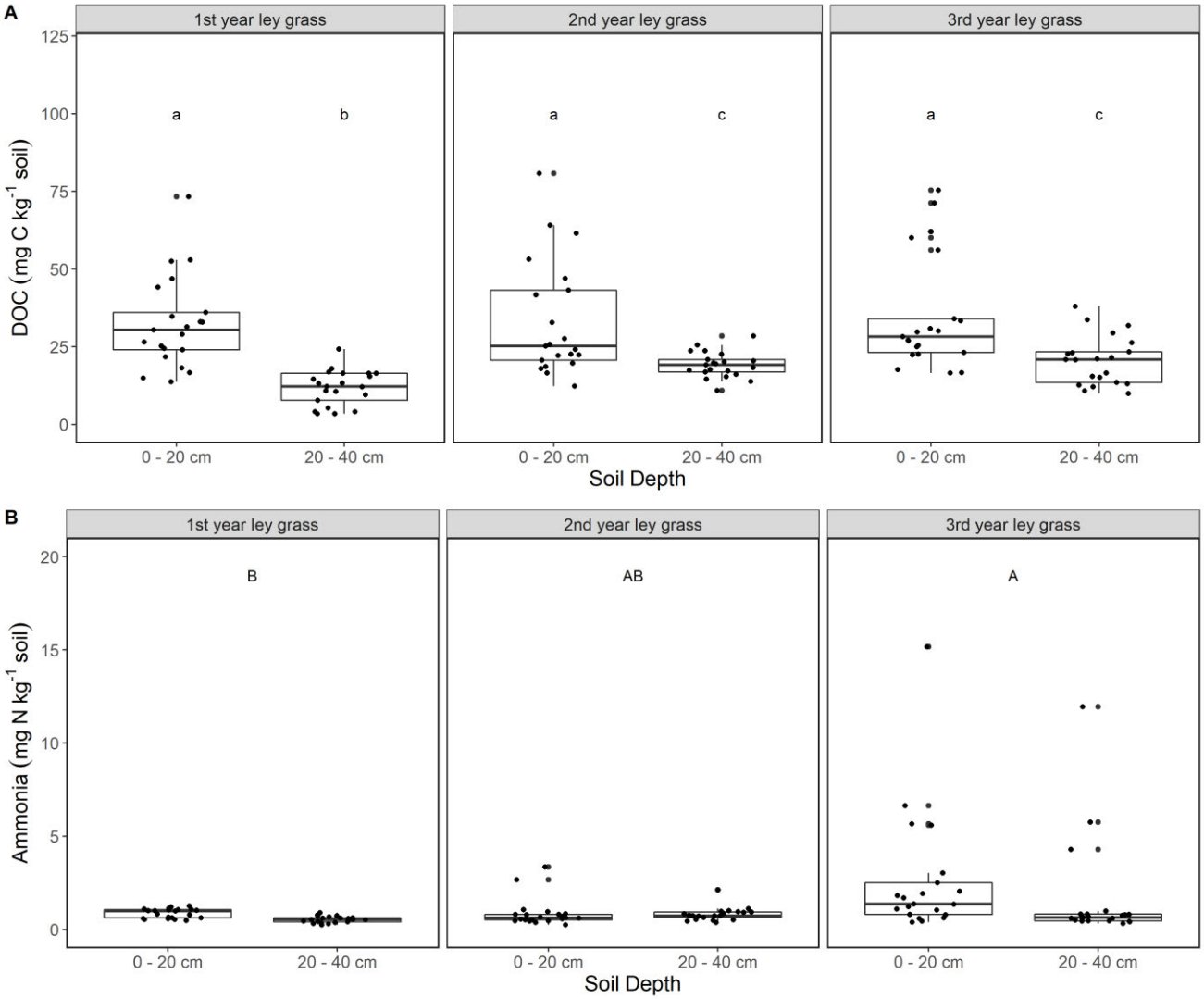

Figure 5.5. Effect of grass year and soil depth on DOC (mg C kg^{-1} soil) (**A**) and ammonium (mg N kg^{-1} soil) (**B**). Different lowercase letters in panel A indicate significant differences between DOC across ley durations and soil depths. Different uppercase letters in panel B, indicate significant differences in ammonium between the years of grass ley ($P < 0.05$).

5.4 Discussion

5.4.1 Effect of soil pH on MAOM-C

MAOM-C was expected to increase with soil pH, due to both biotic and abiotic means. Existing studies along similar pH gradients, attributed a decline in MAOM-C with soil acidification to decreases in MBC and leaching of base cations (Aciego Pietri and Brookes, 2008, (3.7 to 8.3); Chen et al., 2020, (4.8 to 5.29); Ye et al., 2018, (4.5 to 7.5)). In this study, MAOM-C was found to increase with soil pH at 0 – 20 cm, but not at 20 – 40 cm (**Figure 5.1**). Whilst the effect of pH on MBC varied between the grass ley duration (**Figure 5.3**), when analysed across all ley durations, there was no effect of pH on total MBC at 0 – 20 cm, but at 20 – 40 cm MBC declined with increasing pH (**Figure 5.2**). There was no relationship between MAOM-C and soil pH, or MBC and MAOM-C at 20 – 40 cm (**Figure 5.2**). This may be due to the surface application of pH treatments, and the greater spatial separation between soil microbes and C with depth, favouring abiotic retention mechanisms in the subsoils (McFarland et al., 2022). There was also no significant relationship between MBC and MAOM-C at 0 – 20 cm (**Figure 5.2**), suggesting that the increase in MAOM-C with pH at 0 – 20 cm in these soils, may be driven by abiotic factors.

The addition of lime, to raise pH may increase capacity for preservation of C in MAOM via direct sorption and cation bridging. Indeed, there was a positive relationship between MAOM-C and Ca, Mg and Mn within the MAOM fraction at 0 – 20 cm (**Figure 5.2**). There were also significant negative relationships between MAOM-C and Al and Fe within the MAOM fraction at 0 – 20 cm (**Figure 5.2**). Therefore, it is unclear as to whether the observed increase in MAOM-C at higher pHs, was driven by losses of MAOM-C due to leaching of

cations in acidic soils, or by additional retention with Mn oxides, or in cation bridges in alkaline soils.

It is necessary to consider that the observed increase in MAOM-C at 0 – 20 cm may be derived from liming applications themselves, unfortunately it was not possible to discern what proportions of C were lime or organically derived. Over a 15 year period it was estimated that 2.7% of C from liming inputs ($CaCO_3$) remained C (Fornara et al., 2011). Therefore, it is anticipated that this effect would be minimal. Additionally, higher plant C inputs due to greater plant biomass may stimulate microbial activity leading to increases in MAOM-C (Fornara et al., 2011), which is likely to be more prominent at 0 – 20 cm than at 20 – 40 cm.

Whilst ley duration had no effect on total MAOM-C, the relative contribution of MAOM-C to total SOC was affected by both ley duration and soil pH (**Figure 5.3**). MAOM-C made up more of the total SOC in alkaline soils (from pH 6.5 to 7.5) after 1 year of ley grass at 0 – 20 cm. Similarly Chen et al., 2020, observed an increase in POM-C and decrease in MAOM-C, in their most acidic treatment (pH 4.85) compared to the control (pH 5.29). These results are in line with Malik et al., 2018, who suggest that SOC accrual in acidic soils (pH < 6.2) is due to constrained decomposition of POM.

However, in all ley durations there was a decline in the relative contribution of MAOM-C to total SOC at 20 – 40 cm with increasing soil pH. With increasing depth, DOC has been found to be a main contributor to organo-mineral associations irrespective of soil texture, pH and

land use (Schrumpf et al., 2013). Therefore, the decline in the relative contribution of MAOM-C to total SOC in alkaline soils at 20 – 40 cm, may be due to reduced DOC percolation. In this study DOC was lower at 20 – 40 cm than at 0 – 20 cm, within each ley duration (**Figure 5.5**). The pH effect on DOC in these soils resulted in a similar trend across all ley durations. DOC was highest in acidic soils, and declined until a pH of ~ 6.5, before rising again (**Figure 5.3**). This is similar to the trend observed by (Aciego Pietri and Brookes, 2008) along a pH gradient of 3.7 to 8.3. The high DOC in acidic soils suggests that the uptake of DOC by the microbial community was constrained by acidity, and, or inaccessibility, due to sorption with minerals. As soil pH increases, constraints on microbial activity are released causing a reduction in available DOC. In this instance, DOC may have further decreased with increasing pH due to cation bridging with Ca, Mg and sorption with Mn oxides at 0 – 20 cm and incorporation into MAOM (**Figure 5.2**) (Rasmussen et al., 2018). Combined, these processes are likely to have limited the vertical movement of DOC through the soil profile, limiting MAOM-C formation. Despite the changes across the pH gradient, there was no difference in the contribution of MAOM-C to total SOC between the two depths (**Figure 5.4**). With MAOM-C accounting for 50.15 ± 0.93, and 50.67 ± 0.84 % (mean ± SEM, averaged over all ley periods) of total SOC at 0 – 20 and 20 – 40 cm, respectively. This proportion is akin to global estimates of 69.6 ± 22.0 %, in grasslands (Sokol et al., 2022). Therefore, we reject the hypothesis that MAOM-C would make up a greater portion of SOC with depth in these soils.

Soil pH exerts a strong influence on microbial community composition and abundance (Fierer, 2017), and it was anticipated that MBC would be lowest in acidic soils (Aciego Pietri and Brookes, 2008; Chen et al., 2020; Ye et al., 2018), with knock on implications for MAOM-C formation. Whilst there were no relationships between MBC and MAOM-C at

either depth (**Figure 5.2**), the effect of soil pH on MBC between the ley durations might be indicative of microbial community recovery following soil disturbance alongside chronic pH exposure (**Figure 5.3**). The low MBC in acidic soils in year 1 at 0 – 20 cm, may be due to slow regrowth of fungal communities. Acidic soils typically favour fungi (Rousk et al., 2010), which may be particularly sensitive to soil disturbance associated with seeding of a new crop, due to the destruction of fungal hyphae and networks, in the top 15 cm of soil (Kabir, 2005; Mårtensson and Olsson, 2012). Bacterial growth rates have been found to increase fourfold from pH 4.5 to 8.5, at the Rothamsted Research site in the UK (Rousk et al., 2009). However, previous work at the same field trial, as used in this study, found that bacterial biomass significantly decreased with increasing pH (Herold et al., 2012). Whilst Herold, et al., 2012, did not propose a reason for this, the decline in bacterial biomass with soil pH, it may explain the negative relationship between increasing soil pH and MBC at 0 – 20 cm after 3 years of ley grass (**Figure 5.3**). It is not possible to discern the drivers of the temporal trends in MBC, observed in this study.

Whilst there was no relationship between MBC and MAOM-C at either depth, across the entire sampled profile, and all ley durations (0 - 40 cm), there was a significant moderately positive correlation between MBC and MAOM-C ($P < 0.05$, result not shown). Considering the mechanisms of microbial mediated MAOM formation remain unclear (Kleber et al., 2021), and the high contribution of microbial products to MAOM-C (Miltner et al., 2012) it would be erroneous to suggest that microbes have not contributed to the formation of MAOM in these soils. Additionally, Ye et al., 2018, reported concurrent decreases in MBC and MAOM-C, but microbial biomass was not related to MAOM-C in their structural equation model. Further work investigating the microbial response to pH and the implications for MAOM-C accrual would be beneficial in this instance.

5.4.2 Grass-clover ley duration

The reduced soil disturbance during grass ley periods contributes to an increase in SOC (Zani et al., 2021). However, the effects of ley duration on MAOM-C are rarely studied. Zani et al., 2021, found no change in MAOM-C with grass-clover ley duration. Additionally in agricultural grasslands, reseeding events have been found to have no effect on MAOM-C (Carolan and Fornara, 2016; Fornara et al., 2020; Linsler et al., 2013; Paterson et al., 2021). Therefore, it was anticipated that there would be no change in MAOM-C with grass ley duration, as observed in this study (**Figure 5.4**, **Table D.6**). The exact mechanisms for this are unclear, but indicates the resistance, or resilience of abiotic and biotic processes that contribute to MAOM-C formation following soil disturbance. The presence of N plays a role in MAOM-C formation by alleviating nutrient limitations on microbial activity and contributing to organo-mineral association formation (Kopittke et al., 2020). Despite greater ammonium after 3 years of ley than 1 year, possibly due to the establishment of clover, there was no difference in total N between the ley durations, across all the pH treatments (**Figure 5.5**, **Table D.6**) and unlikely to have influenced MAOM-C accrual. Therefore, in this instance MAOM-C may have been unaffected by the soil disturbance associated with ploughing a subsequent seeding of grass-clover leys due to several mechanisms. Firstly any disturbance induced losses of C from MAOM were rapidly replaced or MAOM-C has reached a saturation or equilibrium point (Stewart et al., 2007). Tillage events are likely to have a greater impact on C preserved within larger aggregates (Fornara et al., 2020; Witzgall et al., 2021), than C within MAOM. The narrow rhizosphere:bulk soil ratio in grasslands, and efficient formation of MAOM-C in the rhizosphere (Sokol and Bradford, 2019), may result in the replacement of any MAOM-C that is lost due to soil disturbance. However, further empirical evidence is necessary to elucidate why MAOM-C seems to be relatively unaffected

by soil disturbance associated with tillage events. Potentially in this instance MAOM-C has reached an equilibrium and therefore the grass-clover leys serve as a means to maintain this C content (Johnston et al., 2017).

5.5 Conclusions

This work provides novel insights into the dual effects of soil pH management and grass-clover ley duration on MAOM-C. Results from this work showed that the effect of pH on MAOM-C was depth dependent, with MAOM-C increasing with pH at 0 – 20 cm, and there was no pH effect at 20 – 40 cm. The results from this study suggest that the increase in MAOM-C with pH was due to abiotic retention of C by cation bridging and direct sorption to Mn-oxides. Additionally, the contribution of MAOM-C to total SOC changed with soil pH and ley duration. The changing relative contribution of MAOM-C to total SOC, highlights the need for land management policies focused on C sequestration to be sensitive to both MAOM-C and POM-C. However, across the pH gradient, ley duration had no impact on MAOM-C. The lack of an effect of ley duration on total MAOM-C implies that any losses of MAOM-C due to soil disturbance were fully recovered after 1 year of ley grass. Therefore, ploughing events may not be as detrimental to persistent SOC as previously thought.

Chapter 6 General Discussion

6.1 Overview

SOC is the largest terrestrial pool of C (Lehmann and Kleber, 2015; Smith, 2012), however, significant losses of SOC have occurred due to agricultural intensification and land use change (Smith et al., 2020). The promotion of soil C sequestration, through correct management of agricultural systems, can restore some of this lost SOC (Lorenz and Lal, 2018), and provides an opportunity to mitigate the effects of climate change. In order to increase SOC in the most persistent forms requires an understanding of the mechanisms which confer SOC persistence, the effects of human management on these processes and the response to climate change. The overarching aim of this thesis was to contribute to the understanding of the dynamics of MAOM-C in the context of agricultural grassland management and climate change. Specifically, it explored the use of a relatively simple method to estimate maximum SOC sequestration potential, examined the effect of substrate type, N addition and temperature on substrate C incorporation within MAOM, and the effect of soil pH management and grass ley duration on MAOM-C. This chapter discusses the main findings and limitations of the work and potential avenues for future work.

6.2 Main findings of each chapter

This thesis addresses some knowledge gaps on SOC sequestration within agricultural grasslands. In **Chapter 2,** it was found that the Hassink, 1997, regression equation resulted in the lowest estimation of fine-fraction SOC sequestration potential. As discussed in the chapter, a drawback of this method is that it is more indicative of the mean sequestration potential. Consequently, the quantile regression (QR) at the 90th percentile is recommended as a better representation of the potential sequestration capacity of the agricultural grasslands used in this work. The QR estimate at the 90th percentile found that 76 % of the samples

were below saturation, with a mean saturation ratio of 0.74 (where < 1, is below saturation, and > 1 is oversaturated). This implies that there is potential for additional C sequestration in the studied sites. Nevertheless, this method is constrained by the available data, and it is not possible to say that the sites sampled capture the maximum fine fraction C in the UK. This estimation method does not account for additional constraints on C sequestration imposed by the agri-ecological context of the site, such as management systems, OM inputs and climate, or the biological processes that are now known to influence SOC persistence (see **section 6.3**, for further discussion). Therefore, of the estimates used in the chapter the QR is the most robust, due to statistical benefits compared to linear regression. However, the estimation method is likely too simplistic to capture the mechanisms that are now understood to confer SOC persistence.

Chapter 3 and **Chapter 4** focused on the effects of substrate type and quantity, N addition and temperature on the formation of MAOM-C from labile C substrate. **Chapter 3** examined the effect of glucose quantity and ammonium nitrate (AN) addition in MAOM-C formation. Increased glucose addition rate led to greater absolute substrate ^{13}C recovered in the MAOM fraction, but there was no effect on proportional retention. The role of AN appeared to be a function of C addition rate, with a significant difference in substrate ^{13}C recovered in MAOM only evident in the medium addition rate, and not the low and high glucose addition rates. In **Chapter 4** there was no effect of temperature or substrate on substrate-applied ^{13}C recovered in MAOM fraction. However, in the bulk SOC substrate-applied ^{13}C recovery was significantly affected by substrate and temperature. The substrate ^{13}C recovered in bulk SOC was significantly higher for glucose than acetic acid at 10 and 15°C, but not at 20 or 25°C.

Finally, **Chapter 5**, explored the effects of soil pH manipulation, grass ley duration and soil depth on MAOM-C. The effect of pH on MAOM-C was depth dependent, with MAOM-C increasing with pH at 0 – 20 cm, and there was no pH effect at 20 – 40 cm. The results from this study suggest that the increase in MAOM-C with pH was due to abiotic retention of C by cation bridging and direct sorption to Mn-oxides. Additionally, the contribution of MAOM-C to total SOC changed with soil pH and ley duration. However, ley duration had no impact on MAOM-C.

There were two notable commonalities across the experimental chapters of this thesis. Firstly, in both **Chapter 2,** and **Chapter 5**, there was no evidence that soil disturbance from reseeding or sowing of grass leys had an effect on MAOM-C. Secondly in **Chapter 3, Chapter 4** and **Chapter 5**, there was no relationship between MBC or MAOM-C. The following section considers the main findings in the wider literature in the context of estimating soil carbon sequestration and the role of saturation, MAOM-C formation, and the effects of land management on MAOM-C.

6.3 Estimating soil carbon sequestration and saturation

Soil carbon sequestration, is often presented as a win-win scenario to help reduce atmospheric CO_2 and improve soil quality (Lal, 2004; Smith, 2012). However, the widespread implementation of C sequestration for climate change mitigation has been limited due to concerns of permanence, saturation, difficulties with measuring and verification alongside socio-economic barriers to change in management (such as land ownership, and the level economic investment in equipment required to change established management

systems) (Amundson and Biardeau, 2018; Horwath, 2015; Jones et al., 2017; Smith, 2012). The discussions regarding the global potential of soil C sequestration, after the launch of the "4 per mille – Soils for food security and climate" Initiative (Schiefer et al., 2018; Schlesinger and Amundson, 2019), only highlighted the scientific uncertainties that remain.

In order to use soil carbon sequestration in climate mitigation policies, it is necessary to understand the C sequestration potential of a soil. **Chapter 2**, explored several relatively simple methods of estimating maximum fine fraction C across UK grasslands, and highlighted that differences occur due to estimation method. The quantile regression method resulted in an estimate that was almost double that of the boundary line and linear regression methods. It is important however, to not overestimate the mitigation potential of C sequestration, which may mislead policy makers seeking measures for reducing GHG emissions (Powlson et al., 2011; Schlesinger and Amundson, 2019). Attention must also be paid to the interactions with other biogeochemical cycles, particularly the nitrogen (N) cycle. Additional C may facilitate the mineralisation of N, increasing nitrous oxide emissions which may offset C sequestration benefits (Lugato et al., 2018; Powlson et al., 2011).

It was initially planned to resample the fields used in **Chapter 2.** However, in light of world events following this work it was not possible. Nevertheless, doing so would provide an opportunity to track any changes in soil C concentration in fine fraction. Whilst the fractionation method used in **Chapter 2** was chosen to mirror that of Hassink, 1997, future work should consider isolating a broader MAOM (< 53 to 60 μm) fraction. Doing so would provide an important basis for comparison of MAOM-C within the UK and with other wider

European analysis such as that by Cotrufo et al., 2019. This is likely to provide a better context to make judgements about the opportunities for further MAOM-C accrual.

The estimation method used in **Chapter 2** is based on the theory of soil C saturation, which constrains C accrual due to finite number of binding sites on mineral surfaces (Hassink, 1997; Six et al., 2002). This concept is generally well supported in the literature, and a recent European wide study found that once SOC exceeds 50 g SOC kg^{-1} soil, accrual of MAOM-C is limited (Cotrufo et al., 2019). However, the theory of saturation arose under a different framework of SOC persistence, compared to what is generally considered to confer persistence today. Whilst inherent biochemical recalcitrance plays a role (Kleber et al., 2015), it is no longer considered to be the sole determinant of C persistence. The mechanisms that cause C to persist and accumulate in organo-mineral associations are complex (Kleber et al., 2021).

Saturation theory is considered an inherent soil property independent of limitations imposed by management and climate (Castellano et al., 2015). However, management and climate, have implications for soil structure and environment, nutrient availability, and microbial activity, which are important in the modern understanding of SOC persistence. Recent evidence has shed light on the importance of N in the formation of new organo-mineral associations (Kopittke et al., 2020), and therefore N limitations may have a larger influence on MAOM-C accrual than previously acknowledged. The results in **Chapter 3** suggest that the role of N in retention of fresh labile C in MAOM may be a function of C input. However it remains unclear as to the importance and extent of influence of the C:N ratio of input, versus that of the existing soil (see Wu et al., 2022) with respect of MAOM-C accumulation.

More recently, empirical evidence challenges the notion that soils with a greater quantity of minerals with reactive surface have a higher capacity to preserve C in organo-mineral associations (Feng et al., 2014; Stewart et al., 2007), which is the foundation of estimation methods that use the proportion of fine fraction in a soil and it's C content, to estimate maximum C content. In soils under similar management, but with a clay gradient of 5 to 37%, Schweizer et al., 2021 found no change in total SOC, but differences in the contribution of C within POM and MAOM to total SOC in the high and low clay soils. A greater proportion of total C in the high clay soils was in occluded POM, whilst the majority of C in the low clay soil was in MAOM fraction (Schweizer et al., 2021). Additionally higher MAOM did not coincide with greater mineral surface OM coverage across the clay gradient, therefore the greater MAOM-C in the low clay soils, was due to higher concentration of OC per mineral surface area (Schweizer et al., 2021). These results pose doubt over the accuracy of estimating maximum OC based on the mineral content, and may help explain discrepancies observed whilst using such methods. Indeed in **Chapter 2,** the sites with Leptosols and Luvisols (**Figure 2.2**) had a non-significant relationship between fine-fraction OC and mass proportion of the fine fraction, and yet had the greatest proportion of total SOC within the fine fraction. This may be due to increased OC loading as reported by Schweizer et al., 2021, or due to other pedo-climatic-management factors such as the presence of cations. For instance Luvisols typically have a high base saturation, which can facilitate organo-mineral associations via ligand exchange (Chen et al., 2020).

Typically it is expected that there is greater potential for additional C sorption at depth due to greater available mineral surface area (Abramoff et al., 2021). However the findings of Schweizer et al., 2021, question this concept, which has implications for estimates of soil C

sequestration potnetial which are based on mineral specific surface area, such as Emde et al., 2022. Further analysis across differing pedo-climatic-management conditions are required to corroborate the findings of Schweizer et al., 2021, beyond their study site in Germany.

In light of this, there is a need to consider how saturation theory sits within the current framework of SOC persistance. A more inclusive consideration of soil biotic and abiotic properties within a land management and climatic context, would help to clarify causes of SOC saturation (Craig et al., 2021), and subsequently result in more accurate predictions of maximum MAOM-C. For instance, given the role of the soil microbial community in the accrual and persistence of SOC (Miltner et al., 2012), accounting for processes such as predation, competition and stress, which influence microbial CUE, growth and turnover (Craig et al., 2021), is necessary when considering what confers MAOM-C saturation in a soil. Echoing this, Sokol et al., 2022 proposes a plant and microbial trait-based approach in estimating MAOM-C under future climatic scenarios. The proposal separates plant and microbial traits into those that influence the total OM inputs to the soil (such as plant primary productivity, microbial CUE and growth rate) and those that determine the amount of OM incorporated in MAOM (such as plant allocation of C belowground, litter and rhizodeposit composition, and microbial compound allocation, and cellular composition) (Sokol et al., 2022). Doing so enables the consideration of the effects of climate change on the key traits that influence MAOM-C dynamics. For instance, in **Chapter 4**, neither temperature (influencing OM inputs to soil, primarily through microbial activity) or substrate type (influencing amount of OM entering MAOM) affected the quantity of substrate ^{13}C recovered in MAOM. However, more glucose was recovered in the bulk soil than acetic acid at 10 and 15°C. This additional C retention may drive future microbial activity and biomass production, which influence MAOM-C dynamics.

Improving the understanding of the contextual drivers of saturation would help to guide land management for SOC sequestration which is saturation specific. In **Chapter 2,** fine-fraction OC (g C kg^{-1} bulk soil) was positively correlated with median annual temperature, and negatively with mean annual rainfall (**Table 2.2**). Both of which influence OM inputs to the soil via plant productivity, and leaching of dissolved OM, which alters substrate availability and subsequently microbial activity. Whilst further work would be required with respect to the results of **Chapter 2,** Therefore, in instances where microbial activity was found to have the greatest influence on MAOM-C accumulation, management that promotes more favourable MAOM-C accumulation could be implemented (e.g. alleviating acidity or nutrient deficits) (Craig et al., 2021). Whilst much of the focus of SOC sequestration is on increasing MAOM-C, the long-term persistence of C in POM, although not examined in this thesis should not be overlooked. In some instances, C may accumulate and persist in POM, such as acidic wet conditions (Malik et al., 2018). This was observed in the acidic topsoils in **Chapter 5**, with MAOM-C accounting for less of total SOC (**Figure 5.3**). Consequently, policy aimed at increasing SOC should account for both MAOM and POM-C in addition to ecosystem properties, such as soil pH, nutrient availability and microbial community composition, which contribute to the preferential persistence of C in each form (Lavallee et al., 2020).

Ultimately it is necessary to clarify how the traditional theory of saturation fits within recent frameworks of SOC persistence. What mechanisms confer saturation, and the relative importance of these within different pedo-climatic and management conditions? Efforts to improve the understanding of SOC saturation and sequestration potential, should be mindful

of not overstating the mitigation potential of soil C sequestration, which should be considered alongside a suite of emission reductions in other sectors (Bossio et al., 2020).

6.4 MAOM-C formation

Chapter 3 and **Chapter 4** contribute to the understanding of microbial mediated MAOM-C formation from fresh labile C. In both instances microbial uptake of the substrates used have been shown to outcompete direct sorption with minerals (Fischer et al., 2010), and are representative of common classes of root exudates (Van Hees et al., 2005).

Chapter 3 contributes to current understanding of the role of C:N stoichiometry on MAOM-C formation. Time and circumstances permitting, further development of the data presented in this chapter would have been possible by running further experiments. An avenue worth attention is to explore whether the presence of additional N confers greater MAOM-C accrual and subsequent persistence in the future. With a specific focus on agricultural land management, empirical evidence of the effects of N type (i.e. organic versus mineral substrate additions) on MAOM-C accumulation is essential. Improving the understanding of the N requirements for MAOM-C accrual are necessary given the N limitations often cited as a constraint on accrual. This would provide an evidence base for suggesting the use of a specific type of N fertiliser with respect to MAOM-C formation. However, a full cost-benefit analysis of such changes is necessary prior to any management recommendations, to capture the full costs of such changes (environmental and economic).

It was anticipated that there would be significant relationships between MBC and MAOM-C, as correlations between MBC and MAOM-C, alongside empirical evidence of microbial contribution to MAOM (Miltner et al., 2012) are used to indicate the contribution of the soil microbial community to MAOM-C. However in **Chapter 3, Chapter 4** and **Chapter 5** this was not the case. Whilst this is unexpected, Ye et al., 2018, reported concurrent decreases in MBC and MAOM-C, but microbial biomass was not related to MAOM-C in their structural equation model. Potentially the lack of a significant relationship may be due to the timing of sampling, or predominant formation of MAOM-C via the *ex* vivo pathway. Further investigations would be required to confirm these suggestions. These could involve the use of sterilised soils compared with "live" soils or using soils sampled at different times of the year to capture temporal differences in microbial activity.

Additionally, whilst not explicitly explored in this thesis, but touched on throughout, there is a need for empirical evidence to clarify theorised relationships between microbial CUE and MAOM-C formation. Microbial CUE describes the amount of C allocated to growth versus respiration (Geyer et al., 2019), and therefore determines the amount of microbial by-products generated per unit substrate, which are subsequently available to form organo-mineral associations (Cotrufo et al., 2013). It has been suggested that a higher CUE results in relatively more MAOM-C formation (Cotrufo et al., 2013). Kallenbach et al., 2016, observed a positive relationship between SOM and CUE, but links between MAOM-C and CUE are lacking (Sokol et al., 2022). In instances where higher CUE coincides with reduced gross C uptake, lower respiration and no change in biomass production, it is of no benefit to C accumulation via the microbial pathway (Kallenbach et al., 2019). Despite lower CUE in limed soils, Fornara et al., 2011 attributed the increase in MAOM-C to greater absolute

microbial activity and subsequent greater incorporation into MAOM-C pool. However, in **Chapter 5**, there was no significant relationship between MBC and MAOM-C. Overall the results in this chapter suggested that abiotic mechanisms played a greater role in the increase in MAOM-C in limed soils. This is not to say that there were no changes in microbial activity or CUE, as these were not determined.

Despite differences in reported CUE of glucose and acetic acid, and in whole soil retention of substrate ^{13}C in the bulk soil, there was no difference in ^{13}C recovered in MAOM-C in **Chapter 4**. This suggests similar allocation of C to microbial by-products which went on to form MAOM complexes. Or despite greater glucose derived microbial by-products at 10 and 15°C, incorporation into MAOM was constrained by other means, such as the availability of organo-mineral or organo-organo binding sites. However, as mentioned in that discussion further isotopic analysis to determine substrate C in other soil C pools would be beneficial.

Nevertheless, the links between CUE and MAOM-C formation are complex and there is a need to clarify this relationship, which would greatly contribute to the understanding of microbially mediated MAOM-C formation. Additionally there is a need to consider if other microbial traits are better determinants of MAOM-C formation, with the production of microbial necromass influenced by microbial growth and turnover (Prommer et al., 2019). Such advances in understanding would be beneficial for the inclusion of microbial traits in estimations of the response of SOC to future climatic change (Ye et al., 2019).

6.5 Land management

Recent estimates suggest that ~70% of total SOC in grasslands is made up of C within MAOM (Sokol et al., 2022). Therefore, understanding the impacts of agricultural grassland management on MAOM-C is necessary to inform land management focused on increasing and protecting existing MAOM-C.

Chapter 5 explicitly explored the effect of soil pH manipulation and grass ley duration on MAOM-C. Whilst the impacts of soil acidification (Chen et al., 2020; Ye et al., 2018) and liming (Fornara et al., 2011) on MAOM-C have been previously examined, no study has thus far considered effects on MAOM-C across such a wide pH gradient from one location. The results suggest that MAOM-C accrual was largely driven by abiotic mechanisms in the top 0 – 20 cm, and that ley duration had no effect on MAOM-C, for all pH subplots pooled together. Whilst the work was conducted on a long-term pH manipulation trial which focused on inclusion of grass-clover leys within arable rotation, it provides insights which are likely to be relevant in agricultural grasslands, such as the lack of soil disturbance effect on MAOM-C. The results suggest that liming additions, can contribute to increases in MAOM-C and that any MAOM-C lost due to soil disturbance from ploughing and subsequent sowing of ley grass can be recovered within 1 year. Therefore, recommendations of liming to improve soil pH, and soil disturbance from ploughing events are unlikely to be deleterious to MAOM-C contents.

The lack of a difference in MAOM-C between the ley durations, is similar to the findings in **Chapter 2,** where sward age had no effect on fine fraction C. These results agree with the

literature which has also found no effect of soil disturbance, and grass ley duration on MAOM-C across different temporal scales (Carolan and Fornara, 2016; Fornara et al., 2020; Linsler et al., 2013; Zani et al., 2021). Subsequently reductions in SOC associated from soil disturbance may originate from POM, or C within larger aggregates. In **Chapter 2** it was hypothesised that "*grasslands with an older sward age would have a greater proportion of total SOC stabilised in the fine fraction (< 20 μm) and a lower sequestration potential*", due to greater accumulation of C within the fine fraction with time since soil disturbance. However, given the findings of this chapter and of Zani et al., 2021, in **Chapter 5** it was hypothesised that there would be no effect of grass ley duration on MAOM-C. Whilst the literature and results from this thesis support the limited effects of soil disturbance on MAOM-C, the exact mechanisms for this remain unclear. It is possible that C lost from MAOM due to disturbance of sowing of new swards, or grass ley rotations, is rapidly replaced. This may be due to the narrow rhizosphere:bulk soil ratio in grasslands, and efficient formation of MAOM-C in the rhizosphere (Sokol and Bradford, 2019). Secondly tillage events have a greater impact on C within larger aggregates (Fornara et al., 2020; Witzgall et al., 2021), than C within MAOM, therefore any changes in MAOM-C are insignificant. It may be that effects on MAOM-C are not captured due to the timing of soil disturbance and subsequent soil sampling for analysis. Further investigations which include sampling, before, immediately after, and at several intervals after soil disturbance will help to identify if there is any effect of soil tillage on MAOM-C, and if so, how long it takes for any C losses to be recovered.

Land management is known to influence grassland SOC stocks, however uncertainties remain as to the best management practice. Many studies of management changes are often

implemented in instances where changes are warranted (Conant et al., 2017). However, the general improvement of all management factors (grazing, nutrient management, improved sward mixes) can enhance C stocks (Conant et al., 2017). Whilst the effects of climatic change on MAOM-C have been recently reviewed (Lavallee et al., 2020; Rocci et al., 2021; Sokol et al., 2022), there is a need to reconsider previous research on agricultural land management in context of current understanding of the formation and persistence of SOC as a whole (Chenu et al., 2019). A meta-analysis of existing literature akin to that of Conant et al., 2017, but focused on MAOM-C would help to collate existing knowledge and identify where additional empirical evidence is required to understand how agricultural management influences MAOM-C dynamics.

Overall, the results from this thesis provide insights to guide further work and land management which focuses on increasing and preventing losses of persistent SOC. From the results presented in **Chapter 2** and **Chapter 5** it is evident that MAOM-C is resilient to soil disturbance due to ploughing event, when these are followed by sowing of grass swards. Therefore, ploughing events appear to have relatively little impact on this persistent form of SOC, in the soil types and management systems studied in this thesis. Additionally, despite limited available data on the effects of liming on SOC, the results in **Chapter 5**, show it's positive effect on increasing MAOM-C content. Therefore, the benefits of liming extend beyond enhancing plant productivity to increasing SOC.

The results from **Chapter 3** and **Chapter 4**, further the understanding of the mechanisms that result in the incorporation of fresh labile C into MAOM. Building on this, the examination of the effect of the C:N ratio of fertiliser amendments in field, would help to provide guidance

on the effect of fertiliser regimes on MAOM-C accrual. Additionally, it was found that substrate type had a greater influence on MAOM-C formation than temperature (**Chapter 4**). Building on this knowledge, in field assessments of the effect of varying sward compositions (e.g inclusion of legumes, or deeper rooting species) on MAOM-C accrual, would help to guide recommendations for sward compositions that promote the formation of persistent SOC.

6.6 Conclusions

The two overarching aims of this thesis were to examine the sequestration potential across a representative sample of agricultural grasslands within the UK and contribute to the understanding of MAOM-C formation in the context of land management and climate change. The work presented in **Chapter 2**, focused on the first aim and is the first time the Hassink, 1997, regression equation has been directly compared to one generated from soils in the UK. It was found that the Hassink, 1997, regression equation resulted in the lowest estimation of fine-fraction SOC sequestration potential, and was significantly different to that developed using UK soils. However, this estimation method was developed under a different understanding of SOC persistence and saturation. Therefore, given the current understanding and empirical evidence of C accumulation on mineral surfaces - how does the traditional theory of SOC saturation fit within the modern framework of SOC persistence? The expansion of saturation theory to include biotic and abiotic processes within the context of different land management and climates is necessary to generate better estimates of sequestration potential and guide policy which focuses on this.

The subsequent experimental chapters focused on core aspects of MAOM-C formation with an agricultural land management and climate change perspective. Improving the understanding of the interactions between management-climate and MAOM-C formation is critically important for guiding land management which aims to preserve and, or increase existing MAOM-C. The results in this thesis provide novel insights into the effects of mineral N fertiliser application, substrate type and ambient temperature on the incorporation of labile C in MAOM, and the role of pH management and soil disturbance on MAOM-C accrual. Notably it was found that i) the effect of ammonium nitrate addition on substrate C recovered in MAOM, was dependent on the C addition rate (**Chapter 3**), ii) temperature and substrate type had no effect on substrate C recovered in MAOM but did result in significant differences in substrate C recovered in the bulk SOC (**Chapter 4**), iii) MAOM-C increased with pH at 0 – 20 cm, possibly due to abiotic retention of C by cation bridging and direct sorption to Mn-oxides but there was no relationship at 20 – 40 cm (**Chapter 5**) and iv) there was no evidence to suggest that soil disturbance associated with ploughing and subsequent reseeding of grass swards had an impact on MAOM-C (**Chapter 2** and **Chapter 5**). Whilst these results highlight additional areas for further exploration (as discussed in each chapter and this general discussion). It is evident in this thesis, that MAOM-C is resilient to ploughing events, followed by seeding of grass swards and that liming applications can increase MAOM-C content.

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Appendix A

Appendix for Chapter 2: Estimating maximum fine-fraction organic carbon in UK grasslands

A.1 Supplementary Information for chapter 2.

Table A.1. Bulk-soil properties for each UK site. Values are means of ten replicates in each field, ± one standard error of the mean. Except Harpenden where values are means of five replicates per field. Lettering indicates values that are significantly different, within a site ($p < 0.05$).

Site	Age (years)	BD[a]	pH	C:N	C (g C per kg soil)	C stock (t C ha^{-1})	N stock (t N ha^{-1})
Aberystwyth	2	1 ± 0.01^{a}	5.20 ± 0.05^{a}	9.70 ± 0.05^{b}	26.95 ± 0.63^{b}	73.61 ± 1.73^{b}	7.59 ± 0.16^{b}
	6	0.98 ± 0.04^{a}	4.70 ± 0.04^{bc}	9.68 ± 0.08^{b}	26.7 ± 0.82^{b}	73.86 ± 2.28^{b}	7.62 ± 0.18^{b}
	11	0.82 ± 0.05^{b}	5.12 ± 0.06^{a}	10.46 ± 0.09^{a}	29.72 ± 0.83^{b}	76.97 ± 2.15^{b}	7.36 ± 0.20^{b}
	31	0.74 ± 0.05^{b}	4.99 ± 0.09^{ab}	10.54 ± 0.22^{a}	29.4 ± 1.63^{b}	74.23 ± 4.12^{b}	7.01 ± 0.31^{b}
	33	0.69 ± 0.03^{b}	4.18 ± 0.02^{c}	10.59 ± 0.10^{a}	38.19 ± 1.97^{b}	95.67 ± 4.92^{a}	9.01 ± 0.40^{a}
Crichton	1	0.92 ± 0.03	5.14 ± 0.03^{ab}	12.19 ± 0.08^{ab}	34.66 ± 0.66^{a}	82.40 ± 1.56	6.76 ± 0.11^{b}
	3	0.99 ± 0.07	5.65 ± 0.06^{b}	11.73 ± 0.11^{bc}	29.94 ± 1.37^{ab}	74.63 ± 3.41	6.36 ± 0.26^{b}
	15	0.93 ± 0.05	4.77 ± 0.04^{ac}	9.90 ± 0.88^{c}	30.85 ± 3.06^{b}	79.73 ± 7.91	7.98 ± 0.23^{a}
	20	0.93 ± 0.04	4.54 ± 0.03^{c}	13.21 ± 0.14^{a}	27.26 ± 0.87^{b}	66.62 ± 2.11	5.04 ± 0.15^{c}
Easter Bush	3	1.02 ± 0.04^{abc}	5.45 ± 0.06 ^{ab}	13.03 ± 0.11^{bc}	32.46 ± 1.29^{a}	93.52 ± 3.72^{a}	7.17 ± 0.26^{a}
	5	1.19 ± 0.03^{a}	5.44 ± 0.06^{ab}	12.84 ± 0.21^{bc}	26.41 ± 0.54^{b}	74.45 ± 1.52^{b}	5.80 ± 0.10^{bc}
	5	0.84 ± 0.06^{c}	5.67 ± 0.04^{a}	11.74 ± 0.17^{d}	27.50 ± 1.0^{b}	58.15 ± 2.12^{c}	4.94 ± 0.14^{d}
	6	0.96 ± 0.05^{bc}	5.32 ± 0.06^{b}	12.45 ± 0.13^{c}	30.46 ± 1.93^{ab}	71.16 ± 4.50^{bc}	5.72 ± 0.36^{cd}
	6	1.12 ± 0.05^{ab}	5.81 ± 0.20^{a}	14.15 ± 0.13^{a}	28.95 ± 0.99^{ab}	75.69 ± 2.59^{b}	5.35 ± 0.17^{cd}
	8	1.12 ± 0.03^{ab}	4.99 ± 0.04^{c}	13.43 ± 0.11^{b}	33.03 ± 0.50^{a}	89.43 ± 1.34^{a}	6.66 ± 0.10^{ab}

Harpenden	22	1.37 ± 0.07	7.37 ± 0.04^{a}	12.09 ± 0.2	16.06 ± 0.59^{c}	25.37 ± 0.93^{c}	3.3 ± 0.12^{c}
	68	1.12 ± 0.08	5.85 ± 0.12^{ab}	12.34 ± 0.08	19.8 ± 0.63^{b}	50.49 ± 1.59^{b}	4.06 ± 0.13^{b}
	179	1.09 ± 0.14	5.63 ± 0.06^{b}	12.8 ± 0.26	28.7 ± 1.47^{a}	72.98 ± 3.74^{a}	5.89 ± 0.30^{a}
Hillsborough	1	1.79 ± 0.10	6.31 ± 0.07^{a}	11.25 ± 0.12^{ab}	46.68 ± 2.04	120.16 ± 5.26^{ab}	10.69 ± 0.51^{ab}
	7	1.88 ± 0.08	5.10 ± 0.04^{b}	11.46 ± 0.11^{b}	42.85 ± 1.52	108.86 ± 3.87^{b}	9.51 ± 0.34^{bc}
	16	1.79 ± 0.05	5.33 ± 0.08^{b}	10.87 ± 0.06^{c}	42.36 ± 1.98	111.63 ± 5.21^{ab}	10.27 ± 0.47^{ab}
	23	1.75 ± 0.05	4.76 ± 0.03^{c}	11.33 ± 0.09^{ab}	46.44 ± 1.78	125.43 ± 4.82^{a}	11.08 ± 0.45^{a}
	37	1.69 ± 0.06	5.13 ± 0.06^{b}	10.34 ± 0.77^{ac}	40.90 ± 3.10	86.04 ± 6.52^{c}	8.38 ± 0.24^{c}
Kirkton	1	0.9 ± 0.04	4.78 ± 0.04^{c}	12.13 ± 0.11^{c}	27.90 ± 0.81^{c}	82.03 ± 2.39^{b}	6.77 ± 0.22
	3	0.95 ± 0.04	5.49 ± 0.06^{a}	12.61 ± 0.15^{b}	36.67 ± 1.56^{a}	98.19 ± 4.17^{ab}	7.79 ± 0.31
	5	0.83 ± 0.06	5.15 ± 0.03^{b}	13.56 ± 0.08^{a}	34.83 ± 1.84^{ab}	103.03 ± 5.45^{a}	7.59 ± 0.38
	35	0.97 ± 0.06	4.72 ± 0.07^{c}	11.67 ± 0.13^{d}	30.51 ± 1.48^{bc}	90.50 ± 4.38^{ab}	7.72 ± 0.32
Llangorse	2.5	1.01 ± 0.04	5.14 ± 0.08^{c}	9.21 ± 0.09	17.83 ± 0.42	49.75 ± 1.18	5.40 ± 0.11^{ab}
	5	0.93 ± 0.04	5.44 ± 0.03^{b}	9.40 ± 0.07	18.60 ± 0.45	50.80 ± 1.22	5.40 ± 0.10^{b}
	15	0.94 ± 0.06	5.68 ± 0.03^{a}	9.36 ± 0.17	19.42 ± 0.38	53.70 ± 1.06	5.74 ± 0.12^{ab}
	25	1.06 ± 0.03	5.54 ±0.07^{ab}	9.16 ± 0.87	19.73 ± 2.52	55.10 ± 7.05	6.18 ± 0.34^{a}
Myerscough	2	1.22 ± 0.02^{ab}	4.97 ± 0.05^{b}	13.58 ± 0.24^{bc}	27.47 ± 0.65^{c}	82.25 ± 1.96^{c}	6.07 ± 0.15^{bc}
	6	1.10 ± 0.04^{b}	5.59 ± 0.05^{a}	11.79 ± 0.76^{c}	41.44 ± 2.73^{a}	124.05 ± 8.17^{a}	10.56 ± 0.28^{a}
	13	0.93 ± 0.05^{b}	5.00 ± 0.20^{b}	13.12 ± 0.43^{c}	44.82 ± 2.34^{a}	134.45 ± 7.01^{a}	10.30 ± 0.71^{a}
	34	1.29 ± 0.02^{a}	5.99 ± 0.13a	17.20 ± 1.12^{ab}	37.58 ± 1.45^{ab}	112.46 ± 4.36^{ab}	6.71 ± 0.30^{b}
	48.4	1.44 ± 0.06^{a}	5.77 ± 0.02^{a}	22.10 ± 1.46^{a}	29.86 ± 1.96^{bc}	88.97 ± 5.85^{bc}	4.03 ± 0.08^{c}

Overton	3	0.98 ± 0.09[a]	6.58 ± 0.12[b]	9.76 ± 0.05[b]	32.77 ± 0.84[c]	83.02 ± 2.13[b]	8.51 ± 0.23[b]
	12	0.38 ± 0.03[b]	6.83 ± 0.03[b]	10.18 ± 0.12[ab]	70.18 ± 1.92[a]	81.20 ± 2.23[b]	7.99 ± 0.23[b]
	22	0.71 ± 0.07[ab]	7.36 ± 0.04[a]	10.68 ± 0.39[a]	59.88 ± 3.86[b]	132.75 ± 8.56[a]	12.33 ± 0.39[a]
	50	1.74 ± 0.9[a]	4.63 ± 0.08[c]	10.14 ± 0.14[ab]	51.18 ± 2.84[b]	153.08 ± 8.50[a]	15.08 ± 0.80[a]
Plumpton	1	0.99 ± 0.02[a]	6.34 ± 0.08[b]	10.85 ± 0.08ab	40.92 ± 1.21[b]	122.21 ± 3.61[b]	11.26 ± 0.28[b]
	5	1.08 ± 0.03a	7.15 ± 0.06a	11.27 ± 0.41a	45.55 ± 0.61[b]	132.09 ± 1.77[b]	11.87 ± 0.48[b]
	20	0.72 ± 0.04b	5.38 ± 0.21c	10.54 ± 0.17b	58.08 ± 2.36[a]	163.23 ± 6.62[a]	15.47 ± 0.56[a]

[a]Bulk density (BD), means and SEM of six samples, except Harpenden with two samples per field, corrected for stones.

Table A.2. Fine-fraction (< 20 μm) soil properties for each UK site. Values are means of three replicates in each field, ± 1 standard error of the mean. Lettering indicates values that are significantly different, within a site ($p < 0.05$).

Location	Age (years)	%N	%C	C:N	%Fine-fraction	Organic Carbon (g C per kg bulk soil)[a]	Organic Carbon (% of SOC_{total})
Aberystwyth	2	0.48 ± 0.01[b]	4.16 ± 0.08	8.62 ± 0.08[ab]	19.08 ± 1.04[a]	7.93 ± 0.45[a]	0.30 = 0.01[a]
	6	0.52 ± 0.04[b]	4.14 ± 0.30	8.05 ± 0.17[b]	14.47 ± 1.18[ab]	5.92 ± 0.22[ab]	0.21 ± 0.02[ab]
	11	0.55 ± 0.03[b]	4.89 ± 0.25	8.86 ± 0.06[ab]	18.02 ± 1.51[ab]	8.77 ± 0.56[a]	0.29 ± 0.02[ab]
	31	0.61 ± 0.05[ab]	5.78 ± 0.62	9.51 ± 0.30[ab]	13.78 ± 0.49[b]	8.02 ± 1.15[a]	0.27 ± 0.02[ab]
	33	0.76 ± 0.03[a]	7.57 ± 0.37	9.96 ± 0.23[a]	5.02 ± 0.22[c]	3.81 ± 0.36[b]	0.10 ± 0.01[b]
Crichton	1	1.01 ± 0.06	10.53 ± 0.83	10.40 ± 0.23[ab]	4.00 ± 0.45	4.24 ± 0.69	0.12 ± 0.02
	3	1.15 ± 0.27	11.17 ± 2.28	9.84 ± 0.35[b]	3.28 ± 0.23	3.75 ± 1.00	0.13 ± 0.04
	15	1.02 ± 0.12	9.76 ± 1.20	9.52 ± 0.12[b]	3.52 ± 0.26	3.38 ± 0.31	0.10 ± 0.02
	20	0.82 ± 0.05	9.07 ± 0.72	11.03 ± 0.24[a]	3.37 ± 0.3	3.01 ± 0.09	0.11 ± 0.01
Easter Bush	3	0.65 ± 0.04	7.15 ± 0.50	11.00 ± 0.13[ab]	14.38 ± 1.56[ab]	10.27 ± 1.19[a]	0.30 ± 0.01
	5	0.65 ± 0.04	6.91 ± 0.50	10.57 ± 0.06[bc]	12.17 ± 0.9[ab]	8.34 ± 0.43[ab]	0.32 ± 0.02
	5	0.67 ± 0.02	6.62 ± 0.23	9.83 ± 0.07[c]	9.55 ± 0.73[b]	6.32 ± 0.51[b]	0.23 ± 0.03
	6	0.68 ± 0.03	7.81 ± 0.43	9.85 ± 0.24[c]	9.75 ± 0.23[b]	6.88 ± 1.13[ab]	0.23 ± 0.01
	6	0.72 ± 0.12	7.11 ± 1.31	11.43 ± 0.16[a]	10.58 ± 1.04[b]	8.22 ± 0.70[ab]	0.27 ± 0.02
	8	0.59 ± 0.04	6.07 ± 0.30	10.35 ± 0.28[bc]	16.47 ± 1.3[a]	9.91 ± 0.26[ab]	0.30 ± 0.01

Harpenden	22	0.23 ± 0.01^{b}	1.90 ± 0.04^{c}	8.20 ± 0.26^{b}	36.15 ± 4.77^{a}	6.82 ± 0.75^{ab}	0.42 ± 0.04
	68	0.32 ± 0.01^{b}	3.08 ± 0.06^{b}	9.54 ± 0.21^{a}	22.27 ± 0.92^{b}	6.86 ± 0.28^{ab}	0.36 ± 0.02
	179	0.46 ± 0.03^{a}	4.35 ± 0.36^{a}	9.54 ± 0.12^{a}	20.83 ± 1.64^{b}	9.02 ± 0.91^{a}	0.32 ± 0.01
Hillsborough	1	0.90 ± 0.08	8.97 ± 1.14	9.91 ± 0.34	7.37 ± 0.12	6.86 ± 1.93	0.14 ± 0.03
	7	1.04 ± 0.06	10.23 ± 0.91	9.80 ± 0.31	8.05 ± 0.08	8.15 ± 0.96	0.17 ± 0.02
	16	0.99 ± 0.04	9.36 ± 0.32	9.46 ±0.03	6.33 ± 0.19	5.92 ± 0.13	0.15 ± 0.01
	23	1.15 ± 0.01	11.11 ± 0.13	9.70 ± 0.18	4.58 ± 0.27	5.10 ± 0.36	0.12 ± 0.01
	37	1.04 ± 0.04	10.12 ± 0.35	9.76 ± 0.04	7.15 ± 0.33	7.22 ± 0.10	0.27 ± 0.11
Kirkton	1	0.91 ± 0.03	9.27 ± 0.12^{b}	10.15 ± 0.24^{b}	3.90 ± 0.1	3.62 ± 0.13	0.14 ± 0.01
	3	1.01 ± 0.04	10.63 ± 0.33^{a}	10.56 ± 0.27^{ab}	3.02 ± 0.03	3.20 ± 0.07	0.08 ± 0.00
	5	0.88 ± 0.03	10.23 ± 0.16^{ab}	11.66 ± 0.31^{a}	4.62 ± 0.95	4.75 ± 1.03	0.13 ± 0.02
	35	0.96 ± 0.03	9.22 ± 0.40^{b}	9.64 ± 0.39^{b}	4.23 ± 0.42	3.93 ± 0.51	0.14 ± 0.00
Llangorse	2.5	0.51 ± 0.03^{b}	4.76 ± 0.29^{b}	9.36 ± 0.07	6.00 ± 0.32^{a}	2.83 ± 0.10^{a}	0.16 ± 0.01^{a}
	5	0.88 ± 0.08^{a}	8.29 ± 0.80^{a}	9.43 ± 0.07	2.65 ±0.43^{b}	2.13 ± 0.11^{ab}	0.11 ± 0.01^{ab}
	15	0.67 ± 0.10^{ab}	6.06 ± 0.78^{ab}	9.11 ± 0.24	3.23 ± 1.03^{b}	1.81 ± 0.34^{b}	0.09 ± 0.02^{b}
	25	0.62 ± 0.06^{ab}	5.32 ± 0.54^{b}	8.60 ± 0.05	3.27 ± 0.22^{b}	1.72 ± 0.14^{b}	0.07 ± 0.01^{b}
Myerscough	2	0.63 ± 0.08	6.60 ± 0.76	10.43 ± 0.12	5.23 ± 0.66^{c}	3.35 ± 0.09^{c}	0.12 ± 0.00^{b}
	6	0.49 ± 0.03	4.57 ± 0.29	9.31 ± 0.23	27.50 ± 3.85^{a}	12.39 ± 1.24^{a}	0.31 ± 0.04^{a}
	13	0.50 ± 0.05	4.83 ± 0.60	9.52 ± 0.32	30.88 ± 4.39^{a}	14.45 ± 0.92^{a}	0.30 ± 0.02^{a}
	34	0.47 ± 0.01	4.28 ± 0.13	9.12 ± 0.07	18.72 ± 0.04^{ab}	8.02 ± 0.24^{b}	0.21 ± 0.02^{ab}
	48.4	0.47 ± 0.02	4.47 ± 0.36	9.58 ± 0.36	12.08 ± 0.74^{bc}	5.35 ± 0.14^{bc}	0.17 ± 0.02^{b}

Overton	3	0.42 ± 0.03[c]	3.57 ± 0.31[c]	8.45 ± 0.18[b]	38.65 ± 6.58	13.57 ± 1.77	0.41 ± 0.05
	12	0.88 ± 0.05[a]	8.52 ± 0.59[a]	9.64 ± 0.11[a]	20.70 ± 2.41	17.45 ± 1.75	0.25 ± 0.02
	22	0.61 ± 0.02[b]	6.36 ± 0.18[b]	10.36 ± 0.15[a]	19.85 ± 4.39	12.52 ± 2.50	0.23 ± 0.07
	50	0.63 ± 0.05[b]	6.23 ± 0.29[b]	10.04 ± 0.45[a]	29.50 ± 2.23	18.29 ± 0.86	0.34 ± 0.04
Plumpton	1	0.35 ± 0.02[b]	3.81 ± 0.18[b]	10.87 ± 0.09	19.50 ± 5.61	7.23 ± 1.74	0.18 ± 0.05
	5	0.36 ± 0.04[b]	4.19 ± 0.49[b]	11.76 ± 0.91	6.60 ± 2.08	2.56 ± 0.49	0.06 ± 0.01
	20	0.56 ± 0.02[a]	5.96 ± 0.23[a]	10.75 ± 0.62	8.60 ± 0.3	5.11 ± 0.21	0.08 ± 0.01

%Fine-fraction; mass proportion of fine-fraction in a sample (%). [a] Organic carbon (g C kg^{-1} bulk soil) accounts for the proportion of fine-fraction per kilogram of bulk soil.

Table A.3. Linear regression coefficients for the estimation of maximum fine-fraction organic carbon (g C per kg soil). Lettering indicates slopes that are significantly different ($p < 0.05$).

Site	Slope (± 1 SEM)	*p*	Intercept (± 1 SEM)	*p*	RMSE	*n*	R^2
Aberystwyth	0.33 ± 0.059^{bc}	***	2.28 ± 0.892	*	1.11	15	0.70
Crichton	1.14 ± 0.470^{abcd}	*	-0.44 ± 1.684	Ns	0.79	12	0.37
Easter Bush	0.49 ± 0.094^{d}	***	2.33 ± 1.172	Ns	1.10	18	0.63
Harpenden	-0.02 ± 0.07^{a}	Ns	8.01 ± 1.837	**	1.42	9	0.01
Hillsborough	0.97 ± 0.148^{d}	***	0.16 ± 1.02	Ns	0.84	15	0.77
Kirkton	1.01 ± 0.088^{abcd}	***	-0.11 ± 0.357	Ns	0.26	12	0.93
Llangorse	0.29 ± 0.055^{abc}	***	1.03 ± 0.225	***	0.27	12	0.73
Myerscough	0.40 ± 0.031^{bcd}	***	1.07 ± 0.669	Ns	1.14	15	0.93
Overton	0.12 ± 0.109^{cd}	Ns	12.16 ± 3.142	**	3.35	12	0.11
Plumpton	0.30 ± 0.042^{ab}	***	1.45± 0.573	*	0.82	9	0.88

RMSE: Root mean square error. Level of significance: * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$. NS: not significant.

Table A.4. Linear regression coefficients for the estimation of maximum fine-fraction organic carbon (g C per kg soil) with a forced zero intercept. Lettering indicates slopes that are significantly different ($p < 0.05$).

Site	Slope (± 1 SEM)	*p*	RMSE	*n*	R^2
Aberystwyth	0.47 ± 0.024^{bc}	***	1.357	15	0.96
Crichton	1.02 ± 0.067^{cdef}	***	0.796	12	0.95
Easter Bush	0.67 ± 0.024^{e}	***	1.231	18	0.98
Harpenden	0.26 ± 0.035^{a}	***	2.739	9	0.87
Hillsborough	0.99 ± 0.033^{f}	***	0.842	15	0.99
Kirkton	0.98 ± 0.0197^{def}	***	0.265	12	0.99
Llangorse	0.52 ± 0.035^{abcdef}	***	0.474	12	0.95
Myerscough	0.45 ± 0.016^{b}	***	1.255	15	0.98
Overton	0.52 ± 0.055^{bcd}	***	5.297	12	0.89
Plumpton	0.39 ± 0.030^{ab}	***	1.141	9	0.96

RMSE: Root mean square error. Level of significance: * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$.

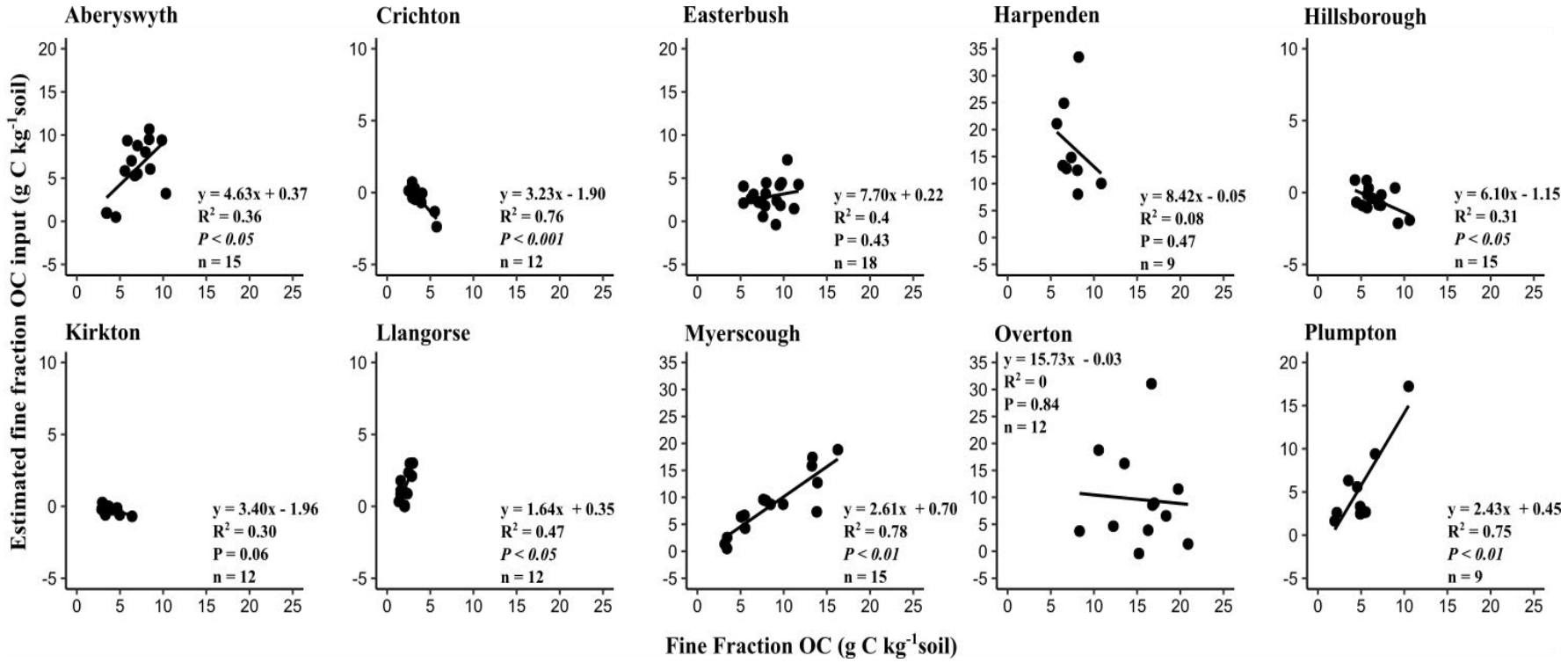

Figure A.1. Estimated fine-fraction OC input (g C per kg soil) compared to measured fine-fraction OC (g C per kg soil) in each of the sites studied. The estimated fine-fraction OC input (g C per kg soil) was calculated by subtracting the maximum fine-fraction OC (g C per kg soil) from the current fine-fraction OC (g C per kg soil). The maximum fine-fraction OC (g C per kg soil) was estimated using the quantile regression equation ($\tau = 0.90$), where maximum fine-fraction OC = 0.92 multiplied by the mass proportion fine-fraction (%).

A.2 Map of sites used in chapter 2.

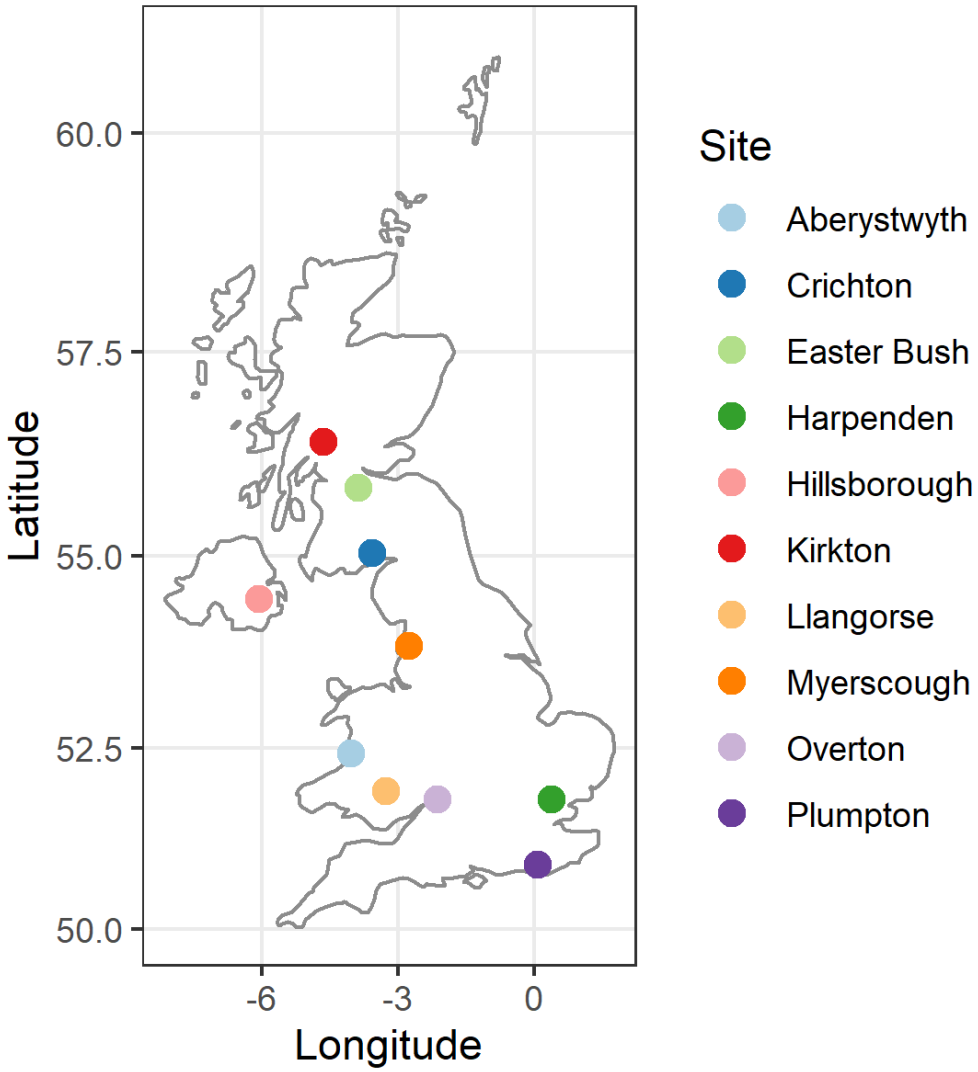

Figure A.2. Grassland locations across the United Kingdom

Appendix B

Appendix for Chapter 3: The effect of glucose quantity and ammonium nitrate on mineral associated organic matter carbon formation

B.1 Supplementary Information for chapter 3.

Table B.1. Atom % ^{13}C values for the treatments in chapter 3. Glucose addition rate were as follows, control (0 mg C g^{-1} soil), low (0.177 mg C g^{-1} soil), medium (4.425 mg C g^{-1} soil), and high (8.847 mg C g^{-1} soil). Ammonium nitrate addition rate equivalent to 0 or 100 kg N ha^{-1}). Results are means ± one standard error of the mean, of each treatment averaged across time (n = 12). Lettering indicates significant differences between mean values, such that values which share a letter are not significantly different ($P < 0.05$).

Glucose addition rate	**Ammonium Nitrate (equivalent kg N ha^{-1})**	**Atm% ^{13}C**
Control	0	1.090 ± 0.002^{a}
Control	100	1.091 ± 0.002^{ab}
Low	0	1.088 ± 0.002^{a}
Low	100	1.093 ± 0.002^{ab}
Medium	0	1.097 ± 0.002^{b}
Medium	100	1.110 ± 0.002^{c}
High	0	1.108 ± 0.002^{c}
High	100	1.107 ± 0.002^{c}

Appendix C

Appendix for Chapter 4: Substrate incorporation into MAOM is independent of substrate type and temperature

C.1 Supplementary Information for chapter 4.

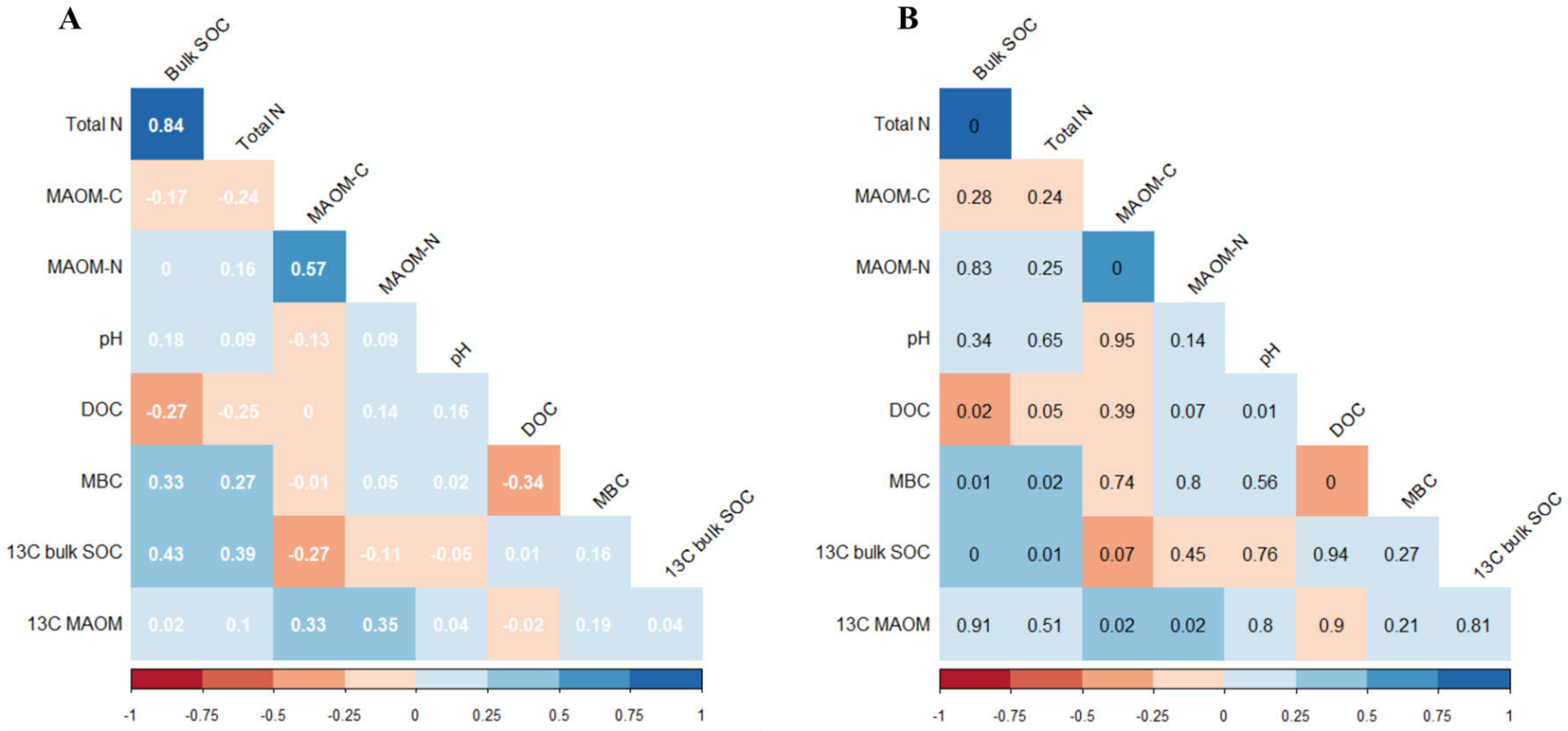

Figure C.1. Correlation matrix of selected measured soil properties (**A**) and *P*-values for each correlation coefficient (**B**). The colour refers to the strength of the correlation, with red indicating negative and blue positive correlations.

Table C.1. Dissolved organic carbon (DOC) and soil pH at each sampling time for each temperature. Values are means and one standard error of the mean, averaged across substrate type (n = 9)

Temperature (°C)	**Sampling Time**	**DOC (mg C kg^{-1} soil)**	**pH**
10	72 hours	23.733 ± 1.32^{a}	6.446 ± 0.01^{abc}
10	10 days	29.554 ± 2.45^{ab}	6.4 ± 0.03^{ab}
15	72 hours	25.004 ± 1.91^{a}	6.487 ± 0.03^{cd}
15	10 days	42.931 ± 4.61^{c}	6.528 ± 0.02^{d}
20	72 hours	43.146 ± 1.39^{c}	6.45 ± 0.02^{abc}
20	10 days	32.839 ± 1.17^{ab}	6.464 ± 0.02^{bcd}
25	72 hours	25.300 ± 1.33^{a}	6.391 ± 0.02^{a}
25	10 days	34.614 ± 1.53^{bc}	6.421 ± 0.01^{abc}

Appendix D

Appendix for Chapter 5: Mineral associated organic matter carbon is differently affected by long-term soil pH amendment, grass ley duration and soil depth

D.1 Supplementary Information for chapter 5.

Table D.1. Regression coefficients for the effect of ley duration (1, 2 or 3 years), soil depth (0 – 20 cm and 20 – 40 cm) and soil pH on MAOM-C. Shown are the regression coefficients, standard errors, t and *P*-values. There was no effect of ley duration, so no additional models were done. Significant *P*-values in bold ($P < 0.05$).

	Estimate	**Std. Error**	**t value**	***P value***
(Intercept)	16.82	3.082	5.474	**< 0.001**
Soil pH	1.710	0.481	3.556	**< 0.001**
Depth (20 – 40 cm)	6.988	4.481	1.559	0.121
Soil pH x Depth (20 – 40 cm)	-1.757	0.697	-2.521	**0.013**

Table D.2. Regression coefficients for the effect of ley duration (1, 2 or 3 years), soil depth (0 – 20 cm and 20 – 40 cm) and soil pH on the contribution of MAOM-C to total SOC. Shown are the regression coefficients, standard errors, t and *P*-values. Subsequent models are post-hoc models run for each ley duration, where terms were dropped if $P > 0.05$. Significant *P*-values in bold ($P < 0.05$).

	Estimate	**Std. Error**	**t value**	***P value***
(Intercept)	15.421	8.603	1.793	0.076
Soil pH	4.963	1.308	3.795	**< 0.001**
Ley (2 years)	21.853	10.85	2.014	**0.046**
Ley (3 years)	36.431	10.732	3.395	**< 0.001**
Depth (20 – 40 cm)	46.092	9.061	5.087	**< 0.001**
Ley (2 years) x Depth (20 – 40 cm)	-4.983	2.765	-1.802	0.074
Ley (3 years) x Depth (20 – 40 cm)	-4.239	2.769	-1.531	0.129
Soil pH x Ley (2 years)	-2.663	1.671	-1.594	0.114
Soil pH x Ley (3 years)	-5.091	1.655	-3.076	**0.002**
Soil pH x Depth (20 – 40 cm)	-6.622	1.377	-4.809	**< 0.001**
Ley – 1 year				
(Intercept)	7.914	10.88	0.727	0.471
Soil pH	6.119	1.661	3.685	**< 0.001**
Depth (20 – 40 cm)	60.751	15.13	4.015	**< 0.001**
Soil pH x Depth (20 – 40 cm)	-8.904	2.333	-3.817	**< 0.001**
Ley - 2 years				
(Intercept)	40.169	9.22	4.357	**< 0.001**
Soil pH	1.84	1.452	1.267	0.213
Depth (20 – 40 cm)	34.612	13.853	2.498	**0.017**
Soil pH x Depth (20 – 40 cm)	-5.598	2.167	-2.583	**0.014**

Table D.2 continued.

	Estimate	**Std. Error**	**t value**	***P value***
Ley – 3 years				
(Intercept)	71.466	8.542	8.366	**< 0.001**
Soil pH	-3.275	1.333	-2.457	**0.019**

Table D.3. Regression coefficients for the effect of ley duration (1, 2 or 3 years), soil depth (0 – 20 cm and 20 – 40 cm) and soil pH on total SOC. Shown are the regression coefficients, standard errors, t and P-values. Subsequent models are post-hoc models run for each *ley duration*, where the interaction term between soil pH and depth were dropped if P > 0.05. Significant P-values in bold ($P < 0.05$).

	Estimate	**Std. Error**	**t value**	***P-value***
(Intercept)	65.415	5.704	11.468	< **0.001**
Soil pH	-1.317	0.877	-1.502	0.136
Ley (2 years)	-22.763	7.223	-3.152	**0.002**
Ley (3 years)	-22.539	7.160	-3.148	**0.002**
Depth (20 – 40 cm)	-26.162	5.949	-4.398	< **0.001**
Soil pH x Ley (2 years)	3.437	1.123	3.062	**0.003**
Soil pH x Ley (3 years)	3.385	1.111	3.047	**0.003**
Soil pH x Depth (20 – 40 cm)	2.487	0.925	2.688	**0.008**
Ley – 1 year				
(Intercept)	58.349	4.707	12.396	< **0.001**
Soil pH	-0.119	0.712	-0.167	0.868
Depth (20 – 40 cm)	-11.614	1.258	-9.229	< **0.001**
Ley -2 years				
(Intercept)	48.915	6.087	8.036	< **0.001**
Soil pH	1.088	0.959	1.136	0.263
Depth (20 – 40 cm)	-40.034	9.145	-4.378	< **0.001**
Soil pH x Depth (20 – 40 cm)	4.747	1.431	3.319	**0.002**

Table D.3 continued.

	Estimate	**Std. Error**	**t value**	***P-value***
Ley – 3 years				
(Intercept)	35.414	6.136	5.772	**< 0.001**
Soil pH	3.182	0.963	3.307	**0.002**
Depth (20 – 40 cm)	-9.389	1.533	-6.125	**< 0.001**

Table D.4. Polynomic regression coefficients for the effect of ley duration (1, 2 or 3 years), soil depth (0 – 20 cm and 20 – 40 cm) and soil pH on MBC. Shown are the regression coefficients, standard errors, t and *P*-values. Subsequent models are post-hoc models run for each ley duration, where terms were dropped if $P > 0.05$. Significant *P*-values in bold ($P < 0.05$).

	Estimate	**Std. Error**	**t value**	***P-value***
(Intercept)	848.612	309.071	2.746	**0.007**
pH	-228.826	99.961	-2.289	**0.024**
pH^2	18.873	7.957	2.372	**0.019**
Ley (2 years)	-920.528	555.996	-1.656	0.101
Ley (3 years)	27.543	543.347	0.051	0.960
Depth (20 – 40 cm)	-46.055	6.653	-6.922	< **0.001**
pH x Ley (2 years)	322.844	177.982	1.814	0.072
pH^2 x Ley (2 years)	-27.098	14.053	-1.928	0.056
pH x Ley (3 years)	29.109	174.213	0.167	0.868
pH^2 x Ley (3 years)	-4.624	13.777	-0.336	0.738
Ley – 1 year				
(Intercept)	888.62	411.68	2.159	**0.038**
pH	-259.35	131.19	-1.977	0.056
pH^2	22.62	10.29	2.198	**0.035**
Depth (20 – 40 cm)	-450.93	542.25	-0.832	0.411
pH x Depth (20 – 40 cm)	169.33	175.05	0.967	0.340
pH^2 x Depth (20 – 40 cm)	-16.38	13.91	-1.177	0.247
Ley – 2 years				
(Intercept)	192.33	8.55	22.494	< **0.001**
Depth (20 – 40 cm)	-53.65	12.09	-4.437	< **0.001**
Ley - 3 years				
(Intercept)	927.31	439.39	2.11	**0.042**
pH	-217.33	140.44	-1.548	0.130
pH^2	15.57	11.07	1.407	0.168
Depth (20 – 40 cm)	-31.95	11.52	-2.773	**0.009**

Table D.5. Polynomic regression coefficients for the effect of ley duration (1, 2 or 3 years), soil depth (0 – 20 cm and 20 – 40 cm) and soil pH on DOC. Shown are the regression coefficients, standard errors, t and *P*-values. Subsequent models are post-hoc models run for each ley duration, where terms were dropped if $P > 0.05$. Significant *P*-values in bold ($P < 0.05$).

	Estimate	**Std. Error**	**t value**	***P-value***
(Intercept)	701.178	77.227	9.079	**< 0.001**
pH	-205.618	24.679	-8.332	**< 0.001**
pH^2	15.525	1.941	7.998	**< 0.001**
Ley (2 years)	26.759	107.096	0.25	0.803
Ley (3 years)	209.038	103.39	2.022	**0.046**
Depth (20 – 40 cm)	-464.343	86.301	-5.38	**< 0.001**
pH x Ley (2 years)	-3.610	34.264	-0.105	0.916
pH^2 x Ley (2 years)	-0.077	2.701	-0.028	0.977
pH x Ley (3 years)	-61.174	33.091	-1.849	0.067
pH^2 x Ley (3 years)	4.428	2.610	1.697	0.093
pH x Depth (20 – 40 cm)	132.119	27.656	4.777	**< 0.001**
pH^2 x Depth (20 – 40 cm)	-9.646	2.181	-4.423	**< 0.001**
Ley (2 years) x Depth (20 – 40 cm)	8.154	3.050	2.673	**0.009**
Ley (3 years) x Depth (20 – 40 cm)	8.366	3.038	2.754	**0.007**
Ley – year 1				
(Intercept)	717.209	77.544	9.249	**< 0.001**
pH	-210.72	24.71	-8.528	**< 0.001**
pH^2	15.924	1.939	8.214	**< 0.001**
Depth (20 – 40 cm)	-492.206	102.137	-4.819	**< 0.001**
pH x Depth (20 – 40 cm)	141.1	32.972	4.279	**< 0.001**
pH^2 x Depth (20 – 40 cm)	-10.355	2.621	-3.952	**< 0.001**
Ley – year 2				
(Intercept)	756.497	124.55	6.074	**< 0.001**
pH	-217.334	39.95	-5.44	**< 0.001**
pH^2	16.006	3.159	5.067	**< 0.001**
Depth (20 – 40 cm)	-560.699	211.743	-2.648	**0.012**
pH x Depth (20 – 40 cm)	162.968	67.28	2.422	**0.021**
pH^2 x Depth (20 – 40 cm)	-11.88	5.279	-2.25	**0.031**

Table D.5. Continued.

	Estimate	**Std. Error**	**t value**	***P-value***
Ley – year 3				
(Intercept)	865.101	105.94	8.166	**< 0.001**
pH	-253.345	34.119	-7.425	**< 0.001**
pH^2	18.973	2.71	7.002	**< 0.001**
Depth (20 – 40 cm)	-305.957	171.696	-1.782	0.083
pH x Depth (20 – 40 cm)	86.767	54.593	1.589	0.121
pH^2 x Depth (20 – 40 cm)	-6.275	4.285	-1.464	0.152

Table D.6. Two-way ANOVA results for bulk soil properties. Ley duration refers to 1, 2 or 3 years of grass-clover ley and depth to soil sampling depth (0 – 20 cm or 20 – 40 cm), significant *P*-values in bold ($P < 0.05$)

Soil property		**Ley duration**			**Depth**			**Ley duration x Depth**		
	Residuals	***df***	***F***	***P-value***	***df***	***F***	***P value***	***df***	***F***	***P-value***
Total SOC	120	2	0.413	0.663	1	137.71	**< 0.001**	2	0.904	0.408
MAOM-C	120	2	0.478	0.621	1	48.442	**< 0.001**	2	0.654	0.522
MBC	118	2	3.181	**0.045**	1	47.683	**< 0.001**	2	0.623	0.538
Prop. of MAOM-C in total SOC	120	2	0.858	0.427	1	0.170	0.681	2	1.258	0.288
DOC	112	2	7.04	**0.001**	1	69.346	**< 0.001**	2	6.096	**0.003**
Total N[a]	120	2	0.461	0.794	1	45.436	**< 0.001**	-	-	-
Nitrates	120	2	1.296	0.277	1	71.286	**< 0.001**	2	3.03	0.052
Ammonium[a]		2	7.232	**0.027**	1	11.352	**< 0.001**	-	-	-

[a] Effects examined by Kruskal-Wallis test due to non-normality after transformation. The effects of grass year and depth were tested separately. Here *F* value refers to chi-squared value.

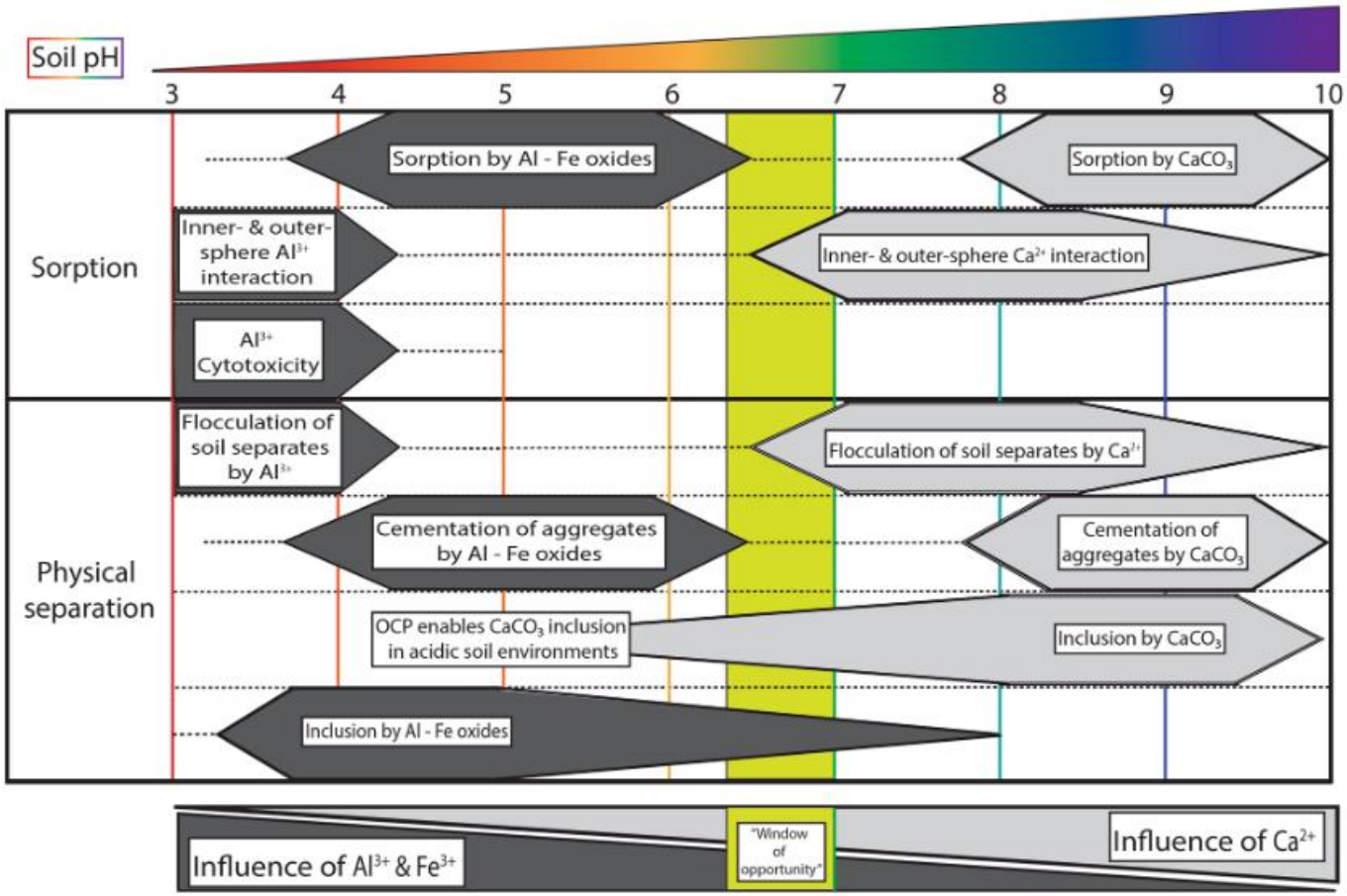

Figure D.1. The effect of soil pH on the role of polyvalent cations in SOC preservation. The "window of opportunity" refers to the optimum pH for microbial decomposition. OCP refers to oxalate-carbonate pathway. In which bacteria biomineralise calcium carbonate ($CaCO_3$), enabling its inclusion within soil crystal matrix. Source : Rowley *et al.*, 2018.

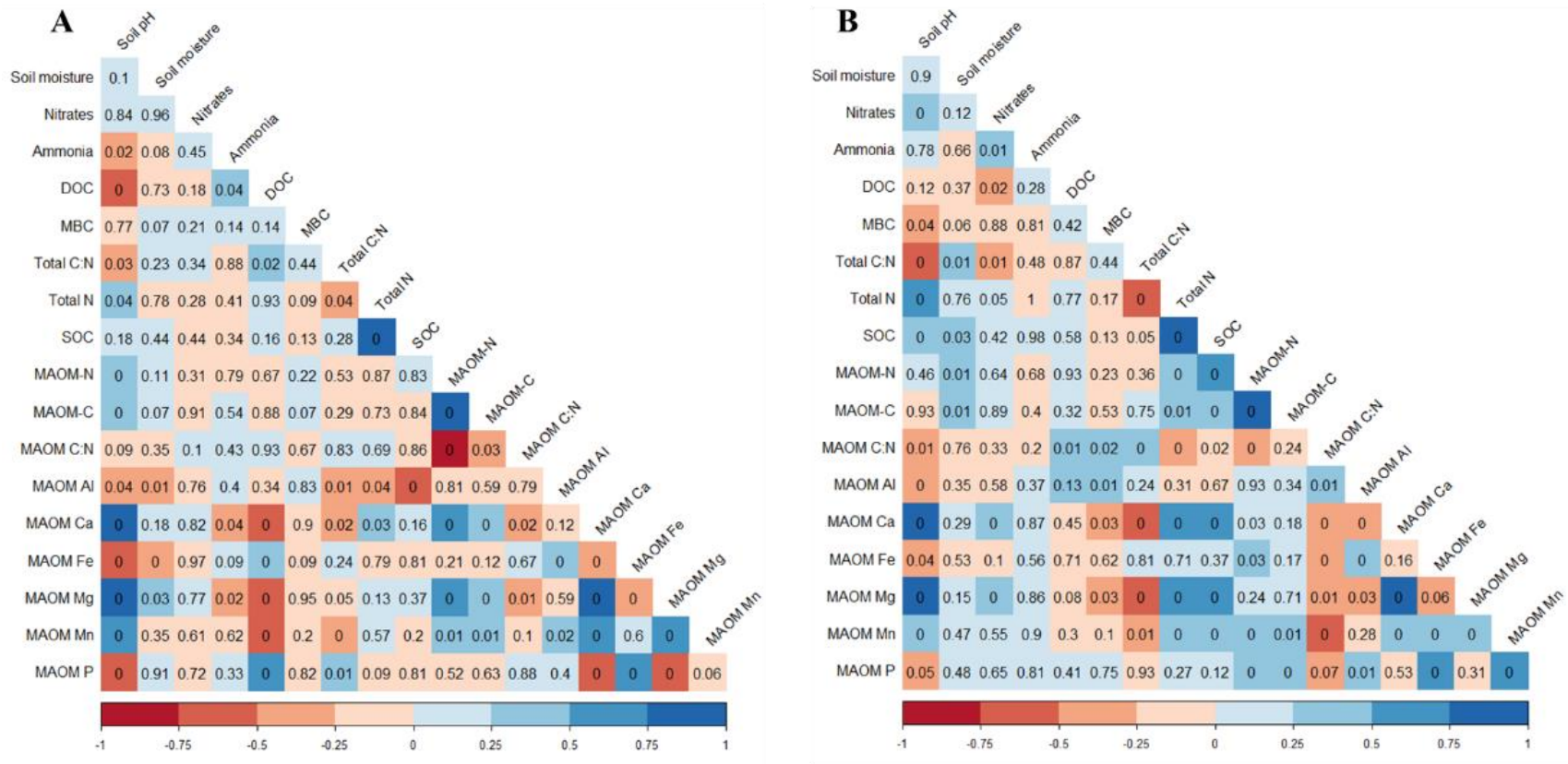

Figure D.2. P-values for correlation matrix across all grass years at 0 – 20 cm (**A**) and at 20 – 40 cm (**B**). Colours represent direction and strength of relationship, negative (red) and positive (blue).