The role of grass-clover leys in soil aggregation and carbon storage: a multi-omics approach

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"The problems are solvable. What is stopping us is ourselves – our economics and values. It is a values debate". "We need to grow empathy not GDP".

MIKE BERNERS-LEE (2019)

Thesis Summary

Intensive conventional agricultural practices, including conventional tillage and short rotations of annual crops, degrades soil organic carbon (SOC) stores, harms soil organisms and degrades macroaggregates important for soil structure and vital ecosystem services. More sustainable regenerative farming practices, for example reintroducing grass-clover leys into arable rotations, need to be adopted urgently to reduce non-renewable inputs and disturbance, increase organic matter inputs, and allow beneficial soil organisms to recover. The main aims of this thesis were to elucidate the mechanistic basis of soil changes caused by grass-clover leys in arable rotations by characterising changes in soil microbial communities, metabolomes and their impacts on soil aggregation and C storage. Introducing a three-year ley into conventionally managed arable fields increased proportions of soil macroaggregates (>2000 μm) by 5.4-fold, achieving similar proportions as the undisturbed adjacent hedgerow. Bulk SOC rose from 20.3 to 22.6 Mg ha^{-1} (0.77 Mg C ha^{-1} yr^{-1}) but was not statistically significant. Macroaggregate-associated and protected OC increased from 2.0 Mg ha^{-1} to 9.6 Mg $ha^{-}1$. Simultaneously, fungal communities significantly changed under ley, with increased prevalence and diversity in the Ascomycota and Glomeromycota phyla, but no significant changes identified in soil bacteria. Biosurfactants, lignans and flavonoids were upregulated in the soil metabolome, possibly associated with aggregate stability. Finally, a one-year earthworm manipulation experiment revealed that soil macroaggregates, and associated OC and N, all increase with increased earthworm numbers, being particularly driven by endogeics. This thesis affirms the benefits of grass-clover leys in arable rotations, evidencing their involvement in improving soil structure and quality associated with changes to the soil metabolome, microbiome and earthworm populations. These results provide justification for the rewarding of farmers for including leys in arable rotations for the biological, chemical and physical benefits to soil health which are in the interest of society.

Declaration

I, Emily Guest, confirm that the thesis is my own work, unless otherwise referenced in the text. I am aware of the University's Guidance on the Use of Unfair Means (www.sheffield.ac.uk/ssid/unfair-means). This work has not previously been presented for an award at this, or any other, University and has not been submitted for any other degree. Chapter 2 has been submitted as a journal article with 'Science of the Total Environment' and is under review at time of submission.

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Chapter 1

1. General Introduction

1.1. Soil and agriculture

With a global population set to reach over 9 billion by the year 2050, the global demand for food production will continue to increase for at least another 30 years (Godfray et al., 2010). However, to ensure that enough food not only can be provided for the present, but for the future, we must ensure that agriculture and soils are managed sustainably. The common agricultural policy of the European Union and its implementation in the UK since the 1970s has led to intensification of agriculture, and increased specialisation. This has led to a 70% decline in mixed arable and livestock farming in Europe from 1975 to 1995 (Ryschawy et al., 2013) as mixed farming became increasingly uneconomic as exemplified by farm business incomes in the UK which averaged only £21,900 for mixed farms but £48,900 for cereal farms in 2020 (Defra, 2021a). Arable production has therefore become focussed on short rotations of the most profitable cereal and oilseed crops, often established by annual ploughing and harrowing (Townsend et al., 2016; Wezel et al., 2014), using large amounts of fertilisers, alongside use of herbicides, fungicides and growth regulators to maximize yields and protect the crops (Ogaji, 2005). In much of the UK lowland, arable fields are now typically cultivated and cropped with conventional tillage in crop rotations annually without a break (Wezel et al., 2014), with cereals typically grown on 67% of the cropping area (Defra, 2020).

However, these intensive short arable rotations are detrimental to soil structure, causing slumping and compaction of soils (Haghighi et al., 2020; Pires et al., 2017), which is difficult and expensive to remediate across fields, in addition to the more localized but more acute effects of controlled traffic using “tramlines” for tractors (Chamen et al., 2015; Langmaack et al., 2002). Soil compaction is commonly addressed by a combination of periodic subsoiling and regular ploughing and harrowing (Townsend et al., 2016), otherwise known as conventional tillage, which can increase soil porosity for crop establishment (Nkakini and Fubara-Manuel, 2012), aiding infiltration, as well as other benefits including weed control. However, this is a short-term solution and is counterproductive in the long-term as tillage accelerates oxidative loss of soil organic carbon (SOC; Taboada et al., 2004), and degrades soil structure including aggregate stability (Portella et al., 2012). The resulting bare unconsolidated soil suffers greater risks of soil erosion and enhanced greenhouse gas (GHG) emissions (Plaza-Bonilla et al., 2014).

Depletion of SOC, which plays a central role in soil multifunctionality including soil structure, water and nutrient-holding capacity, and provides the essential energy and food resources for most soil organisms, has also resulted from selection of high yielding crops that return little residue to soils.

Modern crop varieties of cereals and oilseeds have been bred to enhance partitioning of organic matter (OM) into above-ground biomass (AGB) rather than into the soil through root inputs, with the most widely grown crop, wheat, allocating around 50% of its AGB into grain (AHDB, 2018). The subsequent removal of the majority of this AGB at the point of harvest for both saleable grain (AHDB, 2018) and straw for livestock bedding or fuelling purpose-built power stations that in the UK have the capacity to burn over 1 m tonnes per year have led to little crop residues left in the field (Townsend et al., 2018). The combination of intensive tillage and the growing of annual arable crops that return little OM to soils have led to declines in SOC in arable soils which have fallen to 2.5%, and to below 1.3% in about half of the silt and clay soils in England (King et al., 2005). This combination of soil disturbance and OM depletion also adversely impacts earthworms and other soil organisms important for soil functioning. Tillage decreases root colonisation and symbiosis by arbuscular mycorrhizal fungi (AMF) due to the destruction of mycorrhizal hyphal networks and dispersal and dilution of AM propagules in the topsoil (Kabir, 2005; Wilson et al., 2009). As mycorrhizal hyphal lengths in soil are positively correlated to SOC, most likely through their effects on soil aggregation and SOC sequestration (Wilson et al., 2009), depletion of these symbiotic fungi from fields growing staple crops that associate with these symbionts including maize and wheat (Brundrett, 2009; Wang and Qiu, 2006) likely exacerbates declines in soil quality and functions. Overall, intensive arable cropping drives declines in SOC and beneficial soil organisms that together are causally linked to soil functional degradation, including loss of nutrient and water storage capacity and crop yields (Blanco-Canqui and Lal, 2009; Obalum et al., 2017).

One way to comprehend the enormity of the consequences of soil degradation is to quantify the economic, environmental and human impacts of it. Attempts have been made to estimate the economic value of soils in the provision of ecosystem services (Costanza et al., 2014, 1997; Jónsson and Davídsdóttir, 2016). However, because agriculture only contributes about 0.5% of UK GDP (Defra, 2021b) and 4% of global GDP (World Bank, 2018), economic valuations fail to adequately reflect the infinite value of soils to sustaining both natural ecosystems, food production, human health and well-being. The yearly cost of soil degradation in England and Wales was estimated to be on average £1.2 billion in 2015, mainly due to loss of organic content (47%), compaction (39%) and erosion (12%), with 80% of these losses occurring away from the land contributing to them (Graves et al., 2015). This economic loss was equivalent to 30% of the £4 billion total farm gate-value of UK agricultural production in 2015 (Defra, 2016). A loss of the physical, chemical and biological properties of soil leads to downstream consequences on soil functions and ecosystems, which we heavily rely upon, therefore the sustainable management of agricultural soils is essential in order to maintain these vital ecosystem services (Johnston and Poulton, 2018; Smith et al., 2015). These services include the ability to produce nutritious crops, the storage of SOC and the storage, filtration and slow-release of rainwater,

which are required for food security, safe potable water, climate regulation, and protection of communities from flooding (Blum, 2005; Brevik et al., 2018).

Soil infiltration rates and water storage capacity are amongst the most vital ecosystem services in reducing flood risks and crop production losses, which are intensifying under climate change (IPCC, 2019), due to increasing intensity and frequency of rainfall events and droughts (Groisman et al., 2005; Samaniego et al., 2018). Field flooding can cause catastrophic damage to nearby cities. For example the city of York, England which sits in the middle of the Vale of York, an area of intensive arable farming of which 48% is used to grow cereals (Natural England, 2014), experienced flooding in 2015 that left shops and homes underwater. This caused an estimated £1.3 billion of damage and the need for millions more to be spent on flood defences (Banks, 2016). There is now increasing awareness of the importance of developing natural flood management strategies, including improving soil quality to increase infiltration rates, water storage capacity, and subsurface drainage in such areas liable to flooding (Environment Agency, 2016).

As carbon (C) storage is one of the best all-round indicators of soil quality and functions, especially in mineral soils, increasing soil C sequestration delivers multiple benefits of energy and nutrient sources that supports a greater biodiversity, which in turn enhances soil structure hydrological functioning and nutrient retention (Wagg et al., 2014). Increasing soil C storage is a priority for achieving net zero carbon emissions from agriculture and reducing this sector's contribution to GHG emissions and climate change (Blum, 2005; Lal, 2004a). The sustainable management of agroecosystems to improve soil quality may also aid in the maintenance of yields whilst reducing reliance on chemical inputs and fossil fuel energy associated with intensive tillage (Holland, 2004) and nitrogen fertiliser use, of which the latter accounts for 43% of the environmental footprint of producing a loaf of bread (Goucher et al., 2017). In arable mineral soils, increasing SOC is linked not only to increased OM inputs, but also to improving soil aggregation.

Soil aggregates are crumb-like particles that provide one of the most important components of mineral soil quality and of soil formed by a combination of physical, chemical and biological mechanisms (Churchman, 2010). Water-stable aggregates (WSA) play a key role maintaining structural stability and macropore spaces enabling free drainage in wet soils, as well as stabilizing SOC to enable C sequestration (Jastrow, 1996; Six et al., 1998; Stewart et al., 2009, 2008; Tisdall and Oades, 1982). To better manage arable soils and reverse historical losses of SOC and soil structural degradation requires in depth understanding of the mechanisms and organisms involved in soil aggregation and how to balance crop production with regenerating these core components of soil multifunctionality.

1.1.1. Research on soil aggregation

The five major factors involved in the formation and stabilisation of WSA were already identified in the early 1900s as soil fauna, soil microorganisms, roots, inorganic binding agents and physical and environmental variables (Six et al., 2004). However, the majority of the research into the mechanisms behind soil aggregation started from the 1950s, with more recent papers using modern techniques such as imaging by X-ray microtomography (Toosi et al., 2017) and cryo-electron microscopy (Possinger et al., 2020) and high-throughput DNA sequencing methods (Schloter et al., 2018) to determine the specific role of individual compounds and organisms. One of the most influential papers providing a paradigm for research into the interactions between soil organic matter (SOM) and soil aggregation was by Tisdall and Oades (1982), in developing a hierarchical conceptual framework that has proved useful for both understanding and practically managing land to facilitate soil aggregation. Their paper defined three classes of organic binding agents: transient (e.g., plant- and microbial-derived polysaccharides); temporary (e.g., roots and fungal hyphae); and persistent (e.g., complexes of clay-polyvalent metal-OM). It was suggested that the different classes of binding agents act on different hierarchical stages of aggregation, with stable microaggregates (<250 µm) bound by persistent binding agents, which are subsequently further bound together into macroaggregates (>250 µm) by transient and temporary binding agents.

Among the persistent binding agents identified by Tisdall and Oades (1982) were the SOM components of humus that encompasses humic and fulvic acids, and humins which are defined by extractability in alkali and acids. More recently, Lehmann and Kleber, (2015) have challenged the nomenclature and concepts of humus as being artefacts of the harsh extractants used, and instead proposed a soil continuum model of OM decomposition. This model proposes that decomposer organisms are essential for the production of many elements of SOM, from plant material to smaller transient and persistent binding compounds with distinctive chemical and physical properties (Bronick and Lal, 2005). These include soluble carbohydrates and the hydrophobic protein glomalin, produced by hyphae of arbuscular mycorrhizal fungi, which have been identified as the most important aggregating agents for their resistance against aggregate breakdown (Carrizo et al., 2015).

The concept of soil humus as a recalcitrant secondary product of microbial decomposition processes in soils remains contested, and the soil continuum model has been criticised for its lack of clear mechanistic explanation of how some molecules become protected from mineralisation for long periods of time (Piccolo, 2016). In contrast, the supramolecular structure and origin for humic materials, proposed in the late 1990's overturned the earlier concept of macropolymeric structures, on the basis of detailed biochemical and spectroscopic analyses that support the general consensus that humic molecules are assembled from smaller molecular weight partial degradation products of organic matter (Piccolo, 2016). Whilst the nature and dynamics of soil SOC remains a complex matter to fully resolve,

there is increasing evidence that organo-mineral interactions, typically driven by soil microbiota, play a central role in soil aggregation and soil C sequestration, irrespective of whether the molecules involved are defined as humus or not (Possinger et al., 2020).

Figure 1.1. summarises how the contribution and prevalence of the major classes of binding agents changes with the size and temporal stage of aggregates throughout their formation and degradation, as the existing binding agents control the size and stability of the aggregate. This theory was coined the 'Aggregate Hierarchy Theory', which has been proved to exist in all soils where OM is the major binding agent (e.g. rather than oxides; Oades and Waters, 1991). Surprisingly, this model by Tisdall and Oades (1982) was created mainly intuitively, with little empirical evidence to support it at the time. However, subsequent research (Carrizo et al., 2015; Dignac et al., 2017; Six et al., 2004) has provided increasing support for this widely accepted theory without much deviation.

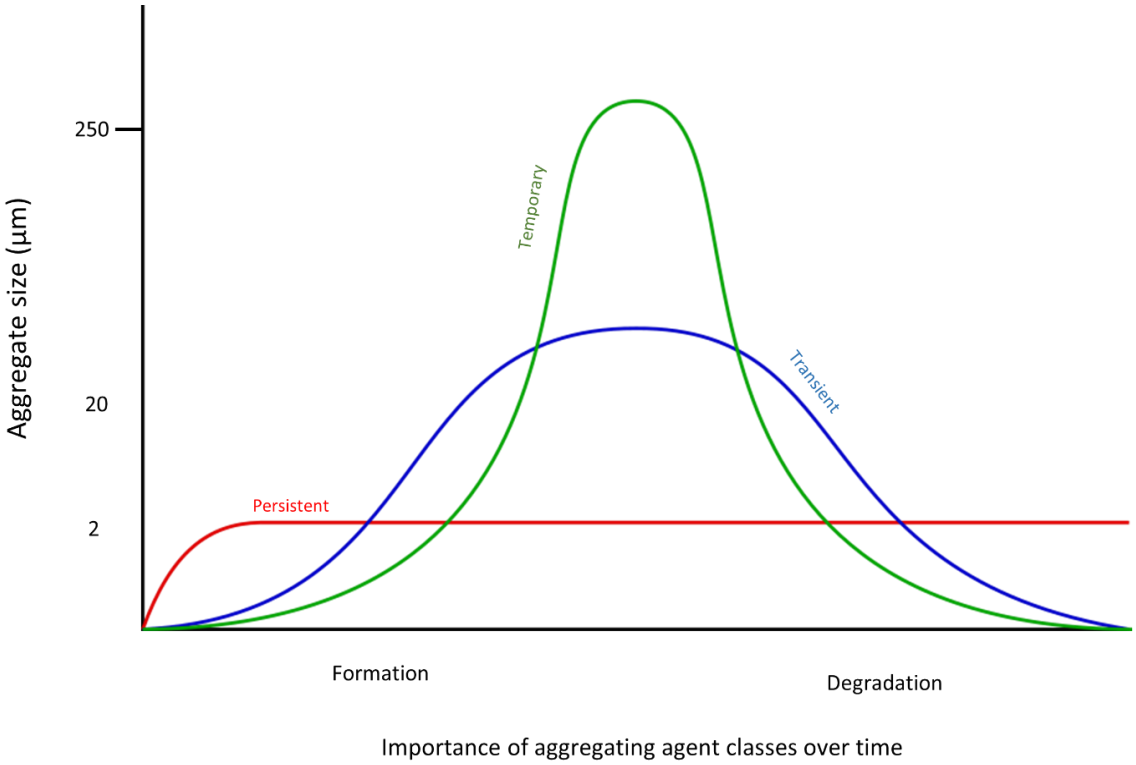

Fig. 1.1. The importance of aggregating agents changes over time throughout the formation and degradation of soil aggregates of different sizes. Persistent binding agents (e.g., humic acid) build up small aggregates quickly and are very resistant to degradation. Transient binding agents (e.g., polysaccharides) build up microaggregates (<250 µm) and are resistant to mechanical disturbance but only slightly resistant to ultrasonic disturbance. Temporary binding agents (e.g., roots and hyphae) are important in producing macroaggregates (>250 µm) but are very sensitive to mechanical disturbance such as tillage. Adapted from Tisdall and Oades (1982).

1.1.2 Microaggregation

Microaggregates are small compound soil structures less than 250 µm in diameter. Decomposer organisms break down organic substances from larger plant and animal residues into smaller, reactive OM which accumulates on mineral surfaces such as clays, forming strong organo-mineral bonds (Lehmann and Kleber, 2015). Polyvalent metals form a strong bond between C and OM to form C-P-OM complexes (<20 µm) which are essential for microaggregate formation and provide resilience against rapid wetting and mechanical damage such as tillage, designating them as the most stable C pool in soils (Six et al., 2000; Tisdall and Oades, 1982).

Many early studies supported the idea that microaggregate formation is initiated by the occlusion of organic debris by mineral particles like clays (Cambardella and Elliott, 1993; Golchin et al., 1994; Jastrow, 1996; Six et al., 1998; Tisdall and Oades, 1982). However, more recent studies by Lehmann et al. (2007) and Possinger et al. (2020) used fine structure spectroscopy to map the distribution of OC in microaggregates. OC was found to be evenly distributed throughout young microaggregates and not solely located in the core, as would be expected from previous theories. This suggests that OC is not initially well protected by aggregation, and that occlusion of OC by mineral particles occurs later in the microaggregate formation stages (Fig. 1.2.).

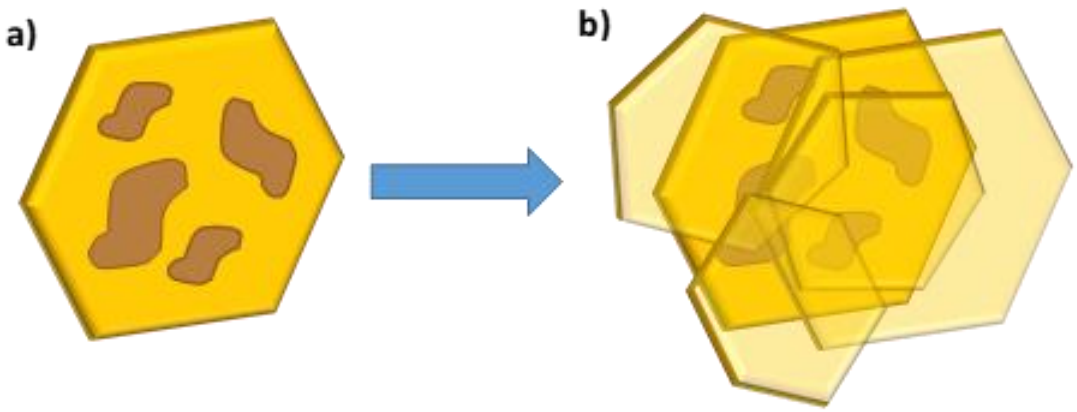

Fig. 1.2. The initial formation of microaggregates. **a)** The preliminary stage of aggregation begins with the accumulation of organic matter on mineral surfaces (e.g., clay) connected by strong organo-mineral interactions. **b)** Organic debris bonded to the mineral surface is occluded by more mineral particles bound by strong organo-mineral interactions. Adapted from Lehmann et al. (2007).

Although Tisdall and Oades (1982) hypothesised that microaggregates are the starting point of aggregate formation, Oades (1984), later proposed a small change to the Aggregate Hierarchy Theory, suggesting that microaggregates can also be formed within macroaggregates later on in the cycle. Carbon can be delivered into these locations for example by hyphae of AMF that can have diameters as small as 2 μm, and often as much as 50 m length per g of soil in grasslands (Leake et al., 2004). When roots and hyphae within macroaggregates decompose, the mucilages produced during the decomposition process and clays encrust the decomposed OM, allowing the formation of microaggregates within previously formed macroaggregates. This was later confirmed by Angers et al. (1997) who traced ^{13}C from labelled wheat straw, which initially accumulated in macroaggregates, but soon became associated with and stabilised within microaggregates. This proves that over time, microaggregates are formed within macroaggregates and it appears these are released as stable free microaggregates upon the breakdown of their surrounding macroaggregate structures, for example by tillage.

1.1.2. Macroaggregation

Macroaggregates are soil crumbs larger than 250 μm in diameter (Tisdall and Oades, 1982). From a study using different intensities of sonic vibration to determine the stability of soil particles, Edwards and Bremner (1964) concluded that macroaggregates are made from an assemblage of weakly-associated microaggregates. This was represented in the Aggregate Hierarchy Model by Tisdall and Oades (1982) where the aggregation of smaller soil particles by temporary (e.g. roots and hyphae) and transient (e.g. plant- and microbial-derived polysaccharides) binding agents were said to be responsible for macroaggregate formation. This idea was corroborated by Jastrow (1996) who showed that particulate organic matter (POM) within microaggregates provides nucleating sites for the growth of fungal hyphae, which deposit transient binding agents in the form of polysaccharides that bind microaggregates together and in so doing form and stabilise macroaggregates. This theory explains why the prevalence, diameter, stability and OC sequestration of macroaggregates is strongly reduced under tillage due to the disturbance of roots and hyphae enmeshing macroaggregates together (Cambardella and Elliott, 1993; Garcia-Franco et al., 2015; Portella et al., 2012; Spohn and Giani, 2011).

Unlike microaggregates, macroaggregate formation and degradation occurs much faster, with a higher turnover rate, meaning it can take one or two decades until the macroaggregate equilibrium is stable, and even longer for SOC to accumulate to reach a new equilibrium (Johnston et al., 2017; Kösters et al., 2013; Poulton et al., 2018). The rate of macroaggregate turnover differs depending on the balance between fresh organic residue and stabilised SOM. In general, an intermediate rate of macroaggregate turnover is ideal, as slow turnover is best for the protection of already-stabilised SOM, but a certain rate is needed for the formation of new aggregates and the protection, rather than release, of OC (Plante and McGill, 2002a, 2002b). However, Six et al. (2004) indicated that it is unlikely that macroaggregate

turnover can ever be so slow in an agricultural ecosystem that it impedes the stabilisation of new OC. These processes of microaggregation, macroaggregation, their interactions, turnover and breakdown are summarised in Figure 1.3. The specific mechanisms involved in the formation of macroaggregates, which complements this, are shown in Figure 1.4.

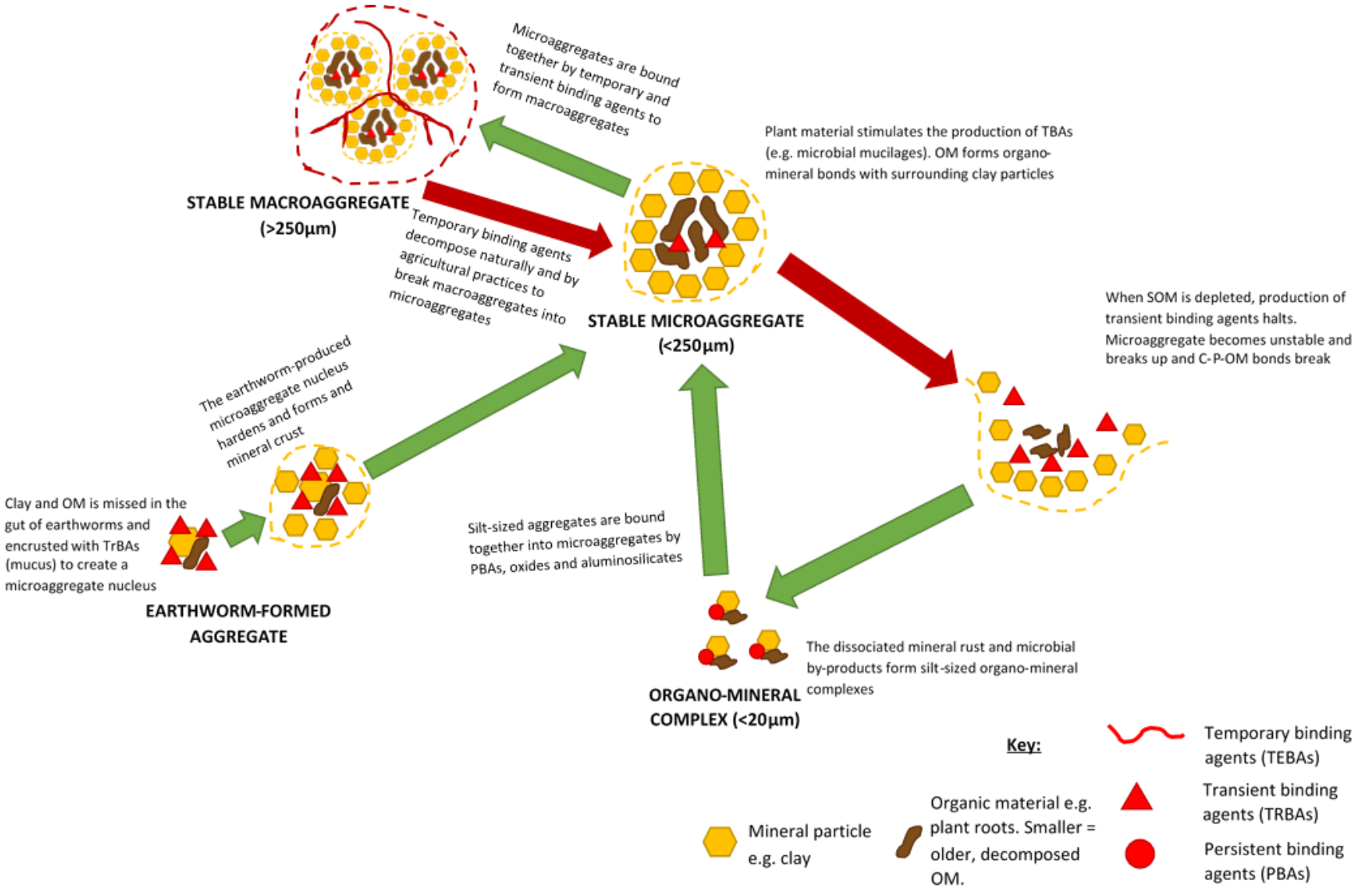

Fig. 1.3. A summary of the processes of micro- and macro-aggregation and disaggregation.

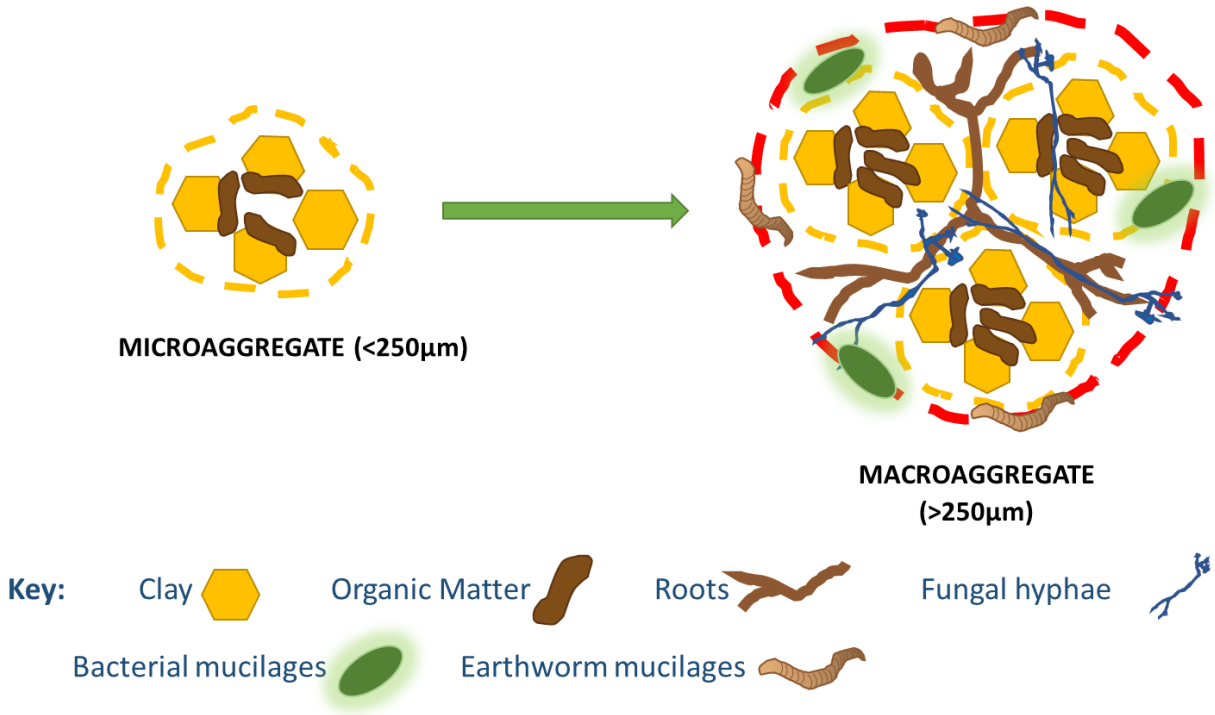

Fig. 1.4. A simplified diagram representing processes of soil macroaggregate formation based on previous published studies. Microaggregates are formed by organic matter bonded to the surface of clays, which are stable but vulnerable to being eroded or washed away. These microaggregates are then joined together by different classes of 'aggregating agents', summarised in the key, to form macroaggregates.

1.2. Biochemical components involved in micro- and macroaggregation

Although it is established experimentally that the aggregation process involves plant roots, soil microorganisms, earthworms and their interactions (Milleret et al., 2009; Piotrowski et al., 2004) the exact mechanisms behind aggregate formation are still incompletely understood (Lehmann et al., 2017a; Zheng et al., 2016), although attention is beginning to be paid towards this much-needed research (Pellegrino et al., 2021). The currently unknown knowledge is constraining the way we develop our soil management systems, through targeted crop breeding and changes to land management, in order to enhance the formation and stabilisation these vital components of soil structure. For example, it is known that soil fungi, particularly AMF, have a positive influence on the aggregation process, but the importance of hyphal enmeshment versus chemical "gluing" of particles by exudates such as glomalin has not been fully resolved. With the recent advances in omics and bioinformatics techniques that can be applied to soils (Bonfante, 2018; Tedersoo et al., 2016), there are now unprecedented opportunities to study microbial community composition, identify particular phyla, genera or species associated with soil aggregation, and to also identify and quantify specific organic compounds potentially involved in these processes.

In this section, the previously known individual biological and chemical components involved in soil aggregate formation and stability are summarised including their methods of identification and quantification (Table 1.1).

Table 1.1. A summary of the key biochemical components thought to be involved in micro- and macroaggregation, including the scale at which they are expected to operate and the methods by which they could be identified.

Compound	Associated biotic factors	Identification method	References
Lipids	Mycorrhiza, roots, soil fauna	phospholipid fatty acid analysis, GCMS	Frostegård et al. (1993); Jandl et al., (2004)
Glomalin	Mycorrhiza	Autoclaving in citrate buffer, Bradford assay Fluorescent antibody	Rillig (2004b); Purin and Rillig (2007); Wright (2000)
Carbohydrates	Soil fauna,	Hot water extraction	Puget et al. (2000)
Polysaccharides	Mycorrhiza, roots, soil fauna	Soil methylation	Cheshire et al. (1983); Bronick & Lal (2005)
Exchangeable cations	Soil fauna,	Cobalt hexamine trichloride	Ciesielski et al., (1997)

1.2.1. Soil fauna

Earthworms and collembolans (small hexapods) are among the most prevalent fauna studied in soil aggregate research (Hallam and Hodson, 2020; Maaß et al., 2015; Sharma et al., 2017). Individuals can be identified down to species level by examination under a light microscope, but DNA barcoding can also be used, which often uses the animal mitochondrial gene 'cytochrome C oxidase subunit 1 (cox1) with subsequent phylogenetic analyses (Shekhovtsov et al., 2018, 2014). As well as their DNA which can be detected by extracting environmental DNA (eDNA) samples, soil fauna will also leave traces of metabolites which can be identified. For example, earthworm casts contain mucilage layers with a mixture of plant and microbial polysaccharides and other organic molecules (Shipitalo and Protz, 1989). Recent research by Yakkou et al. (2021) studied coelomic fluid produced by earthworms, and identified markers including fatty acids and carbohydrates and certain microorganisms which were identified using DNA barcoding approaches.

Earthworms are termed 'ecosystem engineers' as they physically manipulate the soil environment in which they live (Jones et al., 1994). Their activities in the soil, including burrowing, feeding and casting, alters the soil physically, chemically and biologically through changes in soil structure, mineralisation of nutrients and communities of soil microorganisms (Jégou et al., 2001; Lavelle et al., 1998; Sharma et al., 2017). The formation of earthworm burrows increases the prevalence of macropores (Capowiez

et al., 2003), which can be used by plant roots for growth and also the storage and drainage of water, alleviating soil compaction and dramatically improving soil hydrological functioning, especially infiltration rates (Hallam et al., 2020; Yvan et al., 2012).

Earthworms play a large role in the formation of new microaggregates within their casts, which hold together aggregates by gut and microbial mucilages, acting as glues to stick soil particles together (Zhang et al., 2013). Within the gut, OM is encrusted with mucus and plasma, forming a microaggregate nucleus with rejuvenated microbial activity (Barois et al., 1993; Shipitalo and Protz, 1989; Zhang et al., 2013). When excreted, the nucleus becomes stable by drying and hardening with bonding of clay particles with organic debris by plant and microbial polysaccharides (Shipitalo and Protz, 1988). This hardening makes aggregates formed by earthworms sometimes very stable for several years (Shipitalo and Protz, 1988). It has been previously found that earthworm casts are rich in C, polysaccharides and specific cations such as Ca^{2+} and Mg^{2+} (Jouquet et al., 2008) and that earthworm-associated microaggregates are known to be particularly rich in C-P-OM complexes, providing stability for the newly-formed microaggregate (Shipitalo and Protz, 1989).

However, there are a large diversity of other soil organisms that contribute to soil aggregation as shown by the typical numbers found in a cubic metre of soil (Fig. 1.5; Soil Atlas 2015).

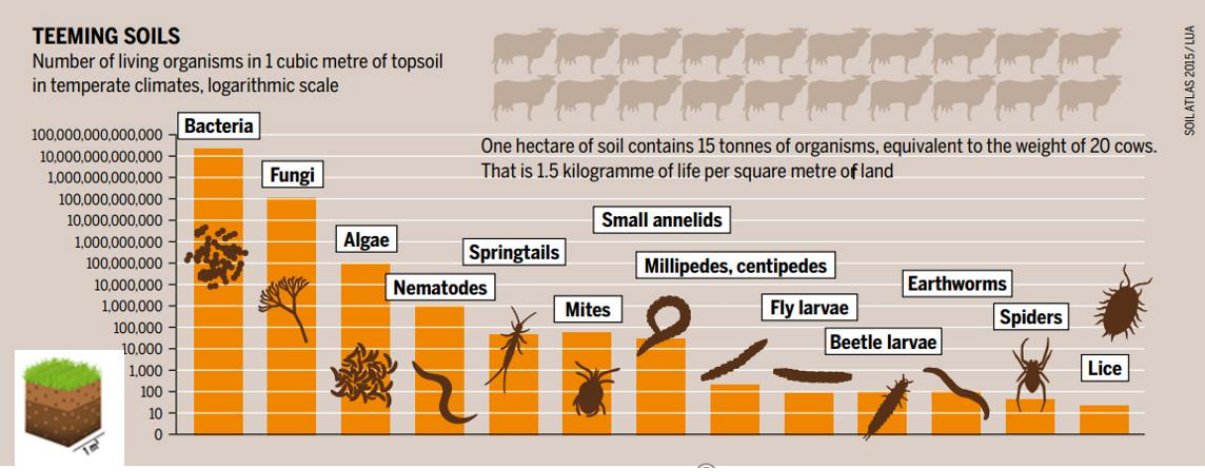

Fig. 1.5. Numbers of organisms (log scale) in a cubic metre of temperate region topsoil, showing the importance of bacteria and fungi, micro and meso faunal numbers (Bartz et al., 2015).

Collembolans, although small, number nearly three orders of magnitude higher numbers than earthworms in temperate soil (Fig. 1.5) and aid soil aggregation in a similar mode to earthworms, where OM is ingested and combined into microaggregates by physical and chemical mechanisms in the gut. Collembolans produce faecal pellets containing large amounts of OM accessible for bacterial and fungal decomposition (Coleman et al., 2017). It is hypothesised that the faeces of soil arthropods assist in soil

aggregation by providing a starting point for the formation of larger aggregates (Culliney, 2013; Lussenhop, 1992). The significant contribution of collembolans and other soil meso- and macro-fauna, including isopods, myriapods, insects and acarids, which influence aggregation at the initial and micro-scale is underestimated due to their small biomass and limitations in sampling methodology (Culliney, 2013; Stork and Eggleton, 1992). It has been estimated that the species richness of some groups of Arthropoda are realistically an order of magnitude greater than estimated (André et al., 2002).

1.2.2. Microbial communities

Microbial communities are a central driver of soil quality (Schloter et al., 2018), with both fungal and bacterial communities, and their associated activities, being considered as both temporary and transient binding agents in the formation of stable soil aggregates (Lehmann et al., 2017b; Johan Six et al., 2002; Tisdall and Oades, 1982). The interaction of soil microbes with mineral surfaces has been recently highlighted as an important driver of SOC sequestration (Kallenbach et al., 2016; C. Liang et al., 2017).

The soil microbial community can be assessed in many ways, with molecular methods, including phospholipid fatty acid (PLFA) analysis and analysis of DNA and RNA sequences, being the most effective ways at characterising and studying microbes without needing to culture, which is not possible for many soil organisms (Hill et al., 2000). As fatty acids are the most abundant class of soil lipids (Weete, 1976), fatty acid profiling is especially effective at determining the abundance of particular microbial groups (e.g. AMF or bacteria) during different stages of soil formation. This has provided evidence that AMF plays a particularly important role compared to bacteria and saprotrophic fungi in the earlier stages of soil development (Welc et al., 2012). Alterations in the microbial community structure as a result of environmental and physical changes can be quantified by phospholipid fatty acid analysis (PLFA), first developed by Frostegård et al. (1993). However, this method must be applied with caution, as some common markers for one group, e.g. AMF, can also sometimes be found in other groups, e.g. bacteria (Frostegård et al., 2011), which can lead to false inferences. Combining gas chromatography and mass spectrometry (GC-MS) forms a powerful analytical tool to identify and quantify different substances by metabolomic approaches, including lipids (Jandl et al., 2004).

The development of methods for DNA extraction and sequencing from soils, and the use of polymerase chain reaction (PCR) amplification of DNA extracts with taxon-specific primers has revolutionized our understanding of soil microbial communities and their phylogenetics. Such methodological advances have rapidly advanced understanding of the cryptic diversity of AMF which are among the most ecologically important fungi in terrestrial ecosystems through their symbiotic associations with around 80% of land plant species (Brundrett, 2009). These fungi associate with roots of many economically important crop plants (Wang and Qiu, 2006), and play important roles in soil aggregation (Ji et al., 2019; Wilson et al., 2009). However, routine reliance on so called AMF-specific DNA primers for molecular identification of fungi in the Glomeromycota phylum, where all AMF were previously placed

phylogenetically (Schüßler et al., 2001) for the past three decades has recently been shown to have a significant flaw. It has since been proposed by Spatafora et al. (2016) that the phylum name be replaced with Mucoromycota, with the majority of AMF being with the sub-phylum Glomeromycotina (Redecker et al., 2013). The limitation of the widely used AMF-specific primers is that they fail to detect an abundant co-occurring group of root symbiotic fungi in the Mucoromycotina subphylum that also form arbuscule-like structures (Orchard et al., 2017b, 2017a). This raises doubts about how to interpret much of the research on mycorrhiza functioning that has relied on Glomeromycota-specific AMF primers to identify AMF communities and their functioning (Orchard et al., 2017b, 2017a; Sinanaj et al., 2021). This problem may be especially acute in arable fields as the Mucoromycotina AMF communities appear to be especially prevalent and favoured by agricultural land management practices that generally impair Glomeromycotean AMF abundance and functioning (Albornoz et al., 2021).

Extracting and analysing RNA, as opposed to DNA, has the advantage of characterising metabolically active organisms and their genes, rather than merely the presence of organisms, which is detected by DNA, which is also more persistent in the environment. Analysis of active microbial communities using RNA gives the opportunity to see more rapid, responsive changes in community structure reflecting changes in activities of organisms as a direct result of changes to land management. Sequencing of the 16S and 18S ribosomal RNA (rRNA) genes to analyse total bacterial and fungal communities, respectively, is particularly useful as these genes are is present in all three domains of life: bacteria, archaea and eukaryotes (Woese et al., 1990), enabling a full view of the soil biodiversity present. Furthermore, this avoids the pitfalls of targeted DNA extraction and PCR amplification, which can give biased results and omit cryptic groups of organisms exemplified by the Mucoromycotina / Glomeromycotina confusion (Sinanaj et al., 2021).

1.2.3. Glomalin

The glycoprotein glomalin was first discovered by Wright et al. (1996), and is produced by a variety of Glomeromycotean AMF. Due to its accumulation in many soils, glomalin can constitute a large portion of the soil C pool (Singh et al., 2013). However, due to it being produced by AMF which are sensitive to changes in land management intensity, the abundance of glomalin in soils is also sensitive to these changes (Rillig et al., 2003; Singh et al., 2013). Interest in this protein heightened when glomalin production was found to be correlated to the formation and water-stability of aggregates (Rillig and Steinberg, 2002; Wright and Upadhyaya, 1998), therefore having a significant effect on overall soil structure and quality (Rillig, 2004). Glomalin is commonly referred to as glomalin-related soil protein (GRSP), first described by Rillig (2004), as most extraction and measurement techniques used routinely do not extract pure glomalin protein. GRSP is further sub-categorised into easily extractable and total "Bradford-reactive soil protein" (BRSP) is also used to name the collection of proteins quantified by Bradford assay (Wu et al., 2012). However, the Bradford reagent is a non-specific dye-binding reagent,

lacking specificity by cross-reacting with compounds like tannins. It therefore provides only a semi-quantitative measure of soil proteins like glomalin, and has been criticized for these limitations (Halvorson and Gonzalez, 2008). Fluorescent antibody assays are potentially more specific for visualizing and quantifying glomalin from hyphae, and associated with roots and soil aggregates (Wright, 2000).

Although, there are issues with the quantification and extraction methods of GRSP, as the extraction processes have been found to also co-extract similar sized proteins of non-AMF origin (Purin and Rillig, 2007; Rosier et al., 2006; Schindler et al., 2007). More recent work by Bedini et al. (2009) also showed a strong positive correlation between greater GRSP concentration and increasing hyphal length of AMF. This led to speculation that the supposed importance of GRSP in aggregate stability could be actually attributed to the enmeshing of soil particles by greater AMF hyphal presence. The postulated involvement of glomalin in enhanced water-infiltration rates and soil aggregate stability was also disproved by Feeney et al. (2004). After these findings, research into the role of glomalin in aggregate stability diminished, with recent publications questioning its origin from mycorrhiza (Lehmann et al., 2017a; Rosier et al., 2006). With the remaining possibility that glomalin plays an important role in aggregate formation and stability, the role of glomalin in soil systems without the presence of AMF needs to be studied in order to quantify the role of the protein independent of other contributing factors.

1.2.4. Roots

Though the main involvement of roots in soil aggregation is by enmeshing of soil particles (Bronick and Lal, 2005), roots also actively exude a range of plant-derived OM inputs including organic acids and enzymes, and release low molecular weight and structural polymers during their decomposition. Bacteria and fungi have evolved to form different niches during the decomposition of this OM (De Boer et al., 2005). Those in the form of simpler, more readily decomposed substrates, such as sugars, amino and organic acids, are degraded almost entirely by bacteria (Jones, 1998), whereas the degradation of more recalcitrant material, such as lignin and cellulose, is more reliant on fungi (De Boer et al., 2005), with lignin constituting almost half of the soil POM (Cambardella and Elliott, 1992).

Living roots contribute disproportionately more OC to soils relative to litter from AGB, due to higher chemical recalcitrance and deposition in deeper soil horizons, which often have higher clay content and lower oxygen levels, facilitating mineral-organic matter complexation (Rasse et al., 2005; Sokol et al., 2019). Therefore, the preferential selection of modern annual crop varieties to partition less OM and energy into root growth, and removal of the majority of AGB in harvested materials (AHDB, 2018) can starve arable soils of OM inputs. The incorporation of plants into arable rotations to increase year-round soil cover, for example through the use of intercropping or cover crops, can enhance the biomass and duration of living root systems and therefore soil quality and SOC sequestration (Cong et al., 2015; Lal,

2015). Cover crops cause a subsequent shift in microbial community structure that favour C and N cycling, beneficial for soil quality (Mbuthia et al., 2015).

1.2.5. Soil organic matter

It is well established that SOM aids the formation and stabilisation of soil aggregates, with often a greater proportion of water-stable macroaggregates seen in older soils with a higher SOM content (Chaney and Swift, 1984; Six et al., 2002). A significant proportion of OM in soils are classed as carbohydrates, with plentiful evidence to support the correlation between the prevalence of soil carbohydrates and increasing soil aggregate size (Puget et al., 1998; Tisdall, 1994; Tisdall and Oades, 1982).

Polysaccharides are polymeric carbohydrates and are produced by all cellular organisms, primarily in the form of mucilages and nutrient mobilisers (Bronick and Lal, 2005). The local concentration of extracellular polysaccharides has been shown to be exceptionally high in the presence of POM which provides nucleating sites to promote the growth of hyphae and their activity. Polysaccharides are known transient binding agents and facilitate the binding of microaggregates to form larger macroaggregates (Costa et al., 2018; Tisdall and Oades, 1982).

1.3. The role of grass-clover leys in arable rotations

Grass-clover leys, temporary grasslands, are commonly integrated into crop rotations in mixed farming systems, (combining crop and livestock production) which helps to maintain soil fertility and provide forage for on-site farm animals (Peyraud et al., 2009; Watson et al., 2006). Compared to continuous arable cropping, leys improve soil structure and quality through biological, chemical and physical interactions driven especially by the perennial plant communities sown, and their interactions with soil organisms (Berdeni et al., 2021; Hallam et al., 2020; Puerta et al., 2018; van Eekeren et al., 2008).

Fields are normally maintained under ley for two-three years. The break from tillage and the development of perennial root systems of evergreen forage experienced during this time enhances the input of organic binding agents such as roots, root exudates, and fungal hyphae which enmesh macroaggregates (Tisdall and Oades 1982; Portella et al., 2012; Rillig et al., 2015). This dramatically increases the OM inputs into soils, with ryegrass-clover sward roots contributing around 1 Mg C ha^{-1} yr^{-1} (McNally et al., 2015) compared to modern wheat varieties, which contribute as little as 0.4 Mg C ha^{-1} yr^{-1} via roots (Sun et al., 2018). As the mean residence time of root C is 2.4 times that of C from AGB due to higher chemical recalcitrance of root tissues (Rasse et al., 2005), differences in root C inputs are amplified when considering recalcitrance and long-term sequestration (Sokol et al., 2019).

Stopping tillage for a few years, together with minimal inputs of agrochemicals such as herbicides, fungicides and minimal use of nitrogen fertilisers, together with the increased OM inputs through living

roots and litter of leys supports recovery of earthworm populations (Jordan et al., 2004; Ponge et al., 2013; Prendergast-Miller et al., 2021; Schmidt et al., 2003; Scullion et al., 2007). Increases in earthworm populations and their burrowing and casting activities enhance soil structure and functions (see Section *1.2.1 Soil fauna* ; Bertrand et al., 2015). Earthworms further accelerate the sequestration and stabilisation of C (Zhang et al., 2013) and burial of residues enhancing disease suppression (Plaas et al., 2019). The introduction of leys also supports changes in the soil microbiome. Leys harbour a greater plant species diversity with a greater variety of rooting strategies important for soil structure (Wu et al., 2015), which in turn supports a greater biological and functional diversity of the microbial communities (D'Acunto et al., 2018; Tiemann et al., 2015; Venter et al., 2016). Functionality of the soil microbiome can provide important ecosystem services through their roles in nutrient cycling and disease suppression (Melero et al., 2008; Peralta et al., 2018).

Despite the existing knowledge of farmers that leys in rotations are good for soil structure, quality and functionality, until very recently, they have fallen out of favour in the UK and most of Europe due to poor economic returns from mixed farming. When fields are in ley, this is a loss of arable land that is not producing saleable crops. However, the UK government 25 year Environment Plan (Defra, 2018) and subsequent Post-Brexit replacement of the Common Agricultural Policy by a new Environmental Land Management Scheme has prioritized farm payments to support public goods and services, including, as a particular priority, improving soil health and growing species-rich leys (Defra, 2021c). Agricultural intensification along with the pressure to constantly produce crops to make profits saw a shift away from mixed farming in the 20th century. With grass-clover leys contributing around 2.5 times more soil C inputs through roots compared to wheat (McNally et al., 2015) and less C return through AGB and livestock manures, this shift resulted in the decline of arable SOC and declines in soil quality (Jarvis et al., 2017; King et al., 2005; Kirk and Bellamy, 2010; Townsend et al., 2018).

1.4. Thesis study system

The research presented in this thesis investigated the effects of introducing grass-clover leys in arable rotations on the biology, chemistry and aggregate structure of soil using experimental ley strips introduced into permanent arable fields with well-documented cropping histories. The study benefitted from the 'SoilBioHedge' experimental leys, established by a NERC-funded Soil Security Programme project based at Leeds University Farm, Tadcaster (53°52'25.2"N 1°19'47.0"W; Fig. 1.5). The design involved the sowing of mown, but not fertilised, grass-clover ley strips in May 2015 into four conventionally tilled arable fields. Rather than converting the whole field to ley, which is usually the case in agriculture, this 'space for time' approach enabled the comparison of soil properties in the leys compared to the adjacent arable fields at the same sampling time, which can be interpreted as changes that would have occurred if leys had been established on a whole-field scale. For my thesis, I sampled the grass-clover ley strips approximately three years after the establishment of the leys (38 months).

The use of this experiment allowed the analysis of many different soil properties after the implementation of a three-year ley into arable rotation, within the timeframe of my PhD.

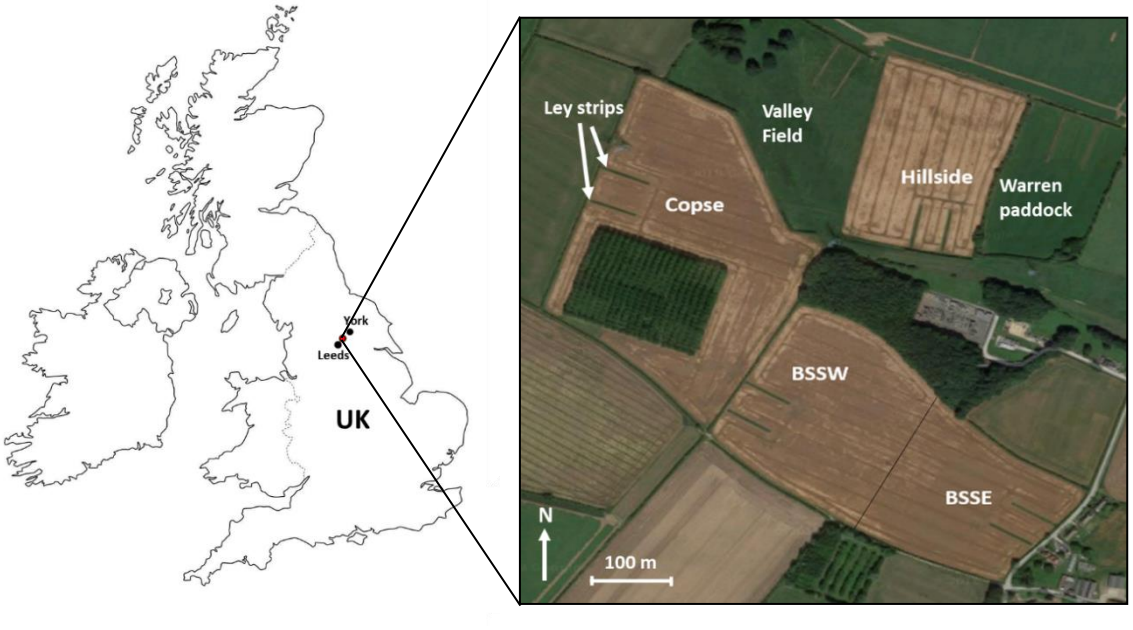

Fig. 1.6. A satellite view of Leeds University Farm, Tadcaster, England during the SoilBioHedge project. The image shows the four sampling fields: Copse, Hillside, BSSE and BSSW, along with the adjacent pasture fields: Valley field and Warren paddock. The leys were 70 m long and 3 m wide and are visible in each sampling field from this Google Earth satellite picture, taken 17/07/2017.

The cropping history of the conventionally managed, ploughed arable fields (Table 1.2) are typical of most arable land in Eastern England and Scotland. There had been constant cropping with short-lived annual crops with conventional ploughing since the fields were last under ley in 1988 (Copse) and 1994 (BSSW and BSSE). Hillside had been in grassland for 11 years until 2009. The proportions of major crops in rotation are also representative, with cereals, especially wheat, dominating the rotation (Defra, 2020). Even after cropping of root crops potatoes and beet, which are particularly damaging to soil structure and earthworm populations, there are no soil restorative phases using leys.

Table 1.2. Cropping history of Leeds University Farm, Tadcaster, UK. Field crop codes: BEET = sugar beet, OSR = oilseed rape, P = pasture, POT = potatoes, SB = spring barley, VPEA = vining peas, WB = winter beans, WW = winter wheat. Grass-clover ley strips were established in each arable field in May 2015.

Field	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016
Copse	WW	WW	WW	WW	POT	WW	WW	VPEA	WW	OSR	WW	POT	WW	OSR	WW	VPEA	WW	WW	SB
Hillside	P	P	P	P	P	P	P	P	P	P	P	WW	OSR	WW	WW	WB	OSR	WW	WW
BSSE, BSSW	WW	WW	WW	WW	POT	WW	OSR	WW	BEET	WW	WW	POT	WW	OSR	WW	VPEA	WW	WW	SB

1.5. Thesis aims, objectives and outline

The introduction of grass-clover leys into continuous cropped arable fields growing annual crops established by ploughing can promote changes in the soil environment including regeneration of biological, chemical and physical properties of soil, such as increases in earthworms and AMF abundances and diversities, which are also known soil aggregating agents. However, the exact mechanisms behind the formation of soil aggregates and SOC sequestration, particularly those promoted by the introduction of grass-clover leys, is still largely uncertain.

The overall aim of this thesis is to gain a greater understanding behind the mechanisms of soil aggregation and C storage that are promoted by the incorporation of grass-clover leys into conventionally ploughed arable rotations, using multi-omic approaches to assess changes in soil physical, chemical and biological properties. The ultimate aim of this research is to identify new methodologies that can be used for monitoring soil quality improvements that deliver improved public goods and services such as those that follow from improved soil structure and C sequestration. This has the potential to enable the rewarding of farmers via the new Environmental Land Management Scheme payments, which will replace the EU Common Agricultural Policy subsidies, and is aiming to deliver more sustainable soil management to benefit the public.

1.5.1. Thesis outline

This thesis is formed of 6 chapters

Chapter 2 is a paper in review at Science of The Total Environment for publication. Co-authors are written as they will appear in publication.

- Chapter 2 introduces the SoilBioHedge project in more detail and investigates the impact of introducing a three-year grass-clover ley into a conventionally ploughed arable rotation on the distribution of WSAs and OC and N storage. Soil structure and OC distribution is compared to the adjacent hedgerow soils as a measure of good quality, undisturbed soils.

- Chapter 3 uses samples taken at the same time point as Chapter 2 to investigate the impact of changes in land management from arable to three-year ley on fungal and bacterial communities. RNA-based methods are used with the aim of sequencing the active microbial communities that may be causing the soil structural and nutrient changes we see in Chapter 2.

- Chapter 4 investigates differences in the soil metabolome between long-term conventionally managed arable fields and three-year leys from the same fields and timepoint as Chapter 2 and 3, with the aim of relating identified metabolites to the changes in microbial communities, plant species and soil fauna that is observed during this change in management.

- Chapter 5 uses a monolith-based field experiment of arable to ley conversion for 1 year in which earthworm populations are manipulated to determine the effect of overall earthworm numbers and individual earthworm ecotypes on soil aggregation and OC and N storage within macroaggregates.

- Chapter 6 is a general discussion, concluding the biological, chemical and physical markers that have been identified and associated with soil aggregation and carbon storage as a result of the introduction of one to three years of ley into an arable rotation throughout this thesis.

Chapter 2

2. Soil macroaggregation drives sequestration of organic carbon and nitrogen with three-year grass-clover leys in arable rotations.

Edited version of submitted manuscript in review with Science of the Total Environment.

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Keywords:

Soil health, water-stable aggregates, regenerative agriculture, soil organic matter

Highlights:

- Three-year leys increased macroaggregates >5-fold in a long-term arable rotation.
- Soil organic C and N increased through accumulation in macroaggregates.
- Macroaggregate-stored organic C is a key indicator of improving soil health.

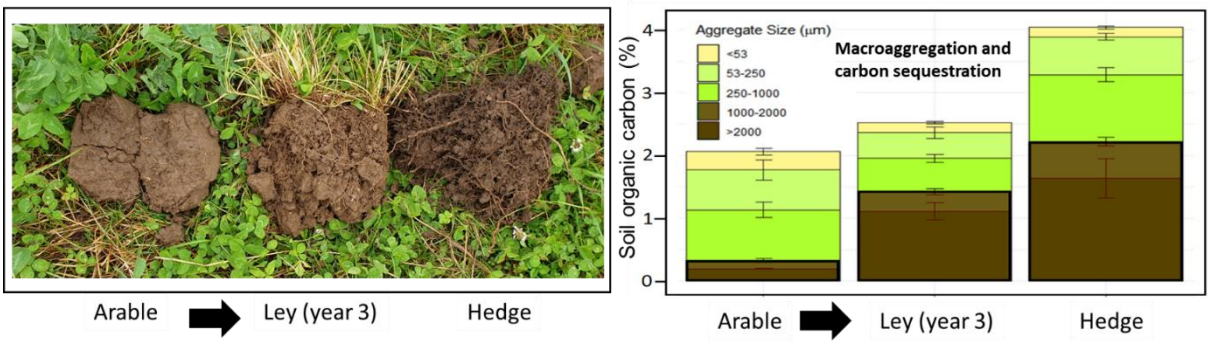
Author Contributions:

Emily J. Guest: Investigation, Writing – Original Draft, Formal analysis, Project administration. **Lucy J. Palfreeman:** Investigation, Writing – Review and Editing. **Joseph Holden:** Funding acquisition, Writing – Review and Editing. **Pippa J. Chapman:** Funding acquisition, Writing – Review and Editing. **Les G. Firbank:** Funding acquisition, Writing – Review and Editing. **Martin G. Lappage:** Investigation, Project administration, Resources. **Thorunn Helgason:** Funding acquisition, Writing – Review and Editing, Supervision. **Jonathan R. Leake:** Conceptualization, Funding acquisition, Writing – Original Draft, Supervision

Abstract

Conventional arable cropping with annual crops established by ploughing and harrowing degrades the larger soil aggregates in which soil organic carbon (SOC) is stored. The urgent need to increase SOC content of arable soils to both improve their functioning, and sequester atmospheric CO_2, has motivated studies into the effects of reintroducing leys into long-term conventional arable fields. However, effects of leys on total SOC accumulation over two-three years have been equivocal. As soil aggregation may be important for carbon (C) sequestration, we investigated the effects on aggregates and SOC of introducing mown grass-clover leys into annually cropped arable fields on a Cambisol soil. In three fields, SOC, total nitrogen (N) and their distributions in different sized water-stable aggregates (WSA) were determined for arable soil and three-year leys and these values were benchmarked against soil from beneath their hedgerow margins. Taking into account soil bulk density, mean SOC (0-7 cm depth) rose from 20.3 to 22.6 Mg ha^{-1} in the arable-to-ley conversion, compared to 30 Mg ha^{-1} in the hedgerows, but this 2.3 Mg ha^{-1} difference (or 0.77 Mg C ha^{-1} yr^{-1}) was not significant (p=0.89). However, the proportion of macroaggregates (> 2000 μm) increased 5.4-fold in the arable-to-ley conversion, recovering to similar abundance as that observed in hedgerow soils. The total SOC (0-7 cm depth) stored in macroaggregates increased from 2.0 Mg ha^{-1} in the arable soils to 9.6 Mg ha^{-1} in the leys (p=0.02), which no longer differed significantly from the 12.1 Mg ha^{-1} under hedgerows (p=0.48). This resulted in a sequestration rate in the macroaggregates three times faster than in the bulk soil, at 2.53 Mg C ha^{-1} yr^{-1}. The improved macroaggregation in the leys drove near parallel increases in total SOC and N within macroaggregates (5.1-5.7-fold respectively), confirming the importance of soil structure for both C and N sequestration. These findings highlight the value of monitoring macroaggregate-bound SOC as a key early indicator of shifts in soil quality in response to change in field management, and the benefits of leys to soil aggregation, C sequestration, and soil functioning, providing justification for fiscal incentives that encourage wider use of leys in arable rotations.

Graphical abstract



2.1. Introduction

Sustainable management of agricultural soils is essential to maintain the natural capital and ecosystem services that healthy soils can provide (Johnston and Poulton, 2018; Smith et al., 2015). These include producing high quality crops, storage of soil organic carbon (SOC), nutrients and water, slow release of rainwater and good water quality for surface and groundwater systems, all of which are vital to meet societal needs (Blum, 2005; Brevik et al., 2018). The requirement to manage soil well to maintain organic matter content, structure, and associated hydrological functioning is even more critical as a result of climate change (IPCC, 2019) increasing frequency and intensity of both drought and extreme rainfall events (Groisman et al., 2005; Samaniego et al., 2018). This increases risks of crop failures, flooding, and soil erosion (Nearing et al., 2004), with serious environmental and human consequences that often exacerbate social inequalities (Sayers et al., 2017). Soil hydrological functioning, and especially water infiltration, percolation and throughflow, are critically dependent on macropores and structural stability on wetting to maintain functional macroporosity (Xiao et al., 2017). If soil disaggregates on rainfall impact or wetting this results in plugging of pore spaces and the generation of surface capping which impedes infiltration and leads to infiltration-excess overland flow (Holden, 2020). This preferentially carries away the finer and lighter soil fractions such as clays and organic matter, further degrading soil quality and functions (Xiao et al., 2017; Yang et al., 2020). Water-stable aggregates (WSA) are therefore a key determinant of soil quality and structure (Churchman, 2010), and also play a central role in carbon (C) sequestration (Tisdall and Oades, 1982; Jastrow, 1996; Six et al., 1998; Stewart et al., 2008, 2009). These crumb-like particles are conventionally grouped into size categories, with the smallest -sized fraction (<53 μm) containing silt and clays that sometimes form very small microaggregates, a middle sized fraction ranging from 53 to <250 μm that comprises microaggregates, and the macroaggregate fraction that joins together silt and clay particles, microaggregates, and OM and ranges from 250- >2000 μm (Six et al., 2000; Totsche et al., 2018).

According to the hierarchical model of aggregate formation proposed by Tisdall and Oades (1982), microaggregates are bound together by persistent binding agents, and then enmeshed together in groups by temporary and transient binding agents, such as roots and fungal hyphae, to form macroaggregates. An important recent advance in understanding these processes has been the evidence that SOC sequestration is driven by microbial interactions with mineral surfaces (Kallenbach et al., 2016; C. Liang et al., 2017). This appears to result from microbially-derived organic carbon (OC) being bound to mineral surfaces within microaggregates that then protect it from decomposition within the larger macroaggregates (Yu et al., 2015) that are assembled mainly by the actions of roots and soil organisms (Six et al., 2000; Denef et al., 2004; Briedis et al., 2012). This directly links the processes of C sequestration to the assembly of macroaggregates by soil organisms, and to the field management practices that impact the ecosystem engineer organisms delivering these functions.

As the binding agents that impart structural stability to macroaggregates are labile and their turnover is accelerated by tillage (Tisdall and Oades 1982; Portella et al., 2012; Rillig et al., 2015), arable cropping normally increases the accessibility and degradation of SOC by soil microorganisms (Ye et al., 2020). Intensification of arable production in the past few decades, for example in Europe, has focussed on short rotations with the most profitable winter cereal and oilseed crops usually established by annual ploughing and harrowing (Townsend et al., 2016; Wezel et al., 2014). These intensive short rotations have depleted SOC and weakened soil structures, increasing disaggregation, slumping and compaction (Haghighi et al., 2020; Pires et al., 2017). Crop selection for enhanced partitioning of organic matter (OM) into above-ground biomass (AGB) compared to below-ground, and removal of this AGB during harvesting, has contributed to soil degradation through reduced OM inputs. For example, the most widely grown crop, wheat, allocates about 50% of its AGB into grain (AHDB, 2018) and barely 50% of straw is added to the soil in the UK (Townsend et al., 2018). The increasing market for straw for livestock bedding and electricity generation in purpose-built power stations has contributed to more than a third of straw being sold from UK arable farms, rather than being left as residues within the field (Townsend et al., 2018). Consequently, OM inputs to arable soils have fallen with intensification, including inputs from root turnover and exudates, as modern wheat varieties contribute as little as 0.4 Mg C ha^{-1} yr^{-1} to soil via roots (Sun et al., 2018), compared to around 1 Mg ha^{-1} yr^{-1} under a ryegrass-clover sward (McNally et al., 2015). Living roots disproportionately contribute to SOC sequestration relative to litter inputs from AGB, due to higher chemical recalcitrance of root tissues and microbial protection, especially in deeper soil horizons (Rasse et al., 2005; Sokol et al., 2019). Consequently, the 20^{th} century shift away from mixed farming in which grass-clover leys typically contribute 2.5 times more C inputs to soil via roots than wheat (McNally et al., 2015) and receive C return of AGB via manure inputs from livestock, is strongly implicated in the decline in arable SOC (King et al., 2005; Kirk and Bellamy, 2010).

Roots and manures have been shown to be important in promoting soil aggregation, C sequestration, and supporting earthworm populations (Tiwari, 1993), which burrow and ingest soil, and release worm-casts. Together, roots of perennial grasses and clovers, and earthworms, interact to generate macroaggregates and macropores, improving soil hydrological functioning and crop growth (Hallam et al., 2020). Conversion of intensively cultivated arable land to short term legume-rich leys has been found to lead to a rapid recovery in earthworm populations (Prendergast-Miller et al., 2021) and to major improvements in soil hydrological functioning, including increased pore space and reduced compaction (lower bulk density), faster infiltration rates via macropores and higher saturated hydraulic conductivity (Berdeni et al., 2021). These biological and structural improvements in soil quality have been correlated with large increases in wheat crop yield resilience to flooding and moderate drought (Berdeni et al., 2021). However, despite the importance of SOM for soil structure and aggregate stability (Li et al., 2021), increases in total SOC by 19 month old leys followed by a direct drilled wheat crop,

studied by Berdeni et al., (2021) were not statistically significant, but nonetheless are strongly implicated in the large biological, soil structural, and crop performance improvements seen.

Several recent studies corroborate that although total SOC tends to increase under two-three year leys introduced into arable rotations, over these short time periods the increases are typically not statistically significant (Gosling et al., 2017; Puerta et al., 2018). In the former case it was concluded that arable to ley conversion is a poor candidate for meeting C sequestration targets in Europe (Gosling et al., 2017). However, detecting statistically significant short-term changes in bulk SOC is constrained by the inherent spatial variability of soils, and the amount accumulated over the ley period will be small compared to the existing stock, but may nonetheless still make important contributions to improving soil functions and health, including C sequestration. This is clearly shown in multi-decadal studies of leys in arable rotations demonstrating significantly higher SOC concentrations that are important for C storage at landscape scales over 50-70 years (Jarvis et al., 2017; Johnston et al., 2017; Poeplau and Don, 2015; Prade et al., 2017). Similarly, an overview of multiple multi-decadal studies shows substantial increases in the rate of SOC sequestration in the early years after management change, including the incorporation of arable/ley rotations, organic amendments or conversion to grassland (Poulton et al., 2018). Thereafter, sequestration rates decline as they progress towards a new saturation-equilibrium (Poulton et al., 2018). This indicates the need to develop more effective measures than bulk SOC to guide our understanding of both the processes and rates of C sequestration in the first two-three years of arable to ley and arable to grassland conversions, and for evaluation of effectiveness of other management practices that may facilitate C sequestration.

One promising approach is to link studies of SOC sequestration to the processes of soil aggregation, which protects this C, by fractionating soil into WSA of different sizes and quantifying the OC pools in these fractions. A recent study of a sandy-silt Cambisol on an arable-to-ley conversion in a field under conventional and organic management in Switzerland, showed strong preferential accumulation of C in large (>2000 μm) macroaggregates in soil 0-6 cm depth (Puerta et al., 2018). In that study, the two-year leys in the conventional and organic rotations that had previously experienced intensive tillage and were frequently mown and highly fertilised with cattle slurry (205 kg N ha^{-1} y^{-1}) showed a significant increase (by 65 and 47%, respectively) in WSA >2000 μm (macroaggregates), which was responsible for the increasing contribution of large macroaggregates (>2000 μm) to total soil C. Previous studies have suggested that N-fixing legumes such as clovers co-sown with grasses increase SOC relative to grass-only swards (Poulton et al., 2018), and there are some indications that co-storage of SOC and N may be linked via constrained C:N ratios (Berdeni et al., 2021).

Here we set out to test the hypothesis that the introduction of a three-year grass-clover ley into replicated long-term, intensively cultivated arable fields on a silty Cambisol in the UK increases the proportion of soil macroaggregates and the storage of OC and N within them.

Our study focussed on four conventionally managed arable fields in which leys were sown in strips in the NERC-funded Soil Security Programme project SoilBioHedge. Arable management continued alongside the leys so that soil could be sampled and compared at the same time for both arable and ley treatments in the same fields. The aims of the study were to assess how the mown, but not fertilised, three-year grass-clover leys influence soil aggregation through the measurement of aggregate size distribution and resulting changes to the distribution of OC and N in these fractions, with the bulk soil total OC and N values being determined by summing the fractions. A previous study on the same soils found modest but not significant increases in bulk SOC and N after 19 months of arable-to ley conversion, followed by a wheat crop (Berdeni et al., 2021). Here, we aim to determine how the distribution of OC and N in soil aggregates changes after three years of arable to-ley conversion, testing the prediction of improved soil macroaggregation resulting in the protection and accumulation of OC and N within macroaggregates. We compared the leys to the arable control and hedgerow margin soils as benchmark start and endpoint comparisons for C saturation potentials, and to evaluate the extent of recovery of structure and chemistry of the soil under ley relative to permanently uncultivated hedgerow soils. Our approach uses a 'space for time' substitution approach whereby differences in soil properties in the leys compared to the adjacent arable controls sampled at the same time are interpreted as change that has occurred over the three years of the leys.

2.2. Methods

2.2.1. Field site and experimental design

Paired grass-clover ley strips (3 m wide, 70 m long and 48 m apart) were sown into four arable fields at The University of Leeds Farm, Tadcaster (53°52'25.2"N 1°19'47.0"W) in May 2015, to study spatial and temporal changes in soil quality (Fig. 2.1). The seed mix used comprised diploid and tetraploid *Lolium perenne* (20%, and 16%, respectively), *Festulolium* spp., 16%, two varieties of tetraploid *Lolium x boucheanum* (12% and 16%), *Trifolium repens* 5%, and *Trifolium pratense* 15%, at an overall seeding rate of 4.2 g m^{-2}. The ley strips were mown four times per year, with grass clippings removed from the leys from June 2016 onwards. In April and May 2016, clippings were returned to the ley.

The four fields had been under continuous arable rotations and conventionally ploughed since they were last under ley in 1988 (Copse) and 1994 (Big Substation West (BSSW) and Big Substation East (BSSE)). One field had been in grassland for 11 years until 2009 (Hillside). The soils in these fields are Calcaric Endoleptic Cambisols (WRB, 2014) but differ slightly in textural class (Hallam et al., 2020), with BSSW and BSSE being silt loams and Copse and Hillside being loam and sandy loam, respectively.

The paired ley strip design enabled direct comparison of the changes to soil properties under ley compared to conventionally tilled arable rotations at the same time, and at the same distance from the field margins. One of each ley pair (Fig. 2.2) was continuous to the field margin (CAL). The other (UAL) was separated by a 2 m wide fallow strip parallel to the hedges, 13 m long and extending 5 m to the left and right of the ley and along which a vertical stainless steel mesh curtain barrier was inserted to the bedrock at about 90 cm depth (Berdeni et al., 2021). This arrangement was designed to prevent the recruitment of earthworms and mycorrhiza from the field margin in the original SoilBioHedge project design.

Fig. 2.1. Map of The University of Leeds Farm, Tadcaster, showing the four sampling fields: Copse, Hillside, BSSE and BSSW. The 70 m long, green paired ley strips are visible in each field from a Google Earth satellite picture, taken on 17/07/2017.

2.2.2. Characteristics of the field hedgerows.

The arable fields were bounded by well-established hedges that are likely to be more than a century old. They were 1.8 m to 4.8 m high and 0.28 m to 1.31 m wide and managed via trimming using a tractor mounted flail mower every one to two years (Holden et al., 2019). They contained a mixture of woody species, dominated by *Crataegus monogyna* (60%) with *Sambucus nigra* (10%) and *Ilex aquifolium* (10%), accompanied by <10% cover each of *Corylus avellana*, *Cornus sanguinea* and *Rosa canina* with occasional *Prunus spinosa*, *Acer campestre*, *Fraxinus excelsior*, *Euonymus europaeus*, and *Rhamnus cathartica* (Holden et al., 2019).

2.2.3. Soil sampling

In July 2018, soil cores 5 cm deep and 100 cm^3 were taken from the top 7 cm of the soil in the middle of each ley strip, 35 m from the hedge, in the arable fields between the paired ley strips, and under the hedge between the paired ley strips (Fig. 2.2), in all four fields, which were growing oilseed rape (*Brassica napus*) at the time of sampling. The soil cores were stored at 4°C for subsequent analysis.

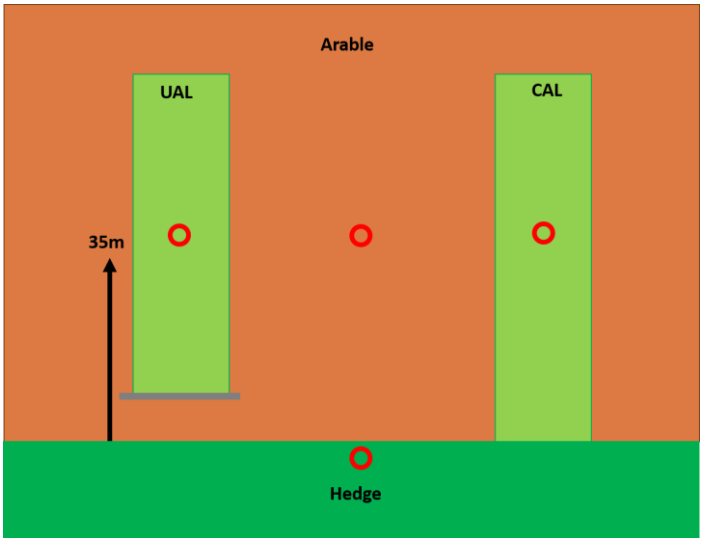


Fig. 2.2. Experimental design for soil collection from The University of Leeds Farm, Tadcaster. UAL = Unconnected Arable Ley, CAL = Connected Arable Ley, Open red circle = soil sample site. Not drawn to scale

2.2.4. Water-stable aggregate fractionation

The fresh soil samples were gently passed through a 2 cm coarse sieve to remove any stones, earthworms, vegetation and large roots. Subsamples of 70 ± 5 g were wet-sieved by hand, using a method adapted from Elliott (1986), through a series of four sieves to obtain five WSA size fractions following Puerta et al., (2018). These were: large macroaggregates (>2000 µm), medium macroaggregates (1000-2000 µm), small macroaggregates (250-1000 µm), microaggregates (53-250 µm) and silt and clay (<53 µm). The latter fraction also contains very small microaggregates (Totsche et al., 2018). Soil was submerged with water 15 mm above the 2000 µm sieve mesh, and the sieve moved vertically back and forth 50 times over a period of 1 minute and 30 seconds. Any floating material and stones were removed and aggregates on the sieve were washed into a pre-weighed aluminium tin. This process was repeated for the remaining soil on a 1000 µm sieve (40 strokes over 1 minute 10 seconds), a 250 µm sieve (30 strokes over 1 minute 20 seconds) and a 53 µm sieve (10 strokes not timed over as long as needed). All fractions were dried in a 105°C oven for 48 hours and weighed.

The following equation was used to calculate the aggregate size distribution by determining the percentage contribution of each aggregate size fraction: $\frac{fraction\ weight\ (g)}{total\ sample\ weight\ (g)} x100$. The data from 'BSSW Hedge' were excluded from the statistical analyses as in these samples the contribution of the >2000 µm aggregate fraction was 10-fold lower than in the other three fields, most likely because the soil was highly disturbed by a rabbit warren with burrows that emerged under the hedge adjacent to the ley strips. This hedge was in poor condition with major gaps, so was excluded as not suitable as an end-point indicator for SOC accumulation. Instead, to ensure adequate replication of samples from typical

well-established hedges, we included samples from the nearby field, Hillside. However, the arable and ley samples from Hillside, which had been in permanent grassland set aside and leys for 60% of the rotation over 48 years (1970-2018) were not included in the statistical analyses as these samples showed much higher >2000 μm aggregate fraction, compared to the other three fields that had been cultivated and cropped annually for 23 years.

2.2.5. Inorganic C removal from dried aggregates

The dried aggregate fractions produced from the WSA sieving were homogenised by mortar and pestle into a fine powder and 90 ± 5 mg of each sample weighed into 1.5 ml Eppendorf tubes. To each tube 500 μl 6M HCl was added, which was stirred using a blunt needle and left for 30 minutes. Additional HCl was added in increments of 100 μl and stirred until the sample stopped effervescing, indicating all reactive inorganic C had been removed. Samples were left for 24 hours in a fume hood to settle, enabling the removal of the supernatant. Samples were then oven-dried at 105°C for 24 hours to remove the remaining HCl.

2.2.6. Organic carbon and nitrogen analysis of dried aggregate fractions

The dried soil samples from which inorganic-C had been removed were homogenised and 30-50 mg transferred into tin boats for analysis of OC and total N percentage and C:N ratios determined by dry combustion in a CN analyser (Vario EL Cube, Hanau, Germany) using acetanilide (3-8 mg) as a standard.

2.2.7. Organic carbon and nitrogen distribution

The aggregate size distribution data was combined with the OC% and N% raw data in the following equation: $\frac{\%\ weight\ of\ aggregate\ fraction \times nutrient\ content\ (\%)}{100}$. This gave a dataset of soil SOC and N concentrations, and their distribution in the different aggregate size fractions.

2.2.8. Determination of soil bulk organic carbon and nitrogen stocks

Previous studies have revealed that surface soil bulk density can be significantly reduced after 18 months of arable to ley conversion (Berdeni et al., 2021), and is much lower in hedgerow soils than arable soils (Holden et al., 2019). Consequently, changes in the concentration of SOC on a soil mass basis do not accurately reflect the changes in total SOC stocks per unit soil volume or area of a field. To determine the effects of land management on the stocks of SOC from 0-7 cm depth, we combined the results of the present study with bulk density measurements made for the same field sites and sampled in the same year to 0-7 cm depth (Shaw, 2018). This previous study had found fine earth bulk density (excluding stones >1000 μm) decreased from the arable (1.41 g cm^3; n=24), to ley (1.33 g cm^3; n= 48) to hedge (1.06 g cm^3; n=12) soils (Holden et al., 2019). Taking into account the actual mass of soil to 7 cm depth in each case, the total SOC and N in Mg ha^{-1} in the top 7 cm of soil were calculated.

2.2.9. Statistical analyses

The WSA distribution data and concentrations of bulk SOC and N were analysed by two-way ANOVA to detect effects of land management treatment on aggregate size fractions, and interactions between these variables. For comparisons of the effects on individual aggregate size soil fractions of land management treatments (arable, CAL, UAL, and hedge) as factors one-way ANOVAs were used. No significant differences were found between CAL and UAL sites in any of the analyses. Therefore, these two treatments were combined into a single ‘ley’ treatment. Where one-way ANOVAs were significant, they were followed by *post-hoc* Tukey multiple comparison tests, to determine the effects of land management on either the WSA size distribution or the OC and N content within them. Fields were not included as a factor, but used as replicates, as all fields were on the same soil types with similar texture (Hallam et al., 2020; Holden et al., 2019) and those included in the final analyses had comparable management histories.

2.3. Results

2.3.1. Water-stable aggregate distribution

A two-way ANOVA revealed a statistically significant interaction of aggregate size and land management (arable, ley, hedge) on the proportion of soil weight in each fraction ($p<0.001$). Three years after the arable soil had been converted to ley there was significantly lower proportions of soil in the <53 μm, 53-250 μm and 250-1000 μm WSA fractions, but greater proportions within the 1000-2000 μm, and especially, in the >2000 μm WSA fractions in ley soil compared to arable (Tukey test, $p<0.05$ in all cases; Fig. 2.3). The most striking difference was in the proportion of large macroaggregates (>2000 μm) accounting for 7.4% of the arable soil but 39.4% of the soil mass in the leys, a 5.3-fold difference (Fig. 2.3). The contribution of large macroaggregates in the ley were similar to that seen in the hedge soil (Fig. 2.3). When the two largest macroaggregate sizes are combined into a >1000 μm fraction, the proportion of soil mass in this fraction was larger in the ley soil at 48.7%, compared to 12.3% in the arable field (Tukey test, $p=0.006$). Similarly, the hedge soils contained much higher proportions of soil in the >1000 μm aggregate fraction than arable soil, at 50.9% of the total soil weight (Tukey test, $p=0.009$). There were no significant differences between the proportions of soil in these macroaggregates in the hedge and ley soils (Tukey test, $p=0.96$).

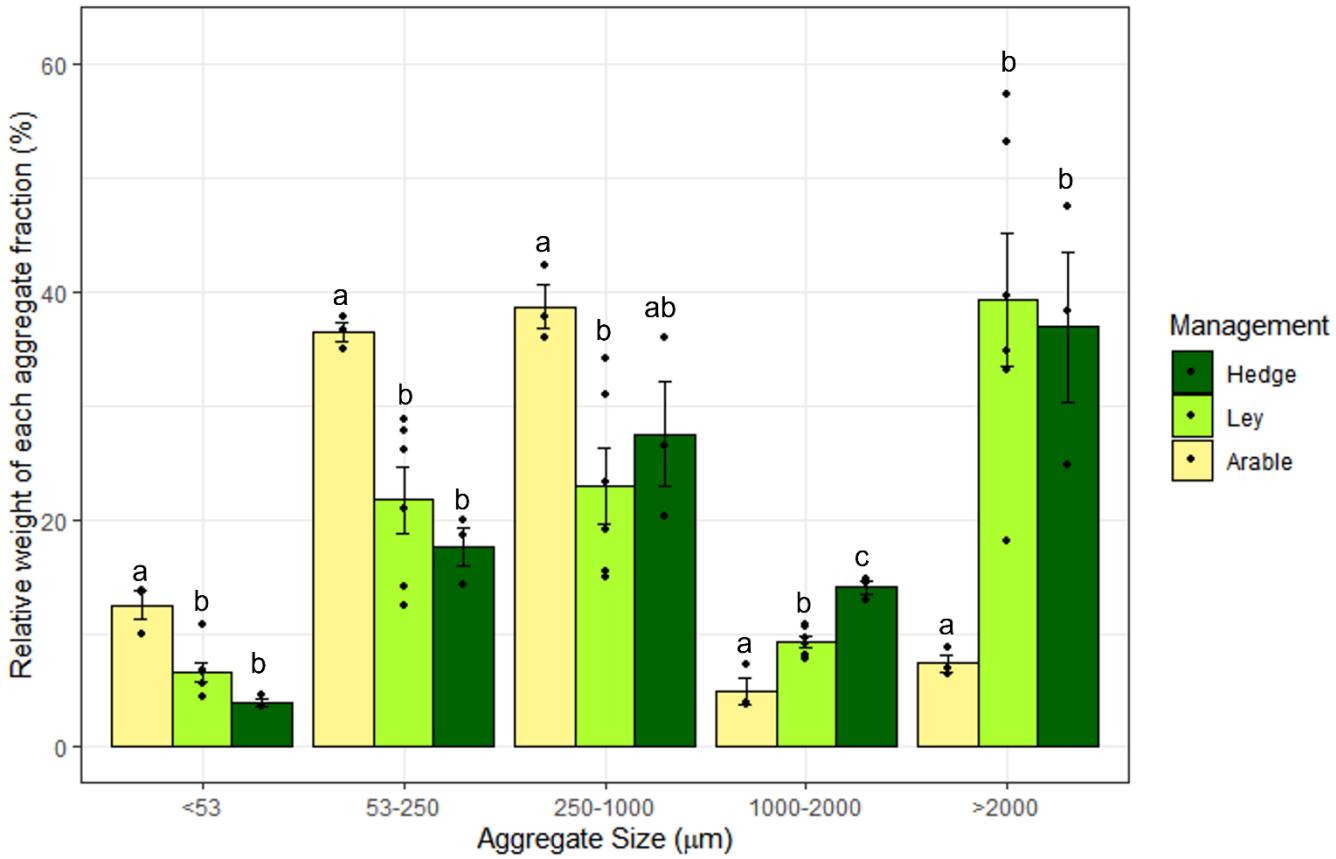


Fig. 2.3. The mean contribution of five different aggregate size fractions to soil mass under three different land managements (arable, three-year ley and hedge) with raw data points. Columns with different letters above them are significantly different from each other within the same aggregate size fraction (Tukey test, $p<0.05$). Error bars represent standard error.

2.3.2. Concentrations of organic carbon and nitrogen

With the exception of the >2000 µm WSA fraction, there was a larger mean concentration of OC in all the aggregate size fractions in the ley compared to arable soil, evidenced by no interaction between aggregate size fraction and OC% in a two-way ANOVA ($p=0.99$), but these increases were not significant (Tukey test, $p>0.05$; Fig. 2.4a). There was also consistently higher OC% stored in hedge soils across all fractions compared to the arable and ley soils, however, this was only significantly higher than the arable soil in the 1000-2000 µm, and the >2000 µm fractions, and in this latter case it was also significantly higher than in the leys (Tukey test, $p<0.05$).

The N concentrations in the aggregate fractions followed a very similar pattern of responses to the OC, suggesting close coupling of OC and total N sequestration in aggregates (Fig. 2.4b). Again, a two-way ANOVA showed no interaction between aggregate size fraction and land management ($p=0.99$). Hedge soils consistently showed the highest N concentrations compared to other land managements, with N

concentrations being significantly greater than those in arable soil in the 1000-2000 µm, and the >2000 µm fractions, where they were again different to the leys. There were also consistently greater N concentrations in the ley soil compared to arable, but these were not significant (Tukey test, p=0.52).

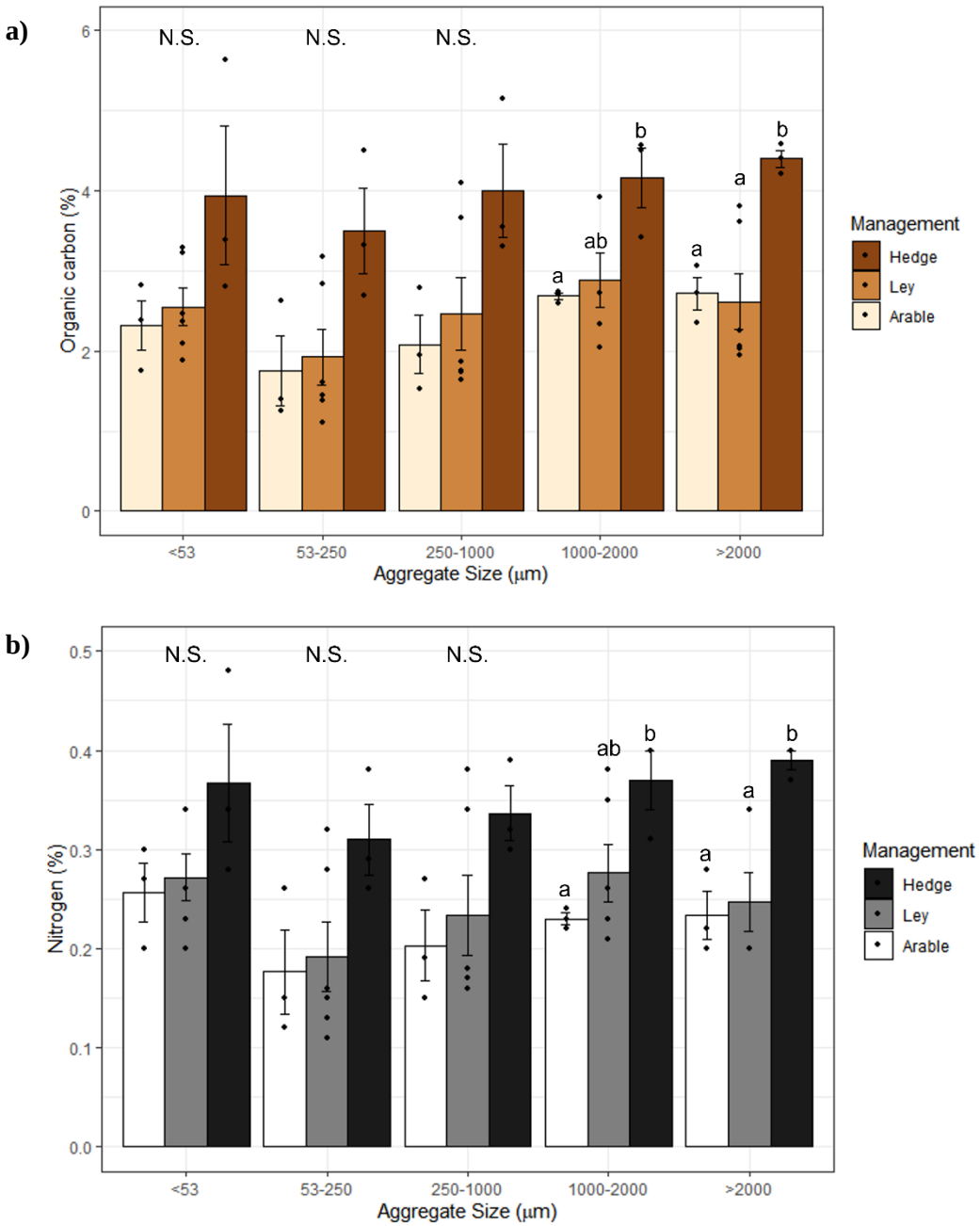


Fig. 2.4. The **a**) organic carbon % and **b**) nitrogen % content of different WSA sizes for three land uses (arable, ley and hedge), showing raw data points. Bars with different letter codes above them are significantly different from each other within the same aggregate size fraction (Tukey test, p<0.05). Aggregate size groups with 'N.S.' above the bars means a non-significant one-way ANOVA result (p>0.05), therefore a post-hoc Tukey test was not performed. Error bars represent standard error.

2.3.3. Ratios of organic carbon and nitrogen concentrations

The OC:N ratios showed modest variation between the WSA fractions in the soils under different management, ranging from 9.0 in the <53 μm aggregates in the arable soils to 11.7 in the 250-1000 μm aggregates in the hedge soils (Fig. 2.5a). There was a significant interaction between aggregate size class and land management (two-way ANOVA, p=0.006). In the three smallest aggregate fractions (<1000 μm) the OC:N ratios increased with increasing aggregate size, with hedge soils having significantly higher OC:N ratios than the arable and ley soils (p<0.05). In contrast, in the two largest aggregate fractions (>1000 μm), the arable soils had the highest OC:N ratios, being significantly higher than that found in the ley soil in the 1000-2000 μm fraction (p<0.05).

Plotting OC against N content and analysis by linear regression showed strong evidence of co-accumulation of OC and N, reflected in the high R^2 values (Fig. 2.5b). The slopes of the fitted regression lines (i.e., the OC:N ratios) systematically increased from arable (9.95) to ley (10.71) to hedge (13.66), with pairwise comparisons revealing no significant difference in the OC:N regression between arable and ley soils (p=0.80), but hedge soil having a steeper gradient than both arable (p=0.03) and ley soils (p=0.02).

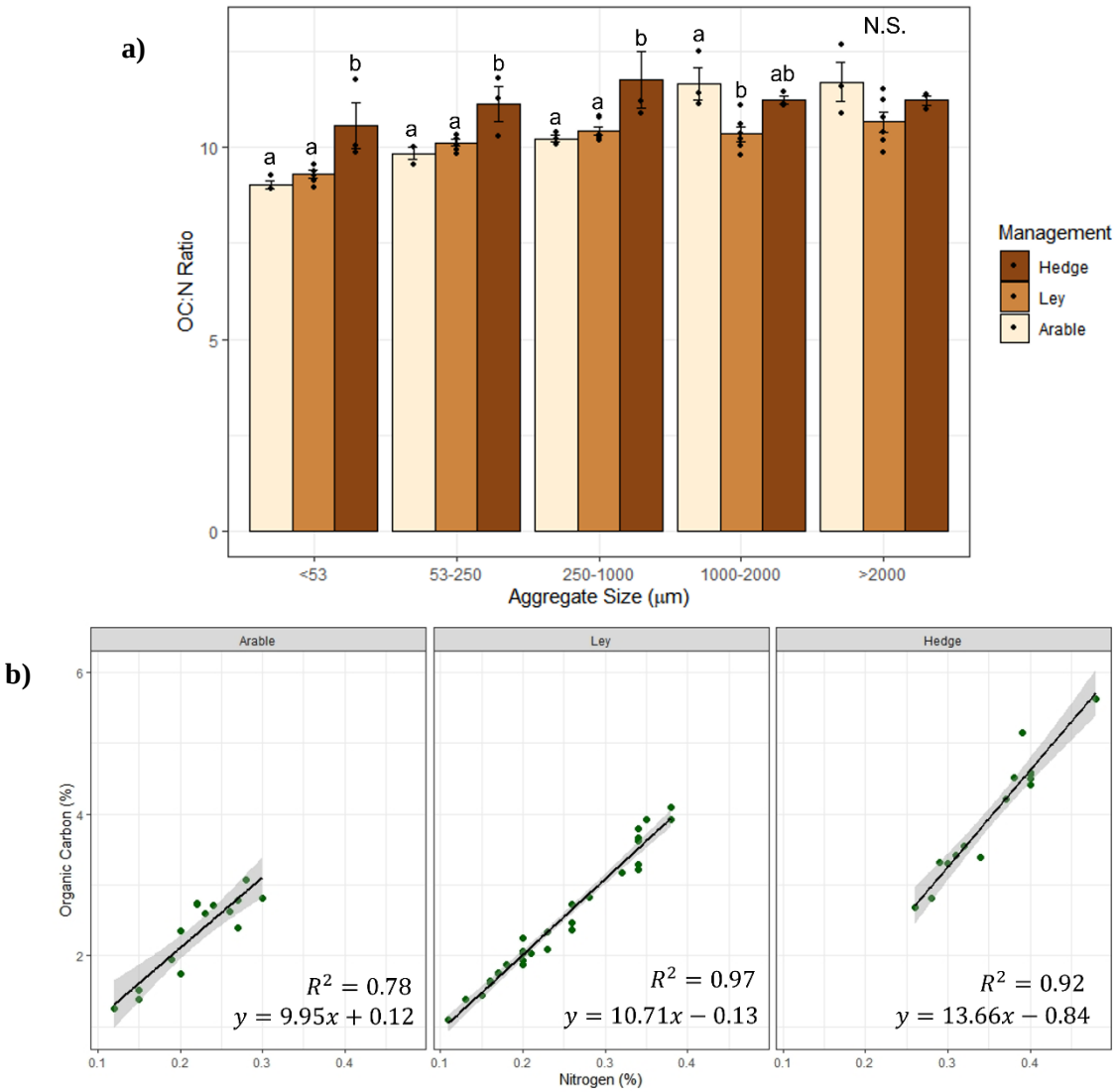


Fig. 2.5. **a)** Bar chart and **b)** linear regressions showing the effects of land management under permanent arable, three-year ley and hedgerow soils on the mean ratios of organic carbon: nitrogen storage, across five aggregate size fractions, with raw data points. Equations of the lines show their gradients, y-intercept, and the adjusted R-squared values. Grey bands either side of the regression line show the 95% confidence intervals.

2.3.4. Organic C and N distribution by aggregate fractions

Since the amounts of soil in each aggregate size fraction and the concentrations of OC and N within those fractions varied between the arable, ley and hedge soils, the proportions of soil mass by size fraction are presented in Fig. 2.6a to aid interpretation of these data. There were major differences in the composition of the different aggregate size fractions between the arable and ley soil, with significantly less soil in the three WSA fractions <1000 µm and significantly more in the two fractions >1000 µm, resulting in the ley being more structurally similar to the hedge than arable soils.

While a one-way ANOVA showed a significant effect of land management on bulk SOC content (p=0.02), there was no significant difference in bulk SOC concentration between arable and ley soils (Fig. 2.6b, Tukey test p=0.77). The bulk SOC concentration in the ley soil (2.43%) was 18% larger than that observed in the arable soil (2.06%), and both had significantly smaller concentrations than hedge soils (4.04%), (Tukey test, p=0.03, p=0.02, respectively; Fig. 2.6b). The ley did, however, have significantly (5.1-fold) greater OC stored within the >2000 μm fraction compared to the arable soil (Tukey test, p=0.03).

Land management also had a significant effect on bulk soil N content (ANOVA, p=0.04), but a post-hoc Tukey test revealed no differences between arable, ley or hedge soils (Fig. 2.6c; p>0.05). Total N (Fig. 2.6c) in the ley (0.23%) was on average 16% greater compared to the arable soil (0.20%) and the hedge soil (0.36%) was 52% greater than the arable. N storage in different WSA sizes (Fig. 2.6c) showed a similar pattern to that of OC, with the ley again having greater N% in the >2000 μm fraction (Tukey test, p=0.03), whereas significantly more N was stored in the smallest microaggregate fraction (<53 μm) of the arable soil compared to the three-year ley (Tukey test, p=0.03).

The SOC stock in the leys was 22.6 Mg ha^{-1} compared to 20.3 Mg ha^{-1} in the arable fields and 30.0 Mg ha^{-1} under the hedges. This shows that the leys accumulated 2.3 Mg C ha^{-1} in the top 7 cm over the three years, which equates to a sequestration rate of 0.77 Mg ha^{-1} yr^{-1} (2.83 Mg CO_2 ha^{-1} yr^{-1}). The latter is the maximum benchmark value for this soil since an unpublished study comparing hedge and adjacent deciduous woodland in the same fields (Marshall-Harries, 2013) found no difference in SOC concentrations. Bulk soil N was also greater in the three-year ley at 2.18 Mg ha^{-1} compared to 2.0 Mg ha^{-1} in the arable soil, with hedge soils storing the most at 2.6 Mg ha^{-1}.

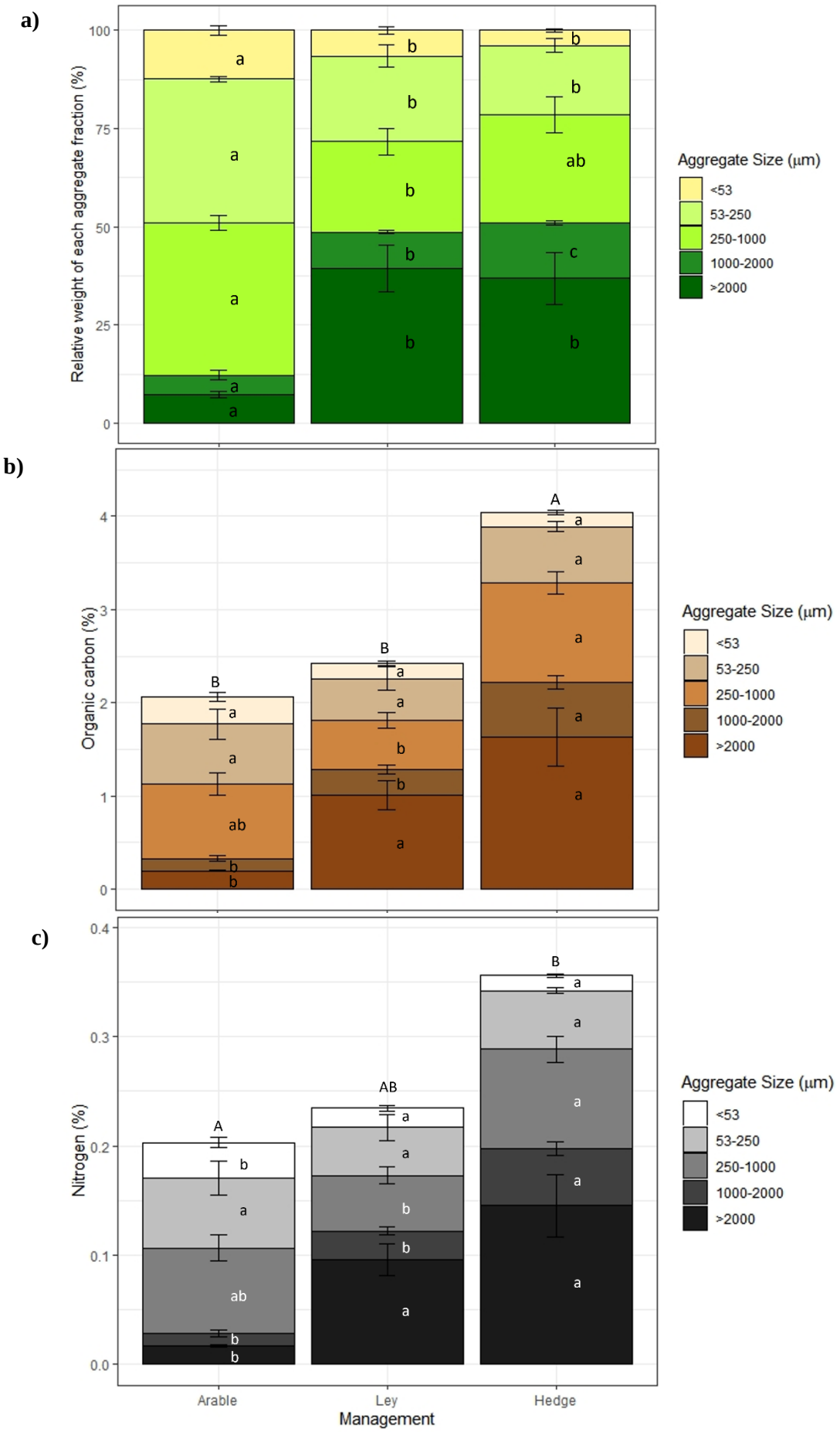


Fig. 2.6. The **a)** soil aggregate size distribution by weight, **b)** SOC and **c)** N storage and where these are stored within the five aggregate size fractions. Aggregate fractions of the same size class with different lowercase letter codes denote significant differences between arable, ley or hedge means (Tukey test, $p<0.05$). Bars with different capital letter codes above them have significantly different total concentrations (Tukey test, $p<0.05$).

2.4. Discussion

Our study corroborates and extends recent research demonstrating that the reintroduction of grass-clover leys into arable rotations for two years can result in remarkably large and rapid changes in soil structure (Berdeni et al., 2021). The results now presented shows that 3 years of ley leads to major reassembly of larger aggregates and this drives changes in OC accumulation, with preferential sequestration into macroaggregates and reductions in the proportion of soil mass in smaller aggregates. From the 'space for time' experimental approach, the differences seen between arable and ley soils are interpreted as changes in soil properties from a three-year arable-to-ley conversion.

2.4.1. Bulk OC and N sequestration

The non-significant 11.3% increase (from 20.3 to 22.6 Mg ha^{-1}) in bulk SOC stock after the arable soil has been under ley for three years is consistent with results seen in other short-term ley and grassland studies. For example, Gosling et al. (2017) reported a non-significant 5% increase in SOC% to 10 cm depth one to two years after introducing grassland into intensive arable cultivation and Puerta et al. (2018) found a non-significant increase of 8% in SOC to 6 cm in a two year grass-clover ley following conventional arable cropping. This non-significant increase in OC sequestration in the bulk soil equates to an annual sequestration rate of 0.77 Mg OC ha^{-1} in the top 7 cm of soil, which is towards the higher limit of measured rates of soil C sequestration through the adoption of restorative land-use practices, including the adoption of conservation tillage and cover crops, ranging from 0.05 to 1 Mg C ha^{-1} (Lal, 2004b).

2.4.2. OC and N in soil water-stable aggregates

Although we see no significant changes in bulk OC stock from arable-to-ley conversion, there are large and highly significant increases in the amounts of SOC in the >2000 μm fraction as a result of arable to ley conversion (Fig. 2.6b). This is consistent with soil macroaggregation being strongly linked to soil C sequestration in mineral soils, where OC bound to microaggregates is further protected within macroaggregates (Aoyama et al., 1999; Mikha and Rice, 2004; Puget et al., 2000; Yu et al., 2015; Zhou and Pan, 2007). Parallel research conducted in the same fields as the present study indicated the importance of the ley and earthworms in the regeneration of soil structure degraded by intensive cultivation (Hallam et al., 2020; Prendergast-Miller et al., 2021). The rapid reassembly of WSA >2000 μm under the three-year leys, with recovery to proportions seen under hedges, was a surprisingly fast recovery and clear indicator of substantial improvement in soil quality. Previous studies of OC accumulation in agricultural soils under different management treatments and OM inputs have suggested that OC saturates in macroaggregates so that the proportion of the soil volume that consists of macroaggregates controls the capacity of soil to store C (Yu et al., 2015). In our study, while the proportion of soil volume in macroaggregates recovered to the proportions seen in hedge soils, the

concentration of OC in these aggregates was far from saturation, but the capacity for this additional C storage had been generated in three years.

The preferential storage of OC within macroaggregates has previously been seen in soils under no-till management compared to conventional tillage (Messiga et al., 2011; Wright and Hons, 2005). Li et al. (2016) also found that soils with enhanced soil aggregation rates stored more C within macroaggregates compared to microaggregates. The lack of soil disturbance in the ley is known to allow for a slower rate of macroaggregate turnover and the formation of new microaggregates within macroaggregates and with them, the accumulation and stabilisation of mineral-associated OC (J. Six et al., 2002; Six et al., 2000, 1999). With more SOC stored within macroaggregates, more OC is protected from microbial decomposition (Balesdent et al., 2000; Plante and McGill, 2002a) by the entrapment of microaggregates within macroaggregates which provide physical protection (Bronick and Lal, 2005; Devine et al., 2014), with external layers being built upon older C stored in the aggregate interior (Santos et al., 1997). If SOC is more protected from microbial decomposition, it is more likely to be stored over a longer period, resulting in a net C sequestration into the soil rather than loss to the atmosphere as CO_2 (Lal, 2004a; Stavi and Lal, 2013). In contrast, the disaggregated, frequently cropped and tilled arable soil leaves OC more available for microbial decomposition and prone to erosion (Graves et al., 2015) via overland flow, which preferentially moves the lighter soil particles rich in OC, including microaggregates. The dominance of microaggregates and silt particles in the arable field also leaves it vulnerable to slumping and compaction, issues which are commonly addressed by a plough and harrow approach (Townsend et al., 2016), which is counterproductive in the long-term.

The leys are highly effective components of a regenerative agricultural system causing rapid reassembly of macroaggregates which must be attributable to the combination of cessation of arable cropping and ploughing, resulting in minimal soil disturbance and an increase in OM inputs predominantly from the roots as in our study, grass clippings were mostly removed after mowing. However, if the mown grass had been consistently left on the ley, OM inputs would have been larger and SOC sequestration rates potentially larger, although the mulch could have impaired photosynthesis, and without nitrogen-offtake the dominance by clover may have declined in favour of grass. Organic carbon inputs, especially via roots and mycorrhizal fungi of the evergreen perennial grass and clover plants, have been found to deliver more than double the C inputs of wheat roots (McNally et al., 2015; Sun et al., 2018). C inputs from roots contribute disproportionately to SOC storage due to the greater chemical recalcitrance of root tissues compared to plant litter, and the interactions between roots, soil microbes and minerals (Rasse et al., 2005). In contrast, tillage causes the degradation of soil structure through the breakdown of roots and hyphae holding macroaggregates together (Portella et al., 2012) and is also responsible for reducing the abundance and activity of soil fauna, earthworms and mycorrhizal fungal networks, all important aggregating agents (Crittenden et al., 2014; Six et al., 2004).

The break in tillage during the ley period will be pivotal in the recovery of the earthworm populations (Edwards and Lofty, 1982). Parallel studies in the same fields (Prendergast-Miller et al., 2021) reported rapid recovery of earthworm populations after 2 years of ley, resulting in a near fourfold increase from 185 earthworms m^{-2} in the arable fields to 732 m^{-2} in the leys in April 2017, exceeding the populations of 514 m^{-2} in the grassy field margins beside the hedges, and 474 m^{-2} in the relatively dry (Holden et al., 2019) hedge soils. Earthworms help to assemble soil aggregates via their casts. The passage of OM through their guts results in physico-chemical forms of OM that have extended residence time in soil, contributing to C sequestration (Zhang et al., 2013). Earthworms also play a major role in the improvement of soil structure and functions (Hallam et al., 2020; Hallam and Hodson, 2020; Yvan et al., 2012). Manipulation of earthworm populations in the same fields as in the present study, through temporary removal and deep-freezing of soil monoliths, clearly demonstrated their importance in the improved soil structural and hydrological functioning seen in the leys (Hallam et al., 2020). After only a year of arable to grass-clover ley conversion, soils supplemented, as opposed to depleted, with earthworms showed a 15% increase in water-stable aggregates >250 μm, increased plant-available water by 21%, increased water-holding capacity and SOM by 9%, and total N by 3.5%. The reassembly of macroaggregates and associated increases in macroaggregate-protected OC appear to drive important functional changes in the soil that reflect improvements in soil health, including enhanced wheat growth on the ley soil (Hallam et al., 2020).

The cessation of tillage is also advantageous for the recovery of mycorrhizal mycelial networks (Garcia et al., 2007), which play an important role in the creation and stabilization of water-stable macroaggregates through enmeshment (Ji et al., 2019; Rillig et al., 2015) and the production of exopolysaccharides (Costa et al., 2018; Sandhya and Ali, 2015). Furthermore, the routine use of glyphosate to kill weeds in the arable fields may additionally impact on the mycorrhizal fungal activities including aggregation, since there are a number of reports of this herbicide adversely affecting these fungi (Druille et al., 2013b, 2013a; Helander et al., 2018). Therefore, the substantial reduction of soil disturbance in the ley through a pause in tillage, enhanced input of OM via perennial roots, and the lack of herbicide use for three years, enables recovery of earthworm populations (Prendergast-Miller et al., 2021), and is likely to enhance mycorrhizal and other microbial communities all responsible for soil aggregation (Caesar-TonThat et al., 2010) and the accumulation of microbial-derived SOM (Kallenbach et al., 2016).

The increase in soil N stocks in the leys through biological N fixation by clover will reduce N fertiliser demand for subsequent cereal crops (McKenna et al., 2018a). As the production and use of N fertiliser accounts for 43% of the global warming potential in the lifecycle analysis of a loaf of bread (Goucher et al., 2017), this ley N fixation has the potential to reduce net emissions from cereal growing. The 0.18 Mg ha^{-1} increase in total N seen after arable soil was put under ley for three years equates to 180 kg ha^{-1}, which is greater than the average yearly N application rates of manufactured fertiliser in England, at

143 kg N ha^{-1} for 2019/20 (Defra, 2021d). Preferential N storage into macroaggregates which are less easily eroded could also result in reduced nitrate leaching into groundwater, as shown in previous studies from the introduction of grasslands into arable crop rotations (Kunrath et al., 2015).

2.4.3. OC:N ratios

It was expected that OC:N ratios would not change between hedge, arable and ley soils, as Holden et al. (2019) and Berdeni et al. (2021) both found no significant effect of land management on C:N ratios at the same study site. Edmondson et al. (2014) also reported no change in soil C:N between arable and pasture, which maintains a similar plant community to leys. However, in this study, we do see significant changes in the OC:N ratio between land management treatments, with the hedge soil having a higher OC:N ratio in aggregates <1000 µm and ley soil having a lower OC:N ratio compared to the permanent arable soil in the 1000-2000 µm fraction (Fig. 2.5a). The correlation of increasing OC:N ratio along the arable-ley-hedge gradient suggests that with increased OC sequestration there is a progressive shift towards less nitrogen being stored for each unit of OC. This may also reflect a gradient of decreasing N inputs from highly nitrogen fertilised arable field, to unfertilised ley with nitrogen-fixing legumes to legume-free, unfertilised hedges.

2.4.4. Comparison of field to hedge soils

Our approach in benchmarking linked changes in macroaggregation and in C and N sequestration in arable-to-ley conversion against the soil properties in the hedges at the field margins provides additional insights and context to the effectiveness of the leys in soil quality restoration and fertility-building. Hedges comprise the most common field boundaries in lowland arable landscapes in the UK, and in much of Europe (Holden et al., 2019). Consequently, soil quality benchmarking in fields against hedgerow soils is particularly useful for evaluating the effects of short- to medium-term management changes on the extent to which agricultural soil properties are recovering towards their local potentials for the particular soil type, landscape, and climatic environment. In many lowland arable landscapes, there are few permanent grasslands to serve as benchmarks for C stock changes, therefore hedgerows provide a more ubiquitous alternative. SOC stocks often change with land management change, but the rate of change progressively decreases towards a new equilibrium for a particular management, soil type, and climate context such as in arable to grassland conversions (Baveye et al., 2018). An earlier unpublished study conducted at the same farm found that soils under the arable field hedges had the same WSA size distributions as soil from the adjacent mature mixed species deciduous woodlands (Marshall-Harries, 2013), supporting the use of hedge soil as an “end point” benchmark of soil macroaggregation. However, the woodland soils contained about 16% more OC in the top 0-7 cm, although this may be due to a lower stone content, as ploughing of the arable fields brings up the shallow limestone bedrock at this site, some of which has been deposited at the edge of the fields and under hedgerows.

2.4.5. Transition to the incorporation of leys into arable rotations

SOC is lost at a much greater rate after returning to arable from grassland than it can be built up from the introduction of grassland (Jensen et al., 2020). Therefore, how soil is managed in between leys is key in maintaining some of their benefits through full cycles of arable rotations. Ploughing can cause a sharp decline in the large WSAs (Low, 1972), the breakdown of which would be paired with the loss of the OC and N they contain, easily reversing the benefits provided by the ley. Ideally, the inclusion of a ley into arable rotations needs to be carried out alongside other regenerative agriculture practices such as direct drilling, or reduced tillage to preserve more of the regenerative effects of the leys on soil health (Chan and Mead, 1988; Puerta et al., 2018), and this may deliver co-benefits of reduced residual weed germination from light-requiring seeds compared to ploughing.

Inclusion of leys into crop rotations needs to be financially sustainable and be economically favourable over continual cropping with full rotations to be successful. Some financial returns can be gained by grazing leys or in stockless systems, through production and selling of silage and other forage products together with fiscal incentives via government managed agricultural subsidies, which has shown to be successful in Sweden (Poeplau et al., 2015). The introduction of the Environmental Land Management (ELM) scheme in England to help achieve Defra's (the UK government's Department for Environment, Food and Rural Affairs) 25 Year Environment Plan goals of net zero C emissions, with the stated goal that all of England's soils are to be sustainably managed by 2030, is supporting actions to reward farmers to encourage C-friendly farming through better soil management (Defra, 2021e), and has identified leys as one component of this strategy.

2.4.6. Application of methodology for soil quality monitoring

From our findings, and previous related work, we conclude that the measurement of OC within macroaggregates compared to bulk SOC is a robust early indicator of soil quality improvement, as this enables detection of statistically significant and functionally important short-term changes in soil structure and quality. While there was no significant difference in bulk SOC stocks between arable and ley soils, we were able to detect large and significant differences in the amount of OC stored within the >2000 μm WSAs (Fig. 2.6). Water-stable macroaggregates are key functional indicators of good soil structure, being associated with reduced bulk density, increased macroporosity, and physical protection of OC against microbial decomposition (Balesdent et al., 2000; Plante and McGill, 2002a). Observations of preferential storage of OC and N in the largest aggregates compared to the bulk soil is a clear indicator that the soil biology, chemistry and structure are regenerating, in the present study, under leys. Wider application of this methodology to assess WSA size distribution and the OC and N% within macroaggregates would be helpful both to guide farmers as to the effectiveness of management changes for soil health. The methodology could be integrated into national monitoring of soil health at

field-to-landscape scales, for example in England's ELM scheme, which is currently developing an indicator framework for assessing improvements in public goods provided on farmland (Defra, 2021e).

Although we measured the distribution of five aggregate size fractions and the OC% and N% within each of them, the key fraction of interest was the largest. Therefore, for more economical and higher through-put processing, it may be appropriate to collect only the >2000 µm wet-sieved fraction and the remaining soil fraction smaller than this and analysing these two fractions for OC% and N%. This will give information on the proportion of soil macroaggregates and how much OC and N are stored within them compared to bulk SOC and N, which are already commonly measured. Further studies are required to test this approach on a wider range of soil textures and types, which can affect macroaggregation processes (Denef et al., 2004; Denef and Six, 2005; Rakhsh et al., 2017), and so determine if the two-fraction (>2000 µm and < 2000 µm) approach would be applicable to the main types of mineral soils used in arable farming. This methodology could potentially help not only determine long-term trends in overall bulk SOC and N, but also detect shorter-term improvements in soil structure and functions which are not picked up from bulk SOC measurements alone.

2.5. Conclusion

Our findings affirm the importance of including grass-clover leys in crop rotations to improve soil structure through increasing the prevalence of macroaggregates. There was no significant difference in bulk SOC and N% or SOC and N stock after the arable fields are under ley for three years. However, this equated to an average annual sequestration rate of 0.77 Mg OC ha^{-1} in the top 7 cm of soil. Compared with adjacent arable soil, we detected a substantially greater OC and N storage within macroaggregates, structures which are known to provide better protection for long-term C sequestration. We propose that monitoring OC and N changes in macroaggregates is a sensitive and useful early indicator of changing soil quality and structure that potentially could be applied in routine soil health monitoring, and for which farmers should be rewarded, but this needs to be tested on a wider range of arable soil types. Further research needs to focus on which other land management practices improve these traits, and whether by only measuring two fractions, the >2000 µm and < 2000 µm WSA, and benchmarking changes relative to undisturbed hedgerow soils would provide an efficient way of routinely monitoring changes in soil functioning in response to land use or management changes.

Declaration of competing interest

We confirm that we have no conflicts of interest to disclose.

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Chapter 3

3. Changes in soil microbial communities from long-term arable cropping to three-year leys, compared to hedgerows.

Abstract

The reintroduction of grass-clover leys into arable rotations involves temporary cessation of ploughing, minimal agro-chemical inputs and establishment of evergreen perennial vegetation, including symbiotic nitrogen-fixing clovers, which together, can drive physical, chemical and biological changes to arable soil. These include regeneration of water-stable macroaggregates and increased organic carbon storage in this soil component, as well as large increases in earthworm populations. Such changes would be expected to be accompanied by substantial shifts in the composition and activities of the soil microbial communities, especially in those groups such as mycorrhizal fungi that are known to be impacted by tillage and fertiliser additions. Here, we investigated responses of active soil bacterial and fungal communities assessed by extraction and sequencing of ribosomal RNA (rRNA) in response to introducing three-year grass-clover leys into four conventionally managed arable fields that had been ploughed and continually cropped for several decades. Microbial communities in soil from the neighbouring hedgerows of the same fields were also assessed as benchmark comparisons for undisturbed soils. Bulk soil RNA was extracted followed by ITS or 16S Illumina Miseq sequencing of the rRNA genes. Fungal communities significantly differed between land managements (arable, ley and hedge; p=0.002). Ley fungal communities were significantly different from those in the arable soil, with a greater relative abundance of Ascomycota but a reduced abundance of Basidiomycota and Mortierellomycota. Ley soil also had a substantially greater relative abundance and diversity of Glomeromycota, with 37 identified species compared to 18 and 13 in the arable and hedge soils, respectively. Hedge soils had a very distinct Glomeromycota community dominated by species in the *Claroideoglomus* genus. Bacterial communities did not vary significantly between arable and ley soil (p>0.05). These findings highlight the importance of fungal community changes from incorporation of leys into arable rotations, and their potential contributions to enhanced soil aggregation which requires further investigation. These results suggest that a reduced disturbance from the arable-to-ley conversion is beneficial for fungal abundance and diversity. However, the leys do not show convergence towards the microbial communities of undisturbed hedgerow soils. Hedgerow soils, despite supporting a high floristic diversity with distinct plant species, support a distinct microbial community dominated by a relatively low diversity of taxa, many of which are poorly represented, or absent, from arable fields and leys.

3.1. Introduction

Well-managed and well-functioning soils provide us with a multitude of ecosystem services for our well-being, including food production, carbon (C) sequestration, water filtration, storage and drainage and hosting biodiversity (Adhikari and Hartemink, 2016). To ensure the continuation of these services, it is important that soil health, function, and quality are understood and managed appropriately to avoid detrimental practices that damage these biological, chemical and physical systems. In evaluation of the impacts of changes in land management, soil microbial communities must be investigated as they are important drivers of soil quality (Schloter et al., 2018). Fungal and bacterial communities, and their associated activities, play a significant role as temporary and transient biological binding agents in the formation of soil aggregates (Six et al., 2002; Tisdall and Oades, 1982), an integral determinant of soil structure and functioning (Churchman, 2010). These organisms play central roles in C sequestration through aggregation and mineral-organic matter interactions (Jastrow, 1996; Six et al., 1998; Stewart et al., 2009, 2008). Recent advances have highlighted the importance of direct microbial interactions with mineral surfaces as a key driver of soil organic carbon (SOC) sequestration (Kallenbach et al., 2016; C. Liang et al., 2017). Microbial communities also play important roles in nutrient cycling, mineralising organic nitrogen and phosphorus compounds, and in disease suppression (Melero et al., 2008; Peralta et al., 2018).

On the other hand, there are also disbenefits of certain microbiota in soils, for example of soil-borne pathogens that can have detrimental impacts upon grain quality and yield, such as *Fusarium graminearum*, a major cause of the cereal disease '*Fusarium* Head Blight' (Del Ponte et al., 2017; Singh et al., 2016; Smith, 2007; Sutton, 2009). The dominance of wheat in short crop rotations has increased the risk of *Fusarium* infection (Champeil et al., 2004), which can also cause mycotoxin build-up in grains which are highly toxic and can present risks to human health, and cause a significant loss in crop value. Take-all, caused by the fungus *Gaeumannomyces graminis* var. *tritici* is another devastating soilborne pathogen that causes root rot to wheat and other related grass crops (Cook, 2003).

Intensification of agriculture has led to conventional farming practices that have been originally developed as a means to reduce crop pathology, but may have adverse impacts on beneficial soil microbiota, like mycorrhizal fungi (Badagliacca et al., 2021; Sun et al., 2016; B. Zhang et al., 2014). For example, continuous arable cropping and short rotations with the most profitable cereal crops, which are often established by annual ploughing and harrowing (Townsend et al., 2016; Wezel et al., 2014). Also, the use of agrochemicals, including the use of mineral fertilisers have increasingly been shown to impact soil microbial communities in arable fields. For example, there is long established evidence of the detrimental effects of phosphorus mineral fertilisers on the suppression of mycorrhization (Abbott et al., 1984; Kahiluoto et al., 2001, 2000; Kuramshina et al., 2020) and emerging evidence for the same effect of nitrogen fertilisers (Blanke et al., 2005). Fungicides and

herbicides are also extensively used in cereal crop production, with the herbicide glyphosate being reported to impair viability of mycorrhiza propagules, but are now being routinely found in high concentrations in most arable soils under conventional management (Druille et al., 2013b, 2013a; Helander et al., 2018; Zaller et al., 2014).

The use of more regenerative farming practices, with lower inputs and less soil disturbance leading to improvements in soil health are being advocated to develop more sustainable food systems (Schreefel et al., 2020; Sherwood and Uphoff, 2000). It is currently unknown whether regenerative agricultural practices that help to restore soil quality and functions are associated with shifts in the active soil microbial communities towards those of relatively undisturbed field margins such as under hedgerows, and if the latter can act as a benchmark reference of the extent of microbiome recovery in arable fields. The particular focus of the work presented in this chapter is on the effects of inclusion of grass-clover leys into arable rotations on the composition and activity of the soil microbial communities after decades of conventional tillage, fertiliser and agrochemical inputs following typical practices of UK agriculture.

The reintroduction of leys into arable rotations clearly changes multiple variables that impact soil microbiomes. A shift from annual cereal roots to perennial grasses increases C inputs via roots, with modern wheat varieties contributing as little as 0.4 Mg C ha^{-1} yr^{-1} (Sun et al., 2018) compared to ryegrass-clover swards which contribute over double, at around 1 Mg C ha^{-1} yr^{-1} (McNally et al., 2015). Multi-species grass-legume leys provide different rooting strategies, including fibrous hairy grass roots important for soil aggregation (Tisdall and Oades, 1979, 1982; Wu et al., 2015), tap-rooted red-clovers, and sparsely branched coarse white and red clover roots with few hairs that are highly dependent on mycorrhiza for uptake of phosphorus (Marschner, 2011). The clover roots will normally form nitrogen fixing nodules with *Rhizobium* bacteria recruited from the soil (Høgh-Jensen et al., 2004). With a combination of increasing OC and N inputs, a reduction of soil disturbance due to a ceasing of tillage, and a reduction or pausing of fertiliser inputs, changes in soil microbial communities, especially in fungi that are sensitive to disturbance, is hypothesised to occur. There is also a potential for the development of disease suppressive soils in leys, as these practices favour the recovery of earthworm populations (Briones and Schmidt, 2017; van Capelle et al., 2012), which are successful at combatting soil-borne diseases (Jorge-Escudero et al., 2021; Lagerlöf et al., 2020). This is through their consumption of litter, including preferential feeding on *Fusarium*-infected crop residues, further facilitating a transition to a lower intensity system with a reduced disease burden (Plaas et al., 2019).

Soil microbial communities rely on specific environments and other species through complex networks and interactions to survive and function, for example interactions between rhizobacteria and clovers (Zhang et al., 2016) and AMF with plant roots (Bonfante, 2018). Consequently, many soil microorganisms are sensitive to changes in land management which alter the plants and other organisms with which they associate, and ecosystems in which they live (Chen et al., 2020; Govaerts et al., 2007;

B. Zhang et al., 2014). Better understanding of the effects of microbial communities and how land management practices affect their functioning is of increasing importance for achieving improved yields with fewer chemical inputs such as through the suppression of plant pathogens (Hilton et al., 2013), and improving soil structure, C sequestration, and hydrological functioning through their roles in soil aggregation (Lehmann et al., 2017b). More knowledge is needed to understand the impact of different agricultural land management practices on the diversity and function of the soil microbiome, utilizing high-throughput molecular-based approaches (Schloter et al., 2018). These methods have the sensitivity to identify and quantify the impacts of short- and long-term changes in land management practices on soil microbial communities (Laudicina et al., 2012; Panettieri et al., 2020; Piazza et al., 2020).

Biological diversity in microbial communities is important, as it is likely to be associated with high functional diversity (Griffiths et al., 1997), essential for the driving of key ecosystem processes such as soil aggregation, C sequestration and reduced erosion risks (Chapin et al., 1997; Cookson et al., 2008; Schloter et al., 2018; Tilman et al., 1997). For specific functional groups of soil microorganisms, increased community diversity has been found to enhance plant productivity through complementarity effects as shown for AMF (Van der Heijden et al., 1998). High biodiversity can also aid in the resilience, reliability and predictability of the provision of ecosystem functions (McGrady-Steed et al., 1997; Naeem and Li, 1997; Oliver et al., 2015; Wagg et al., 2014), a quality that will become increasingly desirable as climate change continues to heighten pressures on ecosystems (Groisman et al., 2005; Samaniego et al., 2018).

The monophyletic phylum, the Glomeromycota encompass all AMF (Schüßler et al., 2001a), apart from those subsequently found to be in the sister group Mucoromycota in which fine root endophytes that form arbuscule-like structures are found (Orchard et al., 2017b, 2017a). Fine root endophytes also form associations with grasses in the *Poaceae* family as well as the important staple cereal crops wheat, maize and oats in this same family (D'Acunto et al., 2018).

It has been proposed by Spatafora et al. (2016) that the phylum name Glomeromycota be replaced by Mucoromycota, with the majority of AMF species being in the sub-phylum Glomeromycotina, containing four orders of AMF 'Glomerales, Archaeosporales, Paraglomerales and Diversisporales consisting of 25 genera (Redecker et al., 2013). However, for the purposes of this study, from now on, the original nomenclature 'Glomeromycota' will be referred to due to the taxonomy assignment methodology used.

AMF have the ability to form symbioses with the majority of the world's land plants, including grasses, clovers, and many crop species such as maize and wheat (Brundrett, 2009; Wang and Qiu, 2006). Oilseed rape (*Brassica napus*), the most widely cultivated crop species in the *Brassicaceae* family (Snowdon et al., 2007), is considered a non-mycorrhizal crop, which, in combination with conventional agricultural practices that disrupt AMF, can lead to a decrease in the AMF gene pool (Berruti et al.,

2018). However, *B. napus* is a highly effective break-crop in rotations with wheat, giving significant yield enhancement in the cereal (Sieling and Christen, 2015). This has encouraged a high frequency of *B. napus* in arable rotations in the UK, prior to the recent neonicotinoid ban, but this has been shown to lead to the build-up of *Brassica*-specific root pathogens that impact oilseed yields (Hilton et al., 2013).

There is evidence that AMF exhibit host preference and that communities differ between different plant species, to the extent that co-existing grass species with combined root systems can support different fungal symbiont communities (Vandenkoornhuyse et al., 2003, 2002). Plant species can thus shape AMF communities and determine which fungal symbionts are dominant in colonizing roots of subsequent plants as shown for legumes preceding wheat (Campos et al., 2018). Microbial host preference indicates the importance of choosing synergistic crop rotation combinations and enhancing diversity of plant species used in rotations for determining the below-ground biological and functional diversity of the microbial communities (D'Acunto et al., 2018; Tiemann et al., 2015; Venter et al., 2016).

Crop rotation is known to have a big influence on the soil microbial community composition (Bünemann et al., 2008; Lopes and Fernandes, 2020), with a higher diversity of crops in rotation supporting a greater microbial diversity and richness (Tiemann et al., 2015; Venter et al., 2016). Soil fungal communities are more affected by crop rotation than bacteria, which increases the fungi: bacteria ratio (X. Liang et al., 2017). The greater resilience of soil bacterial community structure (Navarro-Noya et al., 2013; Peixoto et al., 2006; B. Zhang et al., 2014) reflects their greater capacity to cope with disturbance, likely as a result of fast reproduction rates and occupying microsites within the soil. Crop rotation can also have a significant effect on the presence of plant pathogens, which can in turn effect plant growth and yield. Hilton et al., (2013) found that growing oilseed rape (OSR) as a monoculture compared to in rotation with wheat causes a significant difference in fungal community, predominantly caused by an increase in abundance of two plant pathogens *Olpidium brassicae* and *Pyrenochaeta lycopersici*, which are known to be significantly detrimental to oilseed crop yield. Additionally, Peralta et al., (2018) found that whilst the highest diversity crop rotation did not produce the largest soil bacterial diversity (4% lower than monoculture maize), it did increase the abundance of the disease suppressive functional group *prnD* gene compared to monocultures.

When incorporating legumes into crop rotations specifically, there is contrasting evidence of their effects on microbial diversity and richness, summarised in a meta-analysis by Venter et al. (2016). The incorporation of legumes into rotation with cereal crops is known to successfully increase soil mineral N through N-fixing bacteria and promotes the early infection of cereal (millet and sorghum) roots with arbuscular mycorrhiza (AM), which can in turn increase crop yields (Bagayoko et al., 2000). Mbuthia et al. (2015) also found that growing vetch as a leguminous cover crop increased microbial activity, measured by microbial respiration, associated with enhanced C and N cycling, suggesting the use of a

nitrogen-fixing cover crop as a more sustainable alternative to inorganic N fertilisation. This introduction of legumes into cereal rotation also causes shifts in the community structure of ammonia-oxidising bacteria in the rhizosphere (Alvey et al., 2003).

Another means of increasing above-ground plant diversity, with the potential to encourage microbial diversity, is through the inclusion of grass-clover leys into crop rotation. Grass-clover leys also provide additional benefits such as increasing soil C inputs (McNally et al., 2015), the recovery of earthworm populations (Prendergast-Miller et al., 2021) and improvements in soil hydrological functioning (Berdeni et al., 2021) and soil structure (Chapter 2). Undisturbed grasslands have been found to support large lengths of AMF hyphae often within the range of 50-100 m g^{-1} of soil (Leake et al., 2004), and supporting these hyphae can take about 9% of gross photosynthesis (Leake et al., 2006). However, there is currently limited research into how change in land management from arable to leys affects below-ground microbiota. A recent 25-year study in Norway by Chen et al., (2020) showed that mixed farming production systems had significantly increased microbial biomass, increasing numbers of both fungal and bacterial gene copies compared to crops only. The mixed farming involved spring ploughing and harrowing growing wheat and barley after a two-year grass-clover ley or one-year of barley after a three-year ley compared to permanent arable with mixtures of potatoes, wheat, oats and barley in varying combinations. However, including leys in rotations did not increase microbial richness or diversity. Being similar to grass-clover leys, grasslands are also reported to have significantly different microbial communities compared to arable rotations (Zelles et al., 1995), however different microbial communities develop under legume and grass cover (Zhou et al., 2017).

With the limited research into the effect of leys in arable rotations on microbial communities, evidence of effects on microbial communities as a result of similar changes in land management are worth considering for their potential to indicate likely changes associated with leys. For example, transitions to lower disturbance environments such as from conventional tillage (CT) to no-till (NT) systems, changes from conventional to organic farming, and incorporation of leguminous crops in rotations, may promote specific soil microorganisms such as N-fixing microbes associated with clovers in leys. Other soil properties that might be expected to change with an arable to ley conversion may also affect microbial communities, such as soil moisture, organic inputs and soil pH (Griffiths et al., 2010; Johnsson and Jansson, 1991).

There are conflicting findings concerning the impact of tillage methods on soil microbial biomass. Some report indicate greater microbial biomass in NT than CT systems (Badagliacca et al., 2018a, 2018b; Cookson et al., 2008; Helgason et al., 2009; Zuber and Villamil, 2016) and others finding no change to 7.5 cm depth after 31-years (Mbuthia et al., 2015) or at 0.5 cm and 5-15 cm after 14-years (Acosta-Martínez et al., 2007). As tillage mixes soil layers, it also affects the both the distribution of residues and their rates of mineralisation, with bacteria dominating due to their rapid ability to break down the

more labile organic matter (Govaerts et al., 2007; Wang et al., 2012). On the other hand, NT systems tend to exhibit a higher microbial biomass associated with higher substrate availability (Helgason et al., 2009), and are dominated by fungi, being the key decomposers of polymeric plant residues (Govaerts et al., 2007; Spedding et al., 2004).

There is also contrasting evidence on the effect of tillage on microbial communities, with some studies showing no effect of tillage on microbial community composition (Helgason et al., 2010a) and others showing significant changes between CT and NT (Helgason et al., 2010b). Several studies have shown an increase in the abundance of AMF and saprotrophic fungi (SF) in NT compared to higher intensity tillage systems (Mbuthia et al., 2015; Wang et al., 2012; B. Zhang et al., 2014). Selection for fungi with traits that enable survival of yearly ploughing and cropping is indicated by the dominance of specific genera such as *Funneliformis mosseae* (formerly *Glomus mosseae*), which Helgason et al., (1998) found represented 92% of sequences in ploughed, arable fields, likely due to their ability to sporulate abundantly and colonise quickly.

Focusing on the bacterial community, Navarro-Noya et al., (2013) found that NT most affected bacterial communities compared to residue removal/retention and crop rotation/monoculture. The relative abundance of Actinobacteria, Betapreoteobacteria and Gammaproteobacteria was most affected by tillage, and correlated significantly with total OC, which increased significantly with NT. There are contrasting results on the impacts of tillage on abundances of gram-positive and gram-negative bacteria, with some reporting that NT shows increases in gram-negative bacteria compared to CT (Badagliacca et al., 2021; Wang et al., 2012) and others showing an increase in gram-positive bacterial abundance (Mbuthia et al., 2015). Actinomycetes are a gram-positive mycelial bacteria which is often shown to increase in abundance after a transition to NT from a higher intensity system (Mbuthia et al., 2015; Wang et al., 2012; B. Zhang et al., 2014). Fierer et al., (2007) found that C mineralisation rate, a proxy of C availability, was the best predictor of phylum-level changes in abundance. With increasing C mineralisation rate, they found Acidobacteria abundance decreases, whilst the abundances of β-Proteobacteria and Bacteroidetes increase.

It is clear from the literature that tillage, crop type and rotation are all major factors in influencing soil microbial communities. A combination of NT, crop rotation and the retention of crop residues appears to be beneficial for increasing microbial biomass, activity and diversity compared to conventional farming practices (Govaerts et al., 2007), and are often accompanied by an increase in SOC and total N (Sun et al., 2016). Combining a reduced tillage approach with legume-based crop rotations, a combination of which are similar in land management to incorporating grass-clover leys into rotation, are particular beneficial, supporting higher diversity of soil microbial communities compared to monoculture crops (Lupwayi et al., 1998).

In this study, we investigate how fungal and bacterial communities change between a conventionally tilled, arable field and after conversion to grass-clover ley for three years. We also compare these two sites to soil from underneath the surrounding hedgerows as a benchmark for microbial communities in good quality soil, due to its undisturbed nature. This will enable us to evaluate the extent of recovery in microbial communities in arable soil after three years of ley relative to permanently uncultivated hedgerow soils. However, it must be taken into account that the plant species composition and microclimate under the hedge is very different to the rest of the field, with different plant host species and enhanced dryness (Holden et al., 2019), which undoubtedly causes its own microbial community changes.

Analysis was conducted on cDNA transcribed from RNA, rather than DNA, to target the active microbial community. Using cDNA profiles, we are more likely to identify changes in community composition as a direct result of changes in land management, which could otherwise be overshadowed by dead or inactive organisms (Y. Zhang et al., 2014). We use Illumina sequencing of the 16S ribosomal RNA (rRNA) and ITS rRNA gene to analyse the total bacterial and fungal communities, respectively. From this study, we aim to determine changes in total fungal and bacterial communities after CT arable fields are put under a three-year ley and compare the arable to ley changes to the communities found under permanently uncultivated hedgerow soils in the same fields.

3.2. Methods

3.2.1. Field site and experimental design

The field experimental design is described in greater detail in Experimental Chapter 1 and 2. In brief, paired strips of grass-clover ley (3 m wide, 70 m long, 48 m apart) were sown into four conventionally ploughed, annually cropped arable fields at The University of Leeds Farm, Tadcaster, UK (53°52'25.2"N 1°19'47.0"W) in May 2015 (Fig. 3.1). The seed mix for the ley comprised diploid and tetraploid *Lolium perenne* (20%, and 16%, respectively), *Festulolium* spp., 16%, two varieties of tetraploid *Lolium x boucheanum* (12% and 16%), *Trifolium repens* 5%, and *Trifolium pratense* 15%, sown at a seeding rate of 4.2 g m^{-2}. Each field has a Calcaric Endoleptic Cambisol soil type (WRB, 2014), differing slightly in textural type between fields (Hallam et al., 2020), with both BSSW and BSSE being silt loams and Copse and Hillside being loam and sandy loam, respectively.

The ley strip design allowed the comparison of changes in microbial communities under ley compared to arable rotation under CT simultaneously. One of each of the paired ley strips were connected to the hedgerow (Connected Arable Ley – CAL) whilst the other was disconnected from the hedgerow (Unconnected Arable Ley – UAL), separated by a 2 m wide fallow strip and a 90 cm deep stainless-steel mesh barrier set vertically into the soil. These were originally designed to prevent the migration of earthworms and mycorrhizal fungi from the field margin (Berdeni et al., 2021). When samples were collected in July 2018, the ley strips had been in place for three years and managed by occasional mowing.

3.2.2. Soil sampling

Soil cores (5 cm deep and 100 cm^3 volume) were taken from the top 7 cm of soil in July 2018 (Fig. 3.1) at same time as sampling for aggregate stability and OC concentrations, as reported in Chapter 2, when grass-clover leys were established for three years and two months (38 months). In all four fields, soil core samples were taken in the centre of each paired grass-clover ley strip, in between the paired leys in the CT arable fields, and underneath the hedgerow midway between each paired ley strips (Fig. 3.2). Soil from each core was emptied from the sampling ring and carefully homogenised and stored in a -80°C freezer within 2 hours after collection and transportation to prevent any change or degradation to the RNA. All four fields were growing the non-mycorrhizal oilseed rape (*Brassica napus*) crop at the time of sampling.

Fig. 3.1. Arial map of The University of Leeds Farm, Tadcaster, showing the four sampling fields: Copse, Hillside, BSSE and BSSW. The 70 m-long leys are visible from this Google Earth satellite picture, taken 17/07/2017.

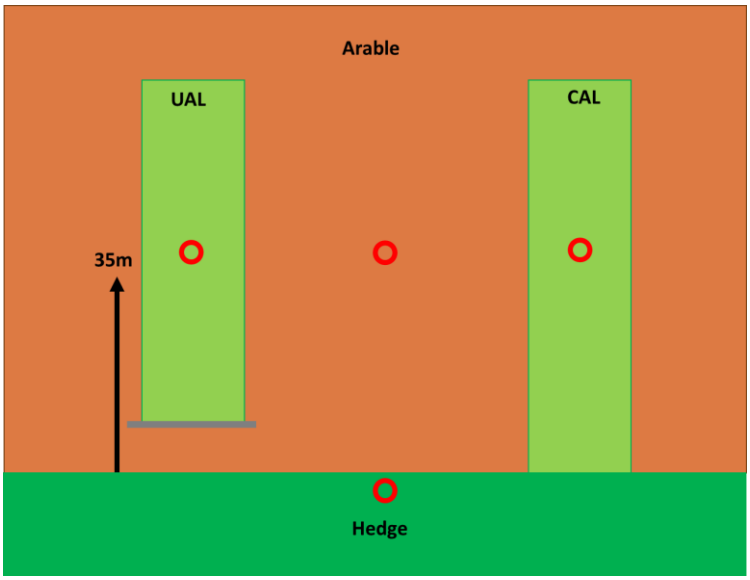

Fig. 3.2. Experimental design of each field at The University of Leeds farm, showing the sampling points (open red circle) in the Unconnected Arable Ley (UAL), Connected Arable Ley (CAL), arable field and hedge.

3.2.3. Soil RNA extraction

A subsample of each frozen soil sample was taken by crumbling the frozen soil and taking a representative subsample throughout the profile, and transferred to the University of York on dry ice and stored in a -80°C freezer. RNA was extracted using RNeasy PowerSoil Total RNA Kit (Qiagen) from each of the soil samples, following the kit protocol (Qiagen, 2017). RNA concentration and quality were quantified by running each sample on a Nanodrop™ 1000 Spectrophotometer (Thermo Scientific), with all samples having high concentrations of RNA (29-148 ng/µl) and high purity, with all samples having 260/280 and 260/230 nm absorbance values over 2, except one sample with a 260/230 nm absorbance value of 1.95. These samples were of sufficient quality (Thermo Fisher Scientific, 2007) to proceed so were stored at -80°C for downstream applications.

3.2.4. DNA clean-up

A DNA clean-up step was performed on the RNA extractions to ensure the removal of any genomic DNA in the samples which could be potentially amplified during subsequent polymerase chain reaction (PCR) amplification and sequencing steps and provide sequences for historically, but not currently active, microorganisms. 50 µl RNA was cleaned using the 'DNA-free DNA removal kit' (Thermo Fisher Scientific, 2012) using DNase treatment, and stored at -80°C.

3.2.5. cDNA Synthesis

DNA-free RNA was converted to cDNA using SuperScript™ IV Reverse Transcriptase (Invitrogen), following the user guide protocol (Thermo Fisher Scientific, 2015). RNA concentrations were normalised to the lowest measured concentration by adding less RNA template and more dH_2O to equal 11 µl (minimum RNA 2.2 µl, maximum RNA 11 µl). Random hexamers diluted to 50 µM were used for the primer (Promega, C1181), with the average primer length being used to calculate concentration. The optional 'remove RNA' step in the protocol was not followed as PCR products were less than 1 kb. cDNA produced from this protocol was stored at -20°C for following PCR amplification.

3.2.6. PCR

Primer sets for different regions of the ribosomal RNA (rRNA) operon were used to target total fungal species or bacterial species. Total fungal community was targeted using a nested PCR approach using fungal ITS genetic markers (ITS1f/ITS4 followed by gITS7-ill/ITS4-ill; Ihrmark et al., 2012). Bacterial species were targeted using a nested PCR with 16SrRNA primers (27F/806r followed by 515f-ill/806r-ill; Caporaso et al., 2011).

3.2.7. DNA clean-up, quantification and normalisation.

Agencourt AMPure XP beads were used to clean up PCR products prior to sequencing. Room temperature beads were mixed with PCR product in a 1:0.8 PCR:Beads ratio before following the remaining product protocol. The DNA concentration of cleaned PCR products was quantified using the QuBit dsDNA HS Assay Kit (Invitrogen). Samples were then normalised to 5 ng/µl in 10 µl ultrapure water for sequencing.

3.2.8. Illumina MiSeq Sequencing

Samples were sent to the Genomics and Bioinformatics Laboratory at the University of York for DNA sequencing by Illumina MiSeq. This process involves recording newly attached bases during the synthesis of DNA strands by the liberation of fluorescent dye, which is imaged and recorded. Forward and reverse FASTQ files for bacterial and fungal data were received with Illumina tags removed.

3.2.9. Fungal processing

FASTQ sequence files were unzipped and processed using the DADA2 (version 3.10) Pipeline using R (version 4.1.0). The DADA2 script used was edited from GitHub (https://benjjneb.github.io/dada2/tutorial.html, accessed: 05.08.2021). Forward and reverse read quality profiles were checked and reads trimmed to where the quality score drops to Q30. This was around 260 base pairs (bp) long for the forward reads and around 200 bp long for the reverse reads. Standard filtering parameters for DADA2 were used and primers trimmed, with 31 bp removed for *gITS7* on forward reads and 20 removed on reverse reads for *ITS4*. Error rates were estimated using the DADA2 algorithm, which were a good fit to the observed rates so data could be used with confidence. The core sample inference algorithm was applied to the filtered and trimmed sequence data and forward and reverse de-noised reads were merged. An amplicon variant table (ASV) was then constructed from the sequence variants and chimeras removed, with 98.3% of the merged sequence reads being non-chimeric, showing data is of high quality. From tracking reads through the DADA2 pipeline, the majority of the raw reads were kept.

Taxonomy was then assigned to the sequence variants using the UNITE reference database (https://unite.ut.ee/repository.php, UNITE general FASTA release for Fungi, version 8.2, release date 2020-02-04, accessed 06.01.2021, Abarenkov et al., 2020). Assigned taxonomy was then manually assessed. Where taxonomic definition was not to genus level, tree building was used to allocate into groups so that if exact identity was not clear, clustering was. Sequence variants with the same assigned taxonomy were manually checked to ensure that they shared 97% sequence similarity, a common measure suggesting sequences are likely from the same species (Gevers et al., 2005). If less than 97% sequence similarity was found, numerical identifiers were used so that these ASVs did not merge during the following aggregation step, which involved merging Operational Taxonomic Units (OTUs) with more

than 97% sequence similarity. The ASV and taxonomy table produced by the DADA2 pipeline along with the metadata table containing information about sample sites and fields, were imported into the phyloseq R package to create a phyloseq object for further analysis of the fungal dataset.

3.2.10. Bacterial data processing

Bacterial FASTQ files were processed using the same DADA2 pipeline, with some differences to the trimming and clustering steps. After assessing the read quality profiles, forward reads were trimmed to 250 bp and reverse reads to 200 bp long, where the quality score dropped below Q30. Primers were trimmed by removing 31 bp for *515f* on forward reads and 21 bp for *806r* on reverse reads. 99.4% of the merged sequence reads were non-chimeric, confirming that the bacterial data is of high quality. Tracking reads through the DADA2 pipeline showed that the majority of raw reads were kept after these processing steps. Taxonomy was assigned to sequence variants using the SILVA reference database (https://zenodo.org/record/1172783, Silva taxonomic training data, Silva version 132, accessed 19.01.2021, Callahan, 2018). The data file for species-level assignment was also used.

As the bacterial dataset was much larger than for fungi, clustering was done in R by sequence similarity to 97% after a phyloseq object had been made. Taxa were then filtered to remove samples with less than 40 reads and normalised to even the sequencing length. Any ASVs not determined to be bacteria at phylum level were removed, eliminating plant related ASVs such as chloroplasts and mitochondria. This script produced a further ASV and taxonomy table which were imported into phyloseq along with the metadata table with sample information for further analysis of the bacterial dataset.

3.2.11. Data processing and Analysis

Data processing and analysis was done in R (version 4.1.0, R Core Team, 2021). Non-metric multidimensional scaling (NMDS) plots, recommended for genomic OTU datasets with null values, such as that produced in this study, were generated using Bray-Curtis similarity matrices using the Vegan R package. Permutational multivariate analysis of variance (PERMANOVA) analyses were run on the Bray-Curtis matrices using the Vegan package (Oksanen et al., 2020) to determine changes in microbial communities between different sampling sites.

3.3. Results

Details about how many reads were outputted from the Illumina Miseq machine to the number of uniquely identified species in both the fungal and bacterial communities are presented in Table 3.1.

Table 3.1. Run statistics regarding total reads from the Illumina Miseq machine, how many passed through the filtering steps in section 2.7, how many ASVs (unique sequences) were identified and how many OTUs considered as unique species after the aggregation step were produced.

	Fungi	**Bacteria**
Total reads	1223495	1523515
After filtering	871257	991153
% Through filtering	71.2%	65.1%
Total ASVs	1476	5314
Total OTUs	876	2176

3.3.1. Changes in Fungal Communities

A Bray-Curtis similarity matrix was constructed on the total fungal data, from which an NMDS ordination was made (Fig. 3.3). The NMDS ordination shows hedge, ley and arable site samples were variable within their group but distinct from each other, and there was no obvious trend in the ley samples becoming more similar to the hedge samples. There are no visible trends suggesting systematic differences between fields, meaning these can be used as replicates as intended. A PERMANOVA on the same Bray-Curtis distance matrix showed that the fungal communities significantly varied at taxa level between land managements (PERMANOVA, p=0.002). A post-hoc PERMANOVA evidenced that each of the three land uses has a distinct fungal community showing that the inclusion of the three-year ley has substantially changed the arable soil fungal community (post-hoc PERMANOVA, p<0.05; Fig. 3.4).

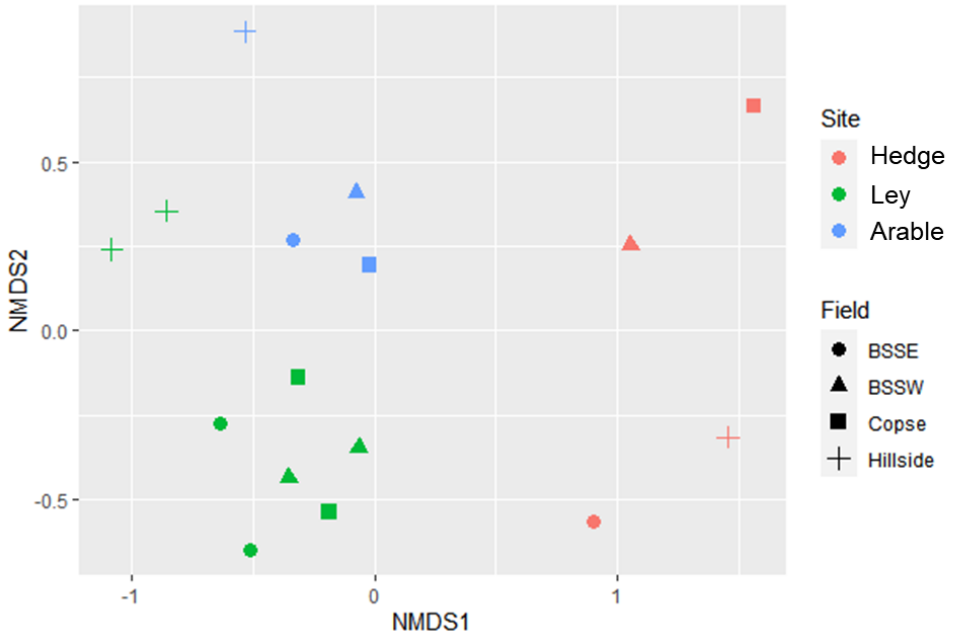

Fig. 3.3. An nMDS ordination on a Bray-Curtis similarity matrix to show differences in fungal OTUs between arable, ley and hedge land uses in four fields.

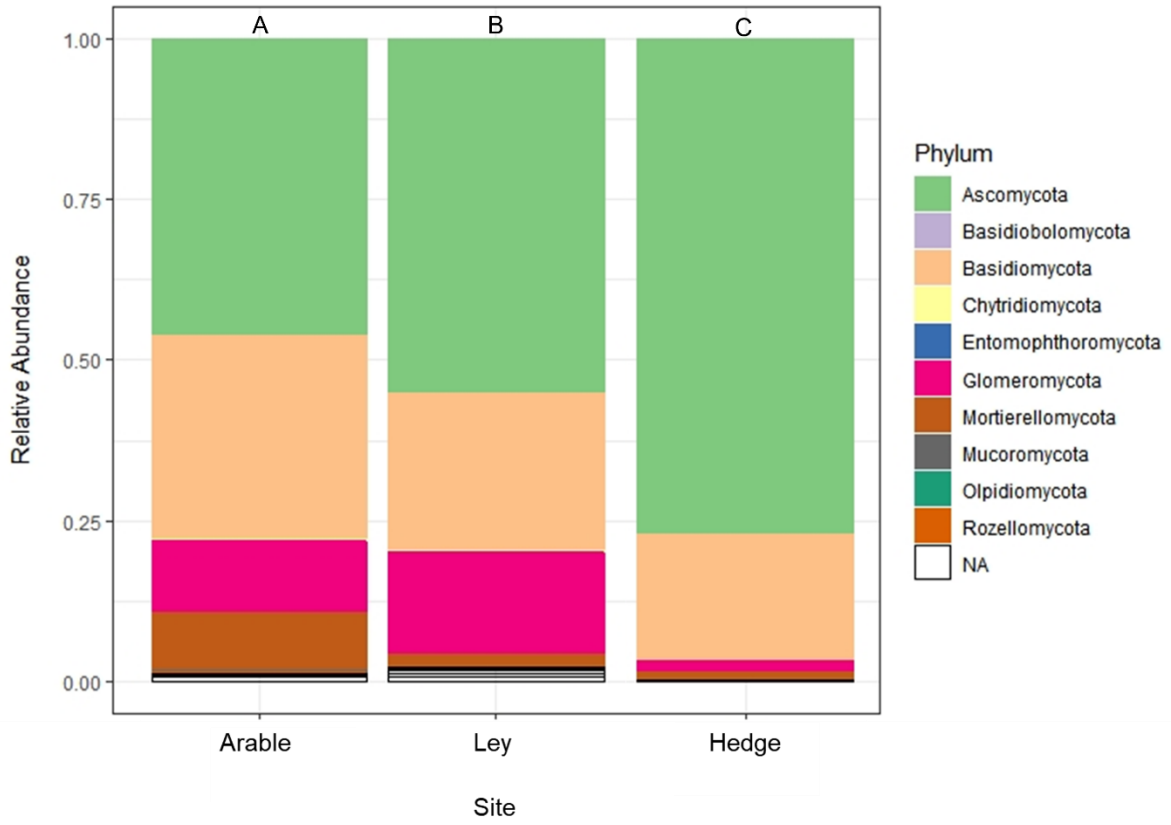

Fig. 3.4. The relative abundance of different fungal phyla in the hedge, ley and arable sites. Bars with different letters above show that communities differ with site at phylum level (PERMANOVA, p=0.002).

The PERMANOVA results can be visualised from the stacked bar chart of the fungal communities, comprising the relative abundance of phyla (Fig. 3.4). The four most prevalent fungal phyla are Ascomycota, Basidiomycota, Glomeromycota and Mortierellomycota. As soil becomes less disturbed along the arable, to ley, to hedge gradient, there is an increase in the relative abundance of Ascomycota, but a decreasing prevalence of Basidiomycota and Mortierellomycota. The one notable exception is the near doubling of relative abundance of Glomeromycota in the ley relative to the arable field (T-test, p=0.003), but very low relative abundance of this group in the hedge soils, paralleling the decline in Mortierellomycota from the arable to hedge.

The numbers of ASVs making up the relative abundance graph by phylum (Fig. 3.4) can be visualised in Table 2, alongside the number of OTUs in each phylum. The number of OTUs gives an idea of the numbers of individual species in each phylum, as OTUs are kept separate and not merged if they have less than 97% sequence similarity. As well as the relative abundance of Ascomycota increasing along the decreasing intensity gradient from arable to ley to hedge soils, the numbers of OTUs also increases from 203 to 283 to 314 species, respectively. However, a greater relative abundance does not always relate to a larger OTU number. For example, the decreasing prevalence of Basidiomycota along the

gradient of less tillage, along with decreasing ASV number, is not followed by OTU number, which is highest in the ley, then the arable soil, then the hedge.

Table 3.2. The total numbers of **a)** ASVs and **b)** OTUs detected in each fungal phylum. As there were double the number of ley samples, with the CAL and UAL ASV numbers are shown for both ley strips, and the average of them both in the merged ley treatment, enabling comparison to the arable and hedge treatments. OTUs observed in each treatment, identified as different species with less than 97% sequence similarity.

a)

Phylum	Arable	Ley	UAL	CAL	Hedge
	ASVs				
NA	2835	6202.5	7015	5390	796
Ascomycota	86693	120212.5	117592	122833	201134
Basidiobolomycota	3	0	0	0	0
Basidiomycota	56329	51254	54241	48267	45440
Chytridiomycota	327	99.5	103	96	341
Entomophthoromycota	0	0	0	0	26
Glomeromycota	21636	32275.5	27936	36615	4380
Mortierellomycota	18906	4375	2978	5772	2910
Mucoromycota	332	0	0	0	0
Olpidiomycota	10	0	0	0	0
Rozellomycota	12	15	30	0	0

b)

Phylum	Arable	Ley	UAL	CAL	Hedge
	OTUs				
NA	28	28	23	13	13
Ascomycota	203	283	218	203	314
Basidiobolomycota	1	0	0	0	0
Basidiomycota	95	106	81	74	82
Chytridiomycota	9	7	5	3	3
Entomophthoromycota	0	0	0	0	1
Glomeromycota	18	37	31	25	13
Mortierellomycota	20	17	15	13	12
Mucoromycota	2	0	0	0	0
Olpidiomycota	1	0	0	0	0
Rozellomycota	2	1	1	0	0

Due to the high interest in and importance of the Glomeromycota phylum, this was investigated at the genus level. A Bray-Curtis similarity matrix was constructed on the Glomeromycota phylum from the fungal dataset, from which an NMDS ordination was made (Fig. 3.5a). The sample from the hedgerow soil in Copse field was anomalous, possibly due to sampling under an atypical woody host plant such as elderberry *Sambucus nigra*, or *Frangula alnus* which occurred between the leys, and so this sample was removed to visualise variation between the other samples (Fig. 3.5b). The NMDS ordination shows that there were significant differences across the Glomeromycota Phylum between sites (p=0.001). Hedge, ley and arable site samples were variable within their group but with hedge samples being very distinct from the arable and ley soils. Arable and ley Glomeromycota are clustered together, but with ley samples tending to be closer to the hedge sites.

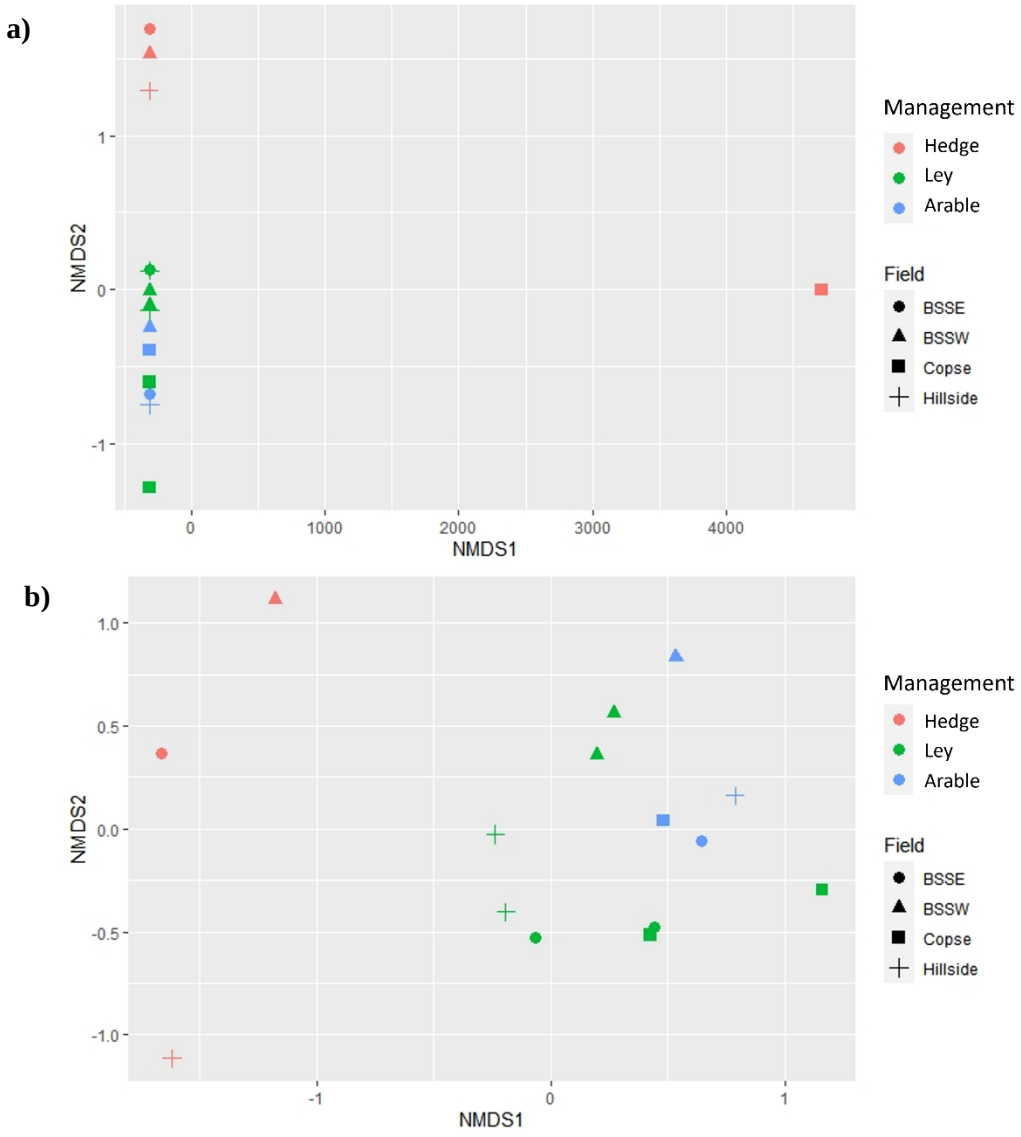

Fig. 3.5. An nMDS ordination on a Bray-Curtis similarity matrix on the Glomeromycota phylum within the fungal dataset to compare differences in OTUs between sites and fields **a)** before and **b)** after removing the anomalous hedge sample from Copse field.

The results of a pairwise PERMANOVA can be visualised in Fig. 3.6, showing that Glomeromycota communities significantly differed between ley and hedgerow soils (p=0.024), but arable soils did not differ significantly from either the ley (p=0.40) or hedgerow (p=0.07) soil communities. The Glomeromycota communities in the ley and arable soils are similar, but with more diversity in the ley soils, with 37 OTUs compared to 18 in the arable field (Table 3.2), and less dominance of particular genera in the ley site. Dominant genera in the arable soil decrease in relative abundance in the ley, with notable declines in *Ambispora, Archaeospora* and *Funneliformis*. Conversely, less relatively abundant genera in the arable soil tends to increase in relative abundance in the ley, most notably in *Glomus, Dominikia, Paraglomus, Septoglomus* and *Acaulospora*. These increases include genera that are much more abundant in the hedgerow soils than arable fields, including *Septoglomus, Glomus, Dominikia*, and the apparent gaining of the dominant genus of the hedgerow soils, *Claroideoglomus*. There is a clear distant community of Glomeromycota in the hedgerow soils, mainly made up of *Claroideoglomus*.

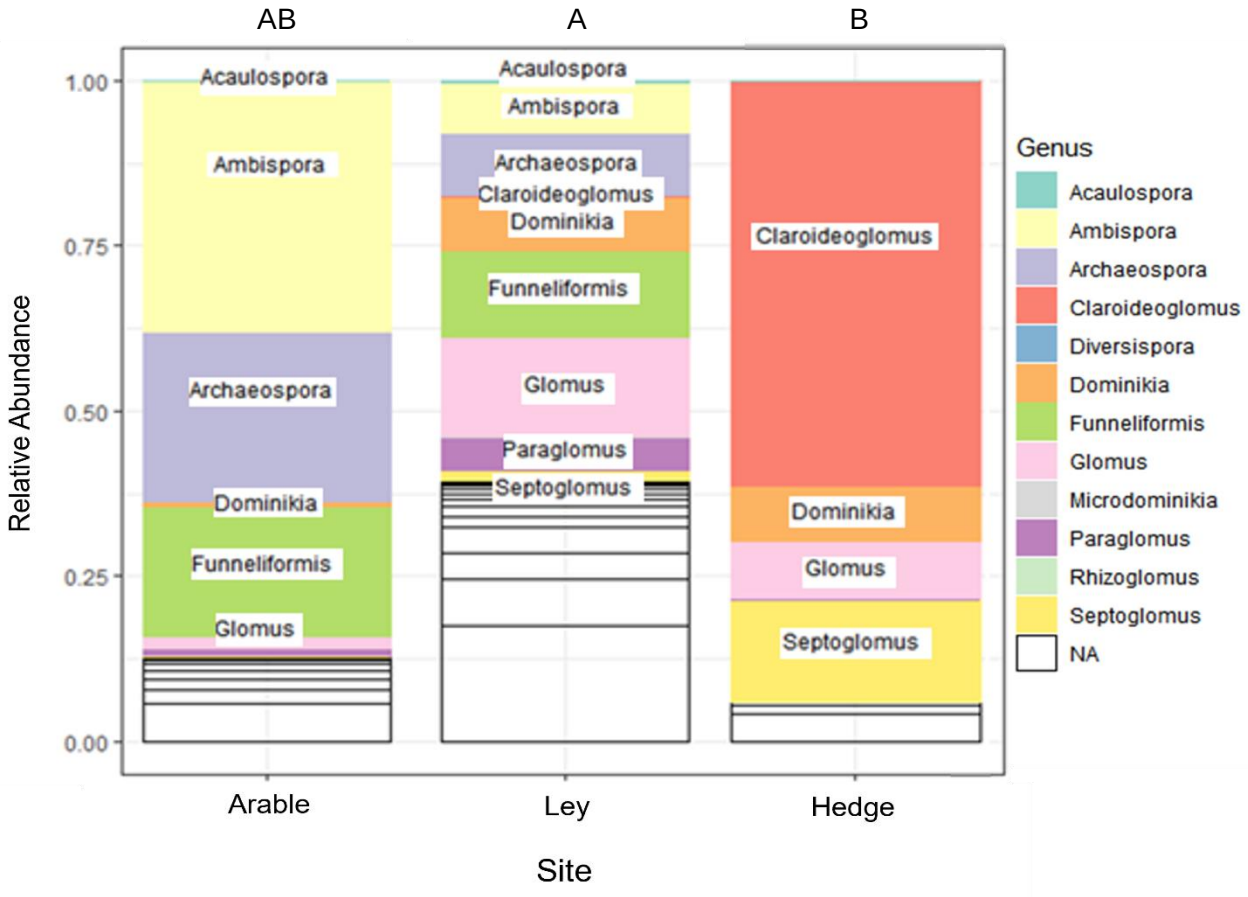

Fig. 3.6. A stacked bar graph showing the relative abundance of different fungal Genera within the phylum Glomeromycota between the hedge, ley and arable sites. Bars with different letters above show that communities vary with site at Genus level (pairwise PERMANOVA, p<0.05).

As a few of the OTUs in the Glomeromycota phylum were not able to be identified down to genus level using a BLAST search, these 'NA' OTUs were assigned numbers coding for different species alongside their smallest identifiable taxonomic rank. Different species were classified by less than 97% similarity, synonymous with the previous aggregations (see section *2.7.1 Fungal processing)*. The ley also increases the relative abundance of a wide range of non-identifiable genera, including 9 OTUs from the *Archaeosporaceae* family in the ley compared to 6 in the arable and similarly 5 OTUs from the *Glomeraceae* family in the ley compared to 2 in the arable soils. There were also 2 OTUs from the *Diversisporales* order in the ley which were unidentifiable to even family level, increasing from 1 in the arable.

The ley promotes diversity, decreasing, but not eliminating arable-dominating genera, whilst increasing the relative dominance of genera that have a greater relative abundance in the hedgerow soils, including the possible recruitment of hedgerow-dominant genera like *Claroideoglomus*.

3.3.2. Changes in Bacterial Communities

An NMDS constructed from the Bray-Curtis similarity matrix of the bacterial data was skewed heavily by the outlier in the Copse field at the Hedge site (Fig. 3.7a), which was also identified as an anomalous sample in the Glomeromycota NMDS plot. This overshadowed the rest of the data, making it difficult to see any associations between the other samples. Therefore, sample 16, "Copse Hedge" was removed as an anomaly from the dataset and a new NMDS plot created, showing that hedge, ley and arable communities were variable within their own management groups, with slight distinction of the hedge community from the ley and arable sites (Fig. 3.7b). A PERMANOVA on this new Bray-Curtis distance matrix evidenced that bacterial community does significantly vary at taxa level between the three types of land management (PERMANOVA, p=0.021), but a post-hoc pairwise PERMANOVA revealing no differences between bacterial communities between any management types (post-hoc PERMANOVA, p>0.05).

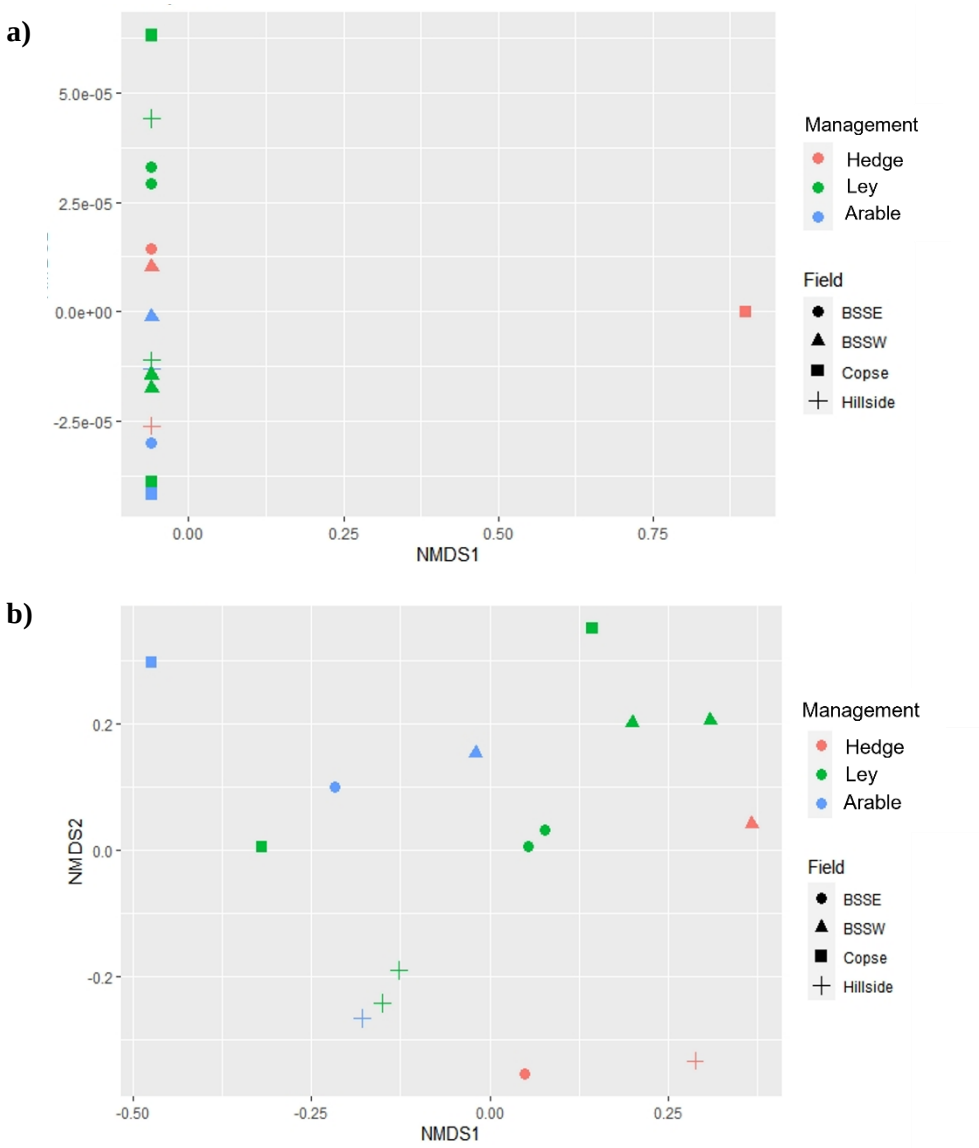

Fig. 3.7. An nMDS ordination on a Bray-Curtis similarity matrix to show differences between bacterial OTUs between sites and fields **a)** before and **b)** after removing the anomaly Copse Hedge.

The differences in bacterial communities can be visualised in a stacked bar chart (Fig. 3.8), showing the relative abundance of each bacterial phylum comprising the total bacterial communities. Proteobacteria are the most dominant bacterial phylum in the hedge, ley and arable bacterial communities, with Actinobacteria, Planctomycetes, Chloroflexi and Bacteroidetes being the other most prevalent phyla.

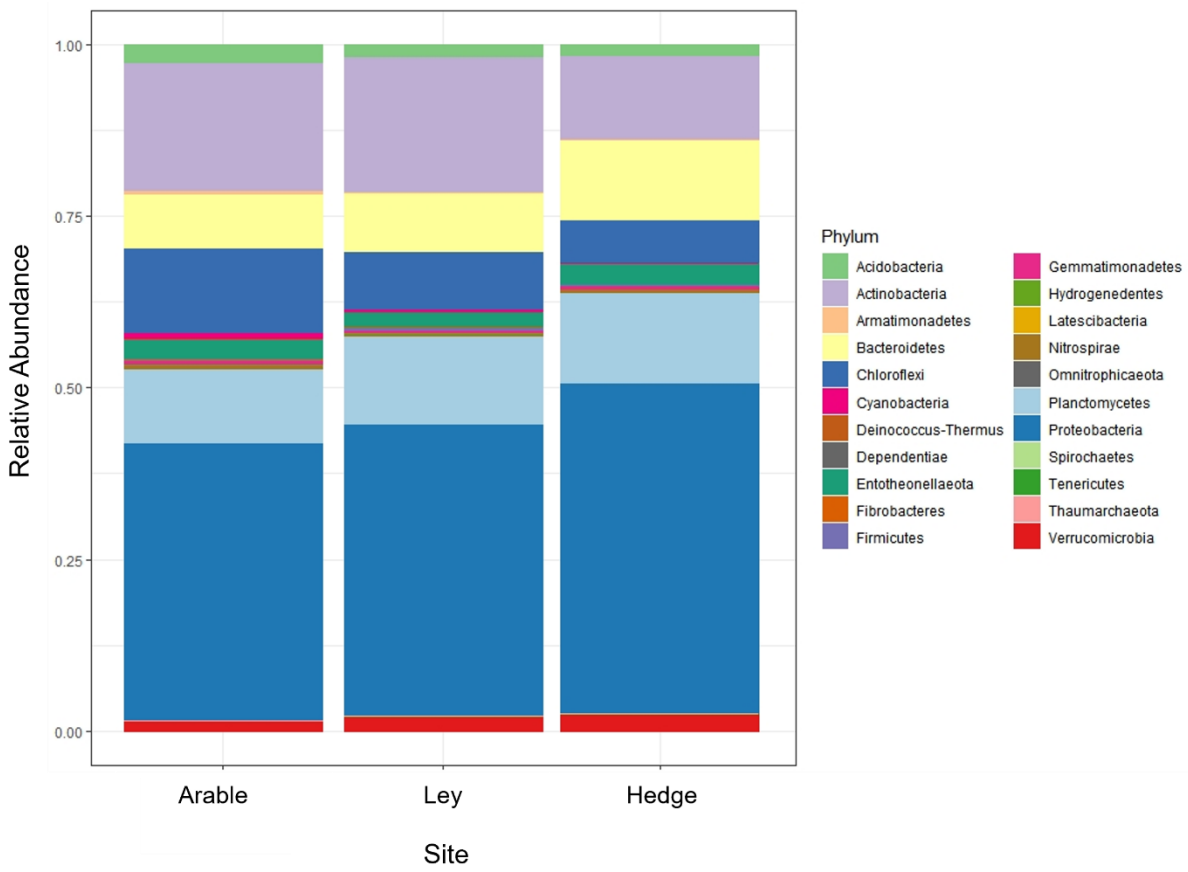

Fig. 3.8. A stacked bar graph showing the relative abundance of different bacterial Phyla between the arable, ley and hedge sites. There are no letters of significance above bars as a post-hoc pairwise PERMANOVA revealed no differences in bacterial community between any land management type (p<0.05).

After exploring each of these most abundant phyla at a more detailed taxonomic rank, little difference was seen between the bacterial communities of the arable and ley soils, numbers of which can be seen in Table 3. Therefore, further analyses of these data are not presented. Nevertheless, although not statistically significant, there are consistent trends with a reduction in disturbance, along the arable-to ley-to hedge gradient, with increasing relative proportions of Proteobacteria and decreasing proportions of Chloroflexi. Hedge soil seems distinct in some phyla, with large increases in Bacteroidetes and decreases in Actinobacteria compared to arable and ley bacterial communities.

Table 3.3. The total numbers of **a)** ASVs and **b)** OTUs detected in each bacterial phylum. As in the fungal ASV and OTU summary (Table 3.2), average ley ASV numbers are shown alongside the individual CAL and UAL management treatments. OTUs refer to number of species within each phylum observed within each management type, separated when sequences had less than 97% similarity.

a)

Phylum	Arable	Ley	UAL	CAL	Hedge
	ASVs				
Acidobacteria	2106	1528.5	1563	1494	1369
Actinobacteria	14996	16182.5	16446	15919	9801
Armatimonadetes	371	156.5	212	101	80
Bacteroidetes	6170	6932.5	6574	7291	15799
Chloroflexi	9794	6863	7040	6686	4743
Cyanobacteria	714	287	269	305	0
Deinococcus-Thermus	6	1.5	0	3	0
Dependentiae	22	9.5	11	8	0
Entotheonellaeota	2222	1683.5	1914	1453	2312
Fibrobacteres	140	109	103	115	109
Firmicutes	144	301.5	104	499	
Gemmatimonadetes	435	357.5	378	337	369
Hydrogenedentes	58	31.5	35	28	68
Latescibacteria	18	0	0	0	25
Nitrospirae	479	338	409	267	368
Omnitrophicaeota	0	1	0	2	3
Planctomycetes	8560	10291.5	10756	9827	8747
Proteobacteria	31952	34752	33554	35950	40015
Spirochaetes	1	2	0	4	0
Tenericutes	4	2.5	3	2	87
Thaumarchaeota	91	41	60	22	21
Verrucomicrobia	1087	1693.5	1573	1814	1777

b)

Phylum	Arable	Ley	UAL	CAL	Hedge
	OTUs				
Acidobacteria	36	41	34	33	28
Actinobacteria	125	173	143	154	128
Armatimonadetes	16	12	12	5	7
Bacteroidetes	155	206	154	172	168
Chloroflexi	142	148	128	130	84
Cyanobacteria	10	3	3	3	0
Deinococcus-Thermus	1	1	0	1	0
Dependentiae	4	4	2	2	0
Entotheonellaeota	9	10	9	8	6
Fibrobacteres	5	5	4	5	6
Firmicutes	8	13	7	8	2
Gemmatimonadetes	26	26	20	17	17
Hydrogenedentes	3	4	3	4	4
Latescibacteria	1	0	0	0	1
Nitrospirae	5	6	6	5	4
Omnitrophicaeota	0	1	0	1	1
Planctomycetes	355	517	420	429	335
Proteobacteria	411	554	456	483	444
Spirochaetes	1	1	0	1	0
Tenericutes	1	2	1	1	1
Thaumarchaeota	3	3	3	2	2
Verrucomicrobia	27	56	45	46	37

3.3.3. Pathogenic species

Data was searched for common pathogenic species in the UK of combinable crops (Defra, 2012), and also specific species described as arising as a direct result of changes in land management (Table 3.4). For example, the two pathogenic species root pathogens *Olpidium brassicae* and *Pyrenochaeta lycopersici* identified by Hilton et al. (2013) as arising as a result of growing oilseed rape as monocultures.

Table 3.4. The total numbers of ASVs of OTUs identified at taxonomic level as known pathogenic species. As in previous tables, the average ley ASV numbers are shown alongside the induvial CAL and UAL management treatments. OTUs refer to sequences with less than 97% similarity, assumed as different species.

Genus	Species	Common Name	Arable	Ley	UAL	CAL	Hedge
			ASVs				
Gaeumannomyces	*arxii*	Take-all	0	0	0	0	2
Botrytis	*cinerea*	Grey mould	16	0	0	0	52
Fusarium	*NA*	Seedling blight	54	3145	583	2562	0
Fusarium	*NA*		0	3792	3601	191	0
Fusarium	*armeniacum*		0	0	0	0	19
Fusarium	*domesticum*		54	66	27	39	4
Fusarium	*solani*		28	42	38	4	32
Pyrenochaeta	*NA*	Root rot	0	106	98	8	0
Pyrenochaeta	*NA*		0	0	0	0	48
Pyrenochaeta	*NA*		2539	6101	3324	2777	0
Pyrenochaeta	*inflorescentiae*		0	0	0	0	480
Pyrenochaetopsis	*decipiens*		696	240	133	107	151
Pyrenochaetopsis	*leptospora*	Found by Hilton et al. (2013)	0	32	11	21	91
Microdochium	*nivale*	Seedling blight	111	2978	2092	886	30
Ustilago	*hordei*	Loose smut	17	66	4	62	0
Oculimacula	*yallundae*	Eyespot	362	697	550	147	0

3.4. Discussion

Our findings provide insight into the active changes in soil fungal and bacterial community structure when grass-clover leys are incorporated into arable fields under CT, highlighting the changes that occur in the below-ground biodiversity as a direct result of incorporating leys into crop rotations. We show that fungal and bacterial communities respond differently to these changes, with fungal communities significantly changing at phylum-level, but bacterial communities not.

The introduction of a three-year grass-clover ley into arable fields under CT resulted in significant shifts in the fungal community structure characterised by relative abundance changes of several individual fungal groups (Fig 3.4), particularly in the *Ascomycota*, *Basidiomycota*, *Glomeromycota* and *Mortierellomycota* phyla. As seen in this study, the *Ascomycota* phylum is often the dominating fungal phylum in the community, and are known to respond positively in response to land restoration (Bastida et al., 2013), reflected by the increasing prevalence of this phylum with a decrease in disturbance along the arable, to ley, to hedge gradient. The prevalence of the *Mortierellomycota* phylum was markedly decreased in response to the introduction of a three-year ley, which is initially surprising as species in this phylum are known to be involved in the metabolism of SOC, which is known to be slightly greater in the ley soil (Chapter 1). However, previous literature by Cuartero et al. (2021) also found the highest abundance for *Mortierellomycota* in the conventional system farming system, compared to organic systems with compost and manure additions.

The relatively high prevalence of fungi in the *Glomeromycota* phylum, containing the ecologically and economically important AMF (Schüßler et al., 2001), found in the arable field is surprising as all arable fields were sown with non-mycorrhizal oilseed rape at the time of sampling. As these sequences were identified through RNA, this suggests the potential for AMF to have the ability to actively persist through periods of time without hosts, consistent with their known ability to tolerate abiotic stresses to an extent and can be a potential tool to restore degraded ecosystems (Lenoir et al., 2016).

However, a higher diversity is often easier to detect with lower dominance (Hughes, 2012), with a likely lack of active mycorrhiza in the arable soil in the sampling year reducing dominance and enabling the ability for detection of Glomeromycota. The RNA-based sampling method used could also be detecting propagules of Glomeromycota in the arable soils rather than solely active vegetative hyphae which is more likely to be prevalent in the ley with host plants that have the ability to form mutualistic associations. It is comprehendible that the sampling point contained AMF propagules, with enough nuclei in a single location to produce a detectable signal during sequencing after PCR, as they are known to survive through dry seasons (Liu et al., 2009). Obviously, many spores from the AMF community must survive crop rotations that contain non-host species or such species would eliminate mycorrhizas from arable fields. Although, this population is likely to still keep a lower diversity than that seen in the ley as it is being formed from a smaller gene pool. There is also the potential for mycorrhizal weeds to

be present in the arable field, although it is not recollected that this was an issue in these fields on the date of sampling.

Enhanced below-ground diversity is seen, particularly in the AMF after arable soil is converted to ley (Table. 3.2b). Enhanced soil biodiversity supports greater provision of soil ecosystem functions (Bender et al., 2016), which can in turn increase the yield of the subsequent crop (Deguchi et al., 2007). A better provision of soil ecosystem functions, such as enhanced water storage and drainage can also aid in maintaining resilience in yields despite climate change (Begum et al., 2019), which is likely to cause more frequent and extreme weather events (Groisman et al., 2005; Samaniego et al., 2018). Changes in AMF communities are known to be associated with changes in land use intensity between arable fields and grasslands (Oehl et al., 2010), with more AMF species and higher diversity in grasslands compared to arable fields, is likely due to the enhanced plant diversity in the ley sward and the presence of mycorrhizal plant species to form active associations. In particular, Oehl et al. (2010) observed *Glomus mosseae* in higher abundance in arable fields compared to grasslands in Cambisols, which is a similar soil type to in this study. This is comparable to the 2466 OTUS in the arable soil compared to 42 in the ley soil of the same species. In contrast, Oehl et al. (2010) found that species like *Glomus aureum* and *Glomus macrocarpum* were more abundant in the grassland compared to arable fields, the latter of which was only detected in the ley fields in this study, but with only 21 OTUs.

Perennial ryegrass and white clover, both of which are present in these leys, are known to host different AMF species, with *Glomus geosporum* showing preference to infect clover roots over grass (Zhu et al., 2000). Although, this species was not present in this study to compare. Presence of the *Funneliformis* genus is important to note, due to its ability to enhance plant growth (Ortas and Ustuner, 2014) and cause a negative effect on the growth of the root rot pathogen *Fusarium oxysporum* (Qian et al., 2015), although this pathogen was not present in this dataset. An enhanced AMF diversity could also be beneficial for the earlier and greater colonisation of mycorrhizal crop roots in the following arable rotation, providing diversity in functional benefits (Verbruggen and Kiers, 2010) and associated benefits to host crops including enhanced yields through their mutualistic symbioses (Smith and Read, 2010).

The fungal dataset was searched for common soilborne pathogens which are the cause of the most common crop diseases in the UK (Defra, 2012). The only presence of take-all disease, one of the most devastating root diseases, was two ASV replicates of the species *Gaeumannomyces arxii* in the hedgerow soils (Table 4), which was identified as a new take-all species by Hernández-Restrepo et al. (2016). The main victim of take-all disease is wheat, but some varieties and certain soil fungi have shown to successfully reduce the soil inoculum (Deacon, 1976; McMillan et al., 2018). However, since there are no ASVs present of any *G. graminis* fungus in either the arable or ley soils, we cannot comment on the biological control of take-all by grass-clover leys from this study. There is also little difference or even a greater presence of other fungal pathogenic ASVs in the ley compared to the arable soil, for

example in the five *Fusarium* species that are identified in these soils. This is surprising given the background research on potential disease suppressive abilities of soils under ley, which are known to support the recovery of both microbial and earthworm populations (Briones and Schmidt, 2017; van Capelle et al., 2012)which are successful at combatting soilborne diseases (Jorge-Escudero et al., 2021; Lagerlöf et al., 2020; Peralta et al., 2018), including *Fusarium*-specific species (Plaas et al., 2019).

In contrast to the fungal communities, the introduction of a three-year ley into arable soil had no significant effect on the soil bacterial community, which has previously been seen to be less affected by changes in crop rotation compared to the fungal community (Hilton et al., 2013; Navarro-Noya et al., 2013; Peixoto et al., 2006; B. Zhang et al., 2014). Chen et al., (2020) also found that the introduction of leys into crop rotations did not increase microbial diversity or richness. Research by Peralta et al., (2018) evidenced that even the highest diversity crop rotation does not produce the largest soil bacterial diversity, even 4% lower than that found in monoculture maize. However, the introduction of legumes into rotations has previously been seen to cause shifts in bacterial community structure (Alvey et al., 2003). A study by Hirsch et al. (2009) suggested that interpreting bacterial diversity in soils bears limited relevance to functionality, as a 50-year bare-fallow section of field supports an active and species-rich bacterial community of similar diversity to that in a field maintained as a grass sward, despite vastly different organic C inputs. In previous studies exploring soil bacterial communities also using the 16S rRNA hypervariable regions for sequencing, bacterial communities across all samples were also dominated by *Proteobacteria* and *Chloroflexi*, but also *Acidobacteria* at Phylum level (Zhao et al., 2014). Zhao et al. (2014) found that bacterial community significantly varies by differing fertiliser regimes (without fertiliser and NPK fertiliser with manure and differing amounts of crop straw) and seasonal changes.

Another point to note from this study is that hedgerow soil is not the refugia for below-ground biodiversity that we might previously expect. Despite the much greater soil structure and quality in hedge soils compared to that found in the field (Chapter 2), hedge soil had much lower diversity and evenness in microbial community structure compared to arable and ley soils, with a tendency to be dominated by fewer species. For example, the *Glomeromycota* phylum in the hedge is mainly dominated by *Claroideoglomus,* which is mainly comprised of the species *C. claroideum,* compared to the ley which is very diverse in genera. *C. claroideum* is a major species previously found in forests and grasslands with an low levels of disturbance (Öpik et al., 2009; Stover et al., 2012; Velázquez and Cabello, 2011; Zangaro et al., 2013).

A potential explanation for the changes in microbial diversity we see here is the intermediate disturbance hypothesis (Osman, 2015), with the arable field experiencing high rates of disturbance due to CT, and rotating crops, keeping the community in an early stage of development. In contrast, the hedge soil experiences very little disturbance in terms of mechanical disturbance, allowing competitive

exclusion for high amounts of resources, i.e., organic matter due to increased organic inputs. However, the hedgerow soils may experience more extreme drying and more wetting-drying cycles compared to field soils. The introduction of a ley into an arable rotation allows for a pause in mechanical disturbance through a period of no tillage and increased organic inputs, allowing the recovery of species than cannot overcome the obstacles faced in a conventional arable environment to multiply. It is difficult to test this hypotheses on microbial diversity in natural soil systems due to the amount of confounding factors involved (Bradley and Martiny, 2007), however some studies have found that intermediate levels of disturbance relate to higher bacterial diversity (Bruce et al., 1995; Walsh et al., 2005). Another potential explanation is, if mainly spore-forming fungi are being identified, these spore communities can be very different to those identified in the roots by DNA approaches, and this difference is more pronounced in undisturbed sites (Helgason et al., 2002, 1999).

Maintaining a better ecological balance by reducing disturbance of the arable soil system, through reducing tillage and improving cropping diversity alongside the introduction of leys containing a mixture of plant species, can supply a broad range of potential hosts to form symbioses with a variety of fungi and enhance the diversity of corresponding benefits they can provide. A simultaneous incorporation of these cover crops and into NT system seems to be the most beneficial method for enhancing AMF spore density and species richness (Säle et al., 2015), which can in turn provide benefits such as increased nitrogen use efficiency and subsequently reducing the need for N fertiliser inputs (Verzeaux et al., 2017).

3.5. Conclusions

We found that the introduction of a three-year grass-clover ley intro conventionally tilled, arable fields had a greater effect on the soil fungal community and did not significantly affect the soil bacterial community. The diversity changes in the *Glomeromycota* community were of particular interest, with the ley providing a much higher *Glomeromycota* diversity, comprising 37 different OTUs compared to 18 in the arable field. Soil under hedgerows was not a hotspot for mycorrhizal fungal diversity, with an intermediate disturbance hypothesis potentially explaining the changes in diversity with a change in land management intensity. Fungal diversity, particularly in the *Glomeromycota* group, could serve as an indicator to assess biological soil quality, however, future studies need to explore the impact of changes in these specific fungal groups on changes in functional diversity, and how this can impact important ecosystem services, including impacts on crop growth, soil carbon storage and nutrient availability that we rely on in agricultural soils.

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Chapter 4

4. Changes in the soil metabolome from long-term arable cropping to three-year leys, comparing to hedgerows

Abstract

Soils underpin the provision of many ecosystem services for food production and human well-being. The formation and stabilisation of large soil aggregates are an important indicator of good soil structure and quality, relating to better soil functionality, including better water storage and drainage capabilities and greater sequestration and protection of organic carbon (OC). However, conventional agricultural land management practices are known to be detrimental to soil structure due to physical breakage of soil aggregates, oxidation of soil OC, and the disturbance of aggregating agents, including earthworms, arbuscular mycorrhizal fungi (AMF) and roots. The inclusion of grass-clover leys into arable rotations is a promising regenerative practice to restore soil structure and quality after decades of arable cropping through the restoration of aggregating agents. However, the exact molecular mechanisms behind their promotion of macroaggregation is uncertain. From previous research, it is known that a variety of organic molecules, including lipids, polysaccharides and phenolic molecules are involved in both the formation and stabilisation of these important soil structures, originating from plant, microbial and bacterial sources. The presence of certain lipids can also be related to specific functional groups of AMF, an important symbiont in crop production due to their ability to enhance soil aggregation, plant nutrition, growth and health. Organic molecules can be detected through multiple methods, but one of the most novel and accurate methods, mass spectrometry, which identifies changes in the soil metabolome through the accurate identification of metabolites through molecular weights, has not previously been used to research the changes in the soil metabolome after arable soil has been put under ley. In this study, we develop and apply a novel approach, using high-throughput matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) of lipidic soil extracts to examine the effects of introducing a three-year ley into annually cropped arable fields on Cambisol soil on the soil metabolome. After the incorporation of the grass-clover ley, there were clear differences in metabolites through the upregulation of m/z bins 403.2 and 439 in the ley compared to arable soils. These mass bins contain masses that correspond to biosurfactants, lignans and flavonoid pathways, but also the presence of herbicides after three years of no direct application. This study gives a starting point for future, more targeted metabolic research into changes in the soil metabolome under changes in land management, providing an insight in the individual organic molecules potentially associated with increased macroaggregation and OC storage which is seen after leys are incorporated into arable rotations.

4.1. Introduction

Maintaining healthy and productive soils is key to achieve global food security. Interactions between plants and soil microbes play a major role in the formation and stabilisation of soil macroaggregates (>250 μm), which are a key component of soil structure and quality (Churchman, 2010) due to their involvement in carbon (C) sequestration (Jastrow, 1996; Six et al., 1998; Stewart et al., 2009, 2008). Alongside plant root and soil microorganism involvement in soil aggregation, organic molecules are also known to be an interacting factor in this process as binding agents (Tisdall and Oades, 1982). The stability of soil aggregates is highly dependent on the amount of organic matter (OM) content, organic molecules including microbial-derived polysaccharides (Haynes and Francis, 1993) and hydrophobic molecules like humic substances (Taylor et al., 1999) and lipids (Dinel et al., 1991).

Polysaccharides materials exuded by both roots and fungal hyphae have gluing and bonding roles in the process of soil aggregate formation, due to their gel-like mucilaginous consistency (Haynes and Beare, 1997). Basidiomycete fungus excretes extracellular mucilages that are rich in polysaccharides and enhance soil aggregation (Caesar-Tonthat, 2002). Extracellular polysaccharides can be formed when other aggregating agents, like particulate organic matter (POM; Bronick and Lal, 2005), are decomposed by microorganisms (Jastrow, 1996). Polysaccharides adsorb strongly onto mineral surfaces (Kay, 2018; Martens, 2000), but are readily mineralisable. For this reason, they are considered transient binding agents, involved in the initiation steps of aggregation but not the long-term stability.

Phenolic molecules, such as phenolic acids, also have a similar role in the early stages of aggregate formation through the formation of complexes with cations (Martens, 2000). Phenolic molecules can originate from the crop plant residues in rotation, as crops with higher phenol content in residues have greater aggregation (Martens, 2000). Plant residues causing an increase in soil phenolic acid concentration, which was highly correlated to an increase in mean weight diameter of soil aggregates (Martens, 2002).

Hydrophobic molecules increase water repellency of soil particles, reducing slaking and dispersion, leading to less soil organic carbon (SOC) loss through reduced aggregate breakdown and the protection of SOC from microbial decomposition (Balesdent et al., 2000; Plante and McGill, 2002a). Lipids are hydrophobic in nature, likely causing their important role in aggregate stability and slaking resistance (Paré et al., 1999). Aggregate stability can therefore be enhanced by the addition of organic materials containing these hydrophobic substances. For example, the addition of urban organic wastes, such as sewage sludge, which contain lipids, making their addition to soils a method of enhancing aggregate stability (Annabi et al., 2007). SOC from certain plant types are also rich in hydrophobic materials, which can enhance aggregate stability through being planted in the soil rather than being added as compost (Ternan et al., 1996). The analysis of soil lipids is also informative as they are biomarkers of

certain functional groups of fungi, including the identification of trends in arbuscular mycorrhizal fungi (AMF) in response to changes in land management.

Organic molecules can be identified and quantified through a variety of approaches, which are summarised in Table 1.1. These include a variety of assays and extractions, including the hot water extraction for carbohydrates (Puget et al., 2000) and the extraction of Glomalin-related soil proteins (GRSP) by Bradford assay (Purin and Rillig, 2007). However, these methods are not always specific, with proteins extracted by Bradford assay also co-extracting proteins not of arbuscular mycorrhizal fungi (AMF) origin (Rillig, 2004).

Traditional culturing techniques to analyse soil microbial communities only allows a very small proportion (<0.1%) of the community to be identified, a problem which is overcome by genomic approaches (Hill et al., 2000; Schloss and Handelsman, 2005). Phospholipid fatty analysis (PLFA) is particularly effective at determining the abundance of particular microbial groups, with fatty acids being the most abundant class of all soil lipids (Weete, 1976). PLFA was first developed by Frostegård et al. (1993), and research using this method has since provided evidence that arbuscular mycorrhizal fungi (AMF) play an valuable role in the early stages of soil formation compared to bacteria and other soil fungi (Welc et al., 2012). However, although the PLFA method is a rapid means of detecting changes in the soil microbial community, it must be used with caution, as some functional markers can overlap between different soil organisms, for example between AMF and bacteria (Frostegård et al., 2011).

More modern and accurate methods include the use of specialist mass-spectrometry equipment that can be used for both stable isotope (^{13}C and ^{15}N) analysis and high throughput lipid analysis using liquid chromatography and gas chromatography/mass spectrometry (GC/MS; Jandl et al., 2004). With mass spectrometry, thousands of organic compounds can be identified simultaneously through the accurate measurement of molecular mass of individual molecules (Dittmar and Paeng, 2009). Another ultra-high-resolution form of mass spectrometry, matrix-assisted laser desorption ionization (MALDI-MS) can be applied to soils (Schmidt et al., 2011). This approach can also be described as ‘metabolomics’ due to its measurement of metabolites (compounds of small molecular weight) which make up the metabolome (Dunn et al., 2005). By combining metabolomic approaches with imaging, these technologies can resolve spatial changes in microbial community structure, the presence of organic molecules and biological processes which are influenced by changes in land management at the nanometre scale (Amstalden van Hove et al., 2010; Herrmann et al., 2007). One of the particular strengths of the MALDI sampling approach is that this can be applied to thin sections of plant tissues (Burrell et al., 2006), and in the future could potentially be extended to use of MALDI-MS imaging (Walker, 2021) on soil aggregates after flash-freezing and cryosectioning to determine spatial mapping of metabolites within them.

Despite the importance of promoting soil aggregate formation and stability, conventional intensive arable cropping, often involving short profitable cereal crop rotations established by ploughing and harrowing (Townsend et al., 2016; Wezel et al., 2014), reduces the abundance of soil macroaggregates through the breakdown of many aggregate binding agents (Tisdall and Oades 1982; Portella et al., 2012; Rillig et al., 2015). This has a consequential result of limiting crop yields, which are limited enhanced drought and flood events and soil erosion, which are exacerbated by a breakdown in soil structure (Blankinship et al., 2016).

Alternative tillage practices, for example conservation tillage and no-till practices, are less damaging to soil biota, enhancing the abundance and diversity of earthworms (Edwards and Lofty, 1982) and mycorrhizal fungi which are both involved in soil aggregation (Johan Six et al., 2002) and C storage (Asmelash et al., 2016; Fernandez and Kennedy, 2015; Wilson et al., 2009; Zhang et al., 2013). PLFA analysis by Helgason et al. (2010) revealed that AMF are particularly affected by tillage, showing the greatest recovery of 40-60% after converting from conventional tillage to no-till practices, compared to communities of bacteria and non-AMF fungi.

Recent studies have suggested that plant roots and associated microbiota may play a particularly dominant role in affecting the soil metabolome in grassland soils (Liu et al., 2020), and for grassland soils in North Wales, land use was amongst a number of environmental variables associated with significant variation in soil metabolomes (Withers et al., 2020). In addition, non-targeted metabolomics studies of the effects of herbicide additions to soils have revealed the rates of metabolism of the added compounds, and potential biomarkers of their impacts on soil microbiomes (Patil et al., 2016). These studies provide a strong conceptual foundation for investigating the effects on soil microbiomes of introducing grass-clover leys into arable rotations. We would expect the changes in land use, ceasing tillage and fertiliser inputs, establishment of perennial root systems, the increase in earthworm populations, and shifts in microbial communities, especially fungi (Chapter 3), and soil aggregation involved in sequestering organic C (Chapter 2), to be reflected in changes in soil metabolites. However, to our knowledge, there is no published research on the impact of introducing grass-clover leys into arable rotations on the soil metabolome. Proof of concept research led by an undergraduate masters student that underpinned the rational for the research presented in this thesis, involved trial metabolomics analysis on the lipid fraction of soil from some of the same arable fields at Leeds University Farm as in the present study, and also from the adjacent deciduous woodland (Crabtree, 2014). Focussing on the extractable lipids in water-stable aggregates of different sizes, Crabtree, 2014) found depletion of a compound with mass-to-charge ratio (m/z) 184 in all field aggregates compared to those in the woodland. This mass is associated with a phospholipid head group (Milne et al., 2003), and interpreted as likely reflecting lower microbial biomass in the arable than woodland soil aggregates, since phospholipids are likely to be most abundant in living organisms rather than in soil residues, but it is possible they are involved in abiotic organo-mineral interactions.

Following on from this previous work, the present study employed metabolomic fingerprinting approaches to determine how the introduction of a three-year grass-clover ley sown into a conventionally ploughed and cropped arable rotation, described in Chapters 2 and 3, impacts the soil metabolome. As there is no previous research on the impact of introducing leys into arable rotations on the soil metabolome, this study uses an untargeted approach as an initial scan to identify any changes in the soil metabolome under changes in land management. This study aims to identify any change in m/z bins under arable-to-ley conversion which can act as a starting point for any targeted future research in this area. Due to the well-known importance of hydrophobic lipid molecules involved in the process of soil aggregation, we will focus on the lipophilic fraction extracted from the soils to target these molecules. Therefore, we do not expect to see non-lipophilic molecules, such as phenolic acids and carbohydrates, which also play a role in soil aggregation and stability.

Together, along with Chapter 2 and Chapter 3, these three chapters will work together to aim to resolve the co-dependence of root-associated soil microbial communities and metabolites in increasing macroaggregate formation and soil carbon storage. This chapter is a follow-on from chapter 3, which investigates changes in soil microbial communities from arable to three-year ley, to determine the mechanistic basis behind their association with increased proportions of soil macroaggregates and the storage of organic carbon (OC) within them.

4.2. Materials and Methods

4.2.1. Field site and experimental design

The SoilBioHedge experimental set up is described in greater detail in experimental Chapter 2, but in brief, paired grass-clover ley strips (3 m wide, 70 m long) were sown into four conventionally ploughed, arable fields at the University of Leeds Farm, Tadcaster, UK (53°52'25.2"N 1°19'47.0"W) in May 2015 (Fig. 4.1). The seed mix for the ley comprised both diploid and tetraploid *Lolium perenne* (20% and 16%, respectively), *Festulolium* species (16%), two varieties of tetraploid *Lolium x boucheanum* (12% and 16%), *Trifolium repens* (5%) and *T. pratense* (15%), sown at a rate of 4.2 g m^{-2}. The soil type in each field was a Calcaric Endoleptic Cambisol (WRB, 2014), but differed slightly in textural type between fields (Hallam et al., 2020), with BSSE and BSSW being silt loams and Copse and Hillside being loam and sandy loam, respectively.

Fig. 4.1. Arial view of the experimental set-up at Leeds University farm, showing the four fields (Copse, Hillside, BSSE and BSSW with 70 m-long ley strips visible in each of them. Picture from Google Earth, taken 17/07/2017.

The ley strip design allowed the comparison of changes in the soil metabolome between land which had been under long-term arable cultivation compared to if the whole field had been under ley, which is the case when they are incorporated into crop rotations. One of each paired ley strip was connected to the hedgerow (CAL – Connected Arable Ley) and one was disconnected by a 2 m wide fallow area and a

90 m deep stainless steel mesh barrier set vertically into the soil. This design was from the original SoilBioHedge experiment to prevent the migration of earthworms and mycorrhizal fungi from the hedgerow and field margin into the ley strips (Berdeni et al., 2021).

4.2.2. Soil sampling

Samples were taken as soil cores (5 cm deep and 100^3 volume) from the top 7 cm of soil in July 2018, when they leys were three years and two months (38 months) old. Samples were taken from 35 m down the CAL and UAL strips, at the same distance in the arable, ploughed area between the two ley strips and from the soil beneath the hedgerow between the ley strips, as shown by the open red circles in Fig. 4.2. This sampling strategy was replicated within the four fields: BSSE, BSSW, Copse and Hillside. Soil from each bulk density core was emptied from the sampling ring, carefully homogenised and stored at -80°C as soon as possible after sample collection to prevent any change or degradation in the soil metabolome.

4.2.3. Soil lipid extraction

The lipophilic and aqueous metabolites were extracted from the soil samples using a method adapted from Frostegård et al. (1991). Soil samples were defrosted and passed through a 2 mm sieve to remove stones, roots and homogenise the soil along natural lines of weakness. Subsamples of 2 g for each sample were weighed into 50 ml Pyrex screw-top test tubes and kept on ice. A 15 ml biphasic methanol:chloroform:UHP (Ultra High Purity)-water mixture at a ratio of 2:1:0.8 was added to each tube and shaken continuously for 30 minutes for lipid extraction. The samples were then spun in a

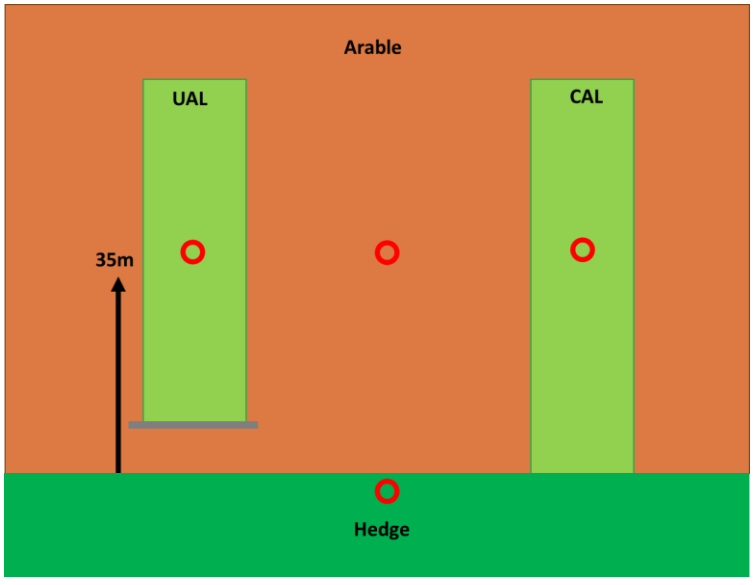

Fig. 4.2. The experimental design of two paired grass-clover ley strips, one Connected Arable Ley (CAL) and one Unconnected Arable Ley (UAL). Open red circles show the sampling locations in each field.

centrifuge at 2,500 rpm for 15 minutes at 4°C, then 1.2 ml of the supernatant was transferred to a 2 ml Eppendorf tube with 320 µl chloroform and 256 µl UHP water to achieve a final solvent ratio of 2:2:1.8. The samples were spun in a centrifuge at 14,000 rpm for 15 minutes at 4°C to obtain two distinct phases: the upper, aqueous phase and the lower, organic phase containing lipids. Each phase was transferred to a separate Eppendorf tube and stored at -80°C ready for analysis.

4.2.4. Matrix-Assisted Laser Desorption/Ionisation Mass Spectrometry Analysis (MALDI-MS) of soil metabolites

The lower organic, lipophilic phase of the biphasic extraction was diluted 1:10 with pure methanol. The lipid phase was analysed on a Waters MALDI SYNAPT G2-MS in positive ionisation mode, to determine how well the extraction and dilution worked with this method. *Alpha*-Cyano-4-hydroxycinnamic acid (5 mg/ml) was used as a matrix for the positive mode, due to its ability to allow the sample to be readily ionised through donation of protons (Burrell et al., 2006). Three 2 µl repeats of each sample/matrix mixture were pipetted onto a 96-sample target plate and allowed to crystallise, by volatilizing the extraction solvent on a warmed hot-plate. Samples were ionised using a MALDI ion source with the laser moving in a spiral pattern concentrated on each sample spot for one minute. Each sample was analysed in triplicate to create technical replicate peaks which were averaged. Further instrument parameters are shown in Table 4.1.

Table 4.1. MALDI SYNAPT G2-MS set up for metabolite extraction.

Instrument Parameter	***Value***
Capillary (kV)	3.00
Sampling Cone (kV)	40
Extraction Cone (kV)	5.00
Source Temperature (°C)	80
Desolvation Gas (L/h)	500
Mass Range (Da)	50-1200
Scan Time (sec)	1.00

4.2.5. Data Processing

The recorded mass spectrum data were centroided and converted into text files for transferring into a Microsoft Excel spreadsheet via an in-house Visual Basic macro. The three technical replicate analyses were combined to calculate mean masses and create the metabolite profile for each sample. The Excel macro also calculated the metabolite masses and total ion counts (TIC) as a percentage using equations by Overy et al. (2005). Calculation of masses and TIC (given as percentage) for each replicate were calculated from the following equations:

"ES+, y <0.00003x+0.0033

ES-, y <0.00003x+0.0044" (Overy et al., 2005)

This took any likely false positives and negatives out of the raw data set by setting a minimum acceptance level of TIC for the peak to be included. A mass unit bin size of 0.2 Da was chosen for the TIC, inside which a peak must appear in all three technical replicates to be included in the final data sheet, further reducing the likeliness of false positives.

The data processing work through resulted in one spectrum per sample with several discrete peaks in each bin. The abundance of the masses present in each bin in each sample can be quantified by the TIC value, which can subsequently be analysed and compared between samples via a t-test.

4.2.6. Data Analysis

An unsupervised Principal Component Analysis (PCA) was conducted using the multivariate analysis software "SIMCA" (Umetrics®) to establish any patterns and differences in the soil metabolomes as a result of different managements (Arable, Ley and Hedge). This unsupervised PCA analysis is a useful analytical tool to determine variation in a dataset whilst reducing dimensionality of the data without site-specific information (Ringnér, 2008). Three pairwise analyses were conducted between arable and each of the two types of ley samples, and between these ley samples. This compared arable and UAL; arable and CAL, and CAL versus UAL to determine main differences between them. Our other studies have shown little evidence for functional differences between the ley strips connected to the field margin and hedges, and those that were unconnected by a barrier and fallow zone. However, it was nonetheless of interest to determine if the arrangements designed to prevent the movement of fungi and earthworms from the hedgerow into the ley has resulted in any differences in the metabolome.

To further interrogate any patterns seen in the PCA, a supervised multivariate analysis was conducted using Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) for pairwise multivariate analysis and O2PLS-DA, which contains an extra component to allow for three and four-way analysis. This supervised analysis gives the model information about the samples, therefore allowing the model to look for similarities and differences between specific groups of samples. Again, three pairwise comparisons were carried out between the paired sites described earlier.

The outcome of this analysis provided a list of mass-to-charge ratio (m/z) bins which are responsible for causing differences between the two treatments being compared. They are ranked in order of the proportion of the total variance they explain between the two treatments. This gives us an idea of the potential molecules which are causing differences in the metabolome between the arable and ley soil.

The top ten ranked mass bins causing the separation between sample groups were selected for putative metabolite identification of metabolites. The detected masses were searched in the public online metabolite database METLIN (Scripps) using the 'simple search' feature. In positive mode, accurate masses were searched through the subtraction of masses of adduct ions +H, +Na and +K. Putatively identified compounds with an error margin in the m/z values of less than 30 ppm were recorded into a

table. Important information including detected and accurate mass, adduct ion, ppm error margin, metabolite mass, name and their related kingdoms, chemical groups and pathways were recorded.

A t-test was used to determine if the changes in land management from arable to ley had caused a significant change in the percentage ion counts of the top ten mass bins causing the statistical separation in metabolome between the two sample groups.

4.3. Results

4.3.1. Unsupervised Analysis of metabolomic fingerprints by Principal Component Analysis

A PCA comparing the metabolomic profiles for all samples and treatments revealed variation between all management types (Fig. 4.3a). This PCA shows clustering of three out of the four hedge samples, grouping and some separation in multivariate space between arable and ley treatments. Interestingly, there is a particular grouping of the four management treatments within the field BSSW, implying that the metabolomic fingerprint in the soil in this field is distinct from the others sampled.

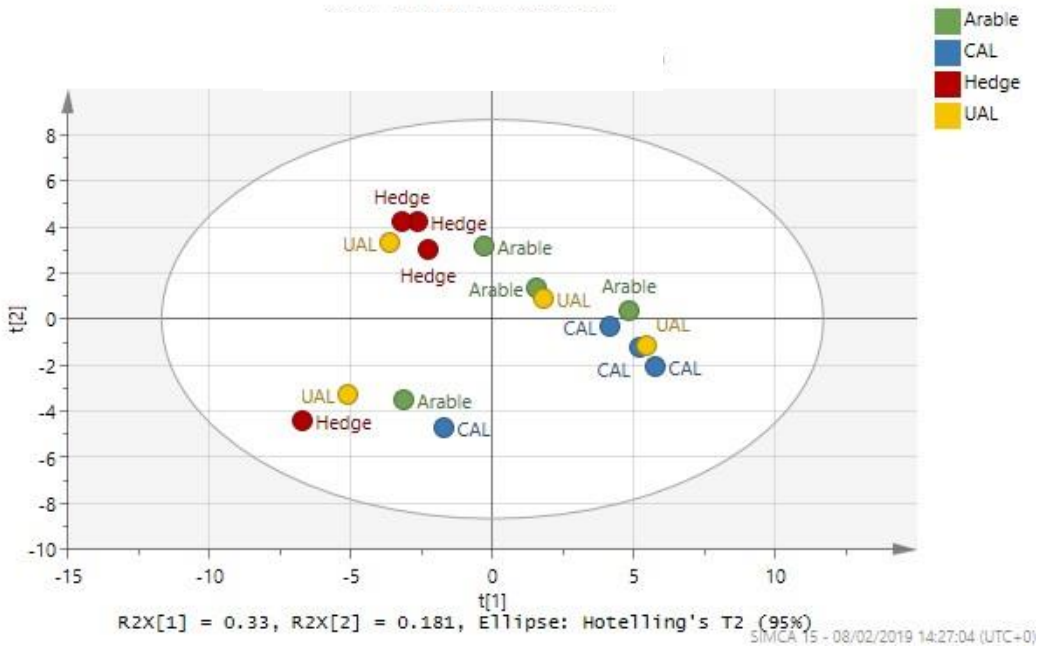

Fig. 4.3. An unsupervised multivariate PCA analysis of the metabolite profiles of Arable, CAL, UAL and Hedge management treatments. Metabolite profiles were of the organic, lipophilic layer analysed in positive ionisation mode.

Focusing solely on the Arable and CAL management treatments of highest interest, a clear separation can be seen between these two distinctly managed soils (Fig. 4.4). The large differences between the arable and CAL treatment justifies further interrogation of these metabolomes using a supervised orthogonal partial least-square discrimination analysis (OPLS-DA), to determine whether the

differences in soil management are potentially responsible for the differences between the metabolomes.

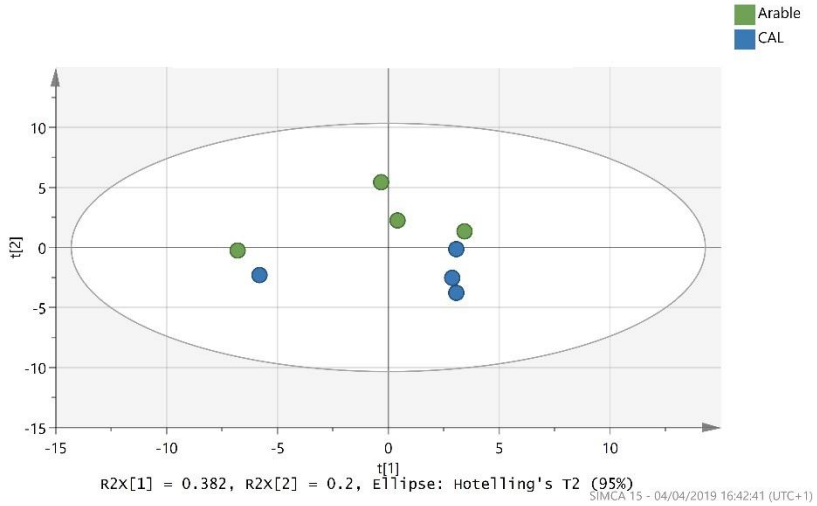

Fig. 4.4. An unsupervised multivariate PCA analysis of the metabolite profiles of Arable and Connected Arable Ley (CAL) management treatments. Metabolite profiles were of the organic, lipophilic layer analysed in positive ionisation mode.

4.3.2. Supervised multivariate analysis of metabolomic fingerprints by Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA)

In the OPLS-DA, the variation within management treatment, between the biological replicates of both treatments is much smaller than what was observed in the PCA plots. This contributes to the grouping of the treatments being clearer overall than the unsupervised analysis. Substantial separation is again evident between the Arable and CAL managements, as it was in the PCA plot (Fig. 4.5a).

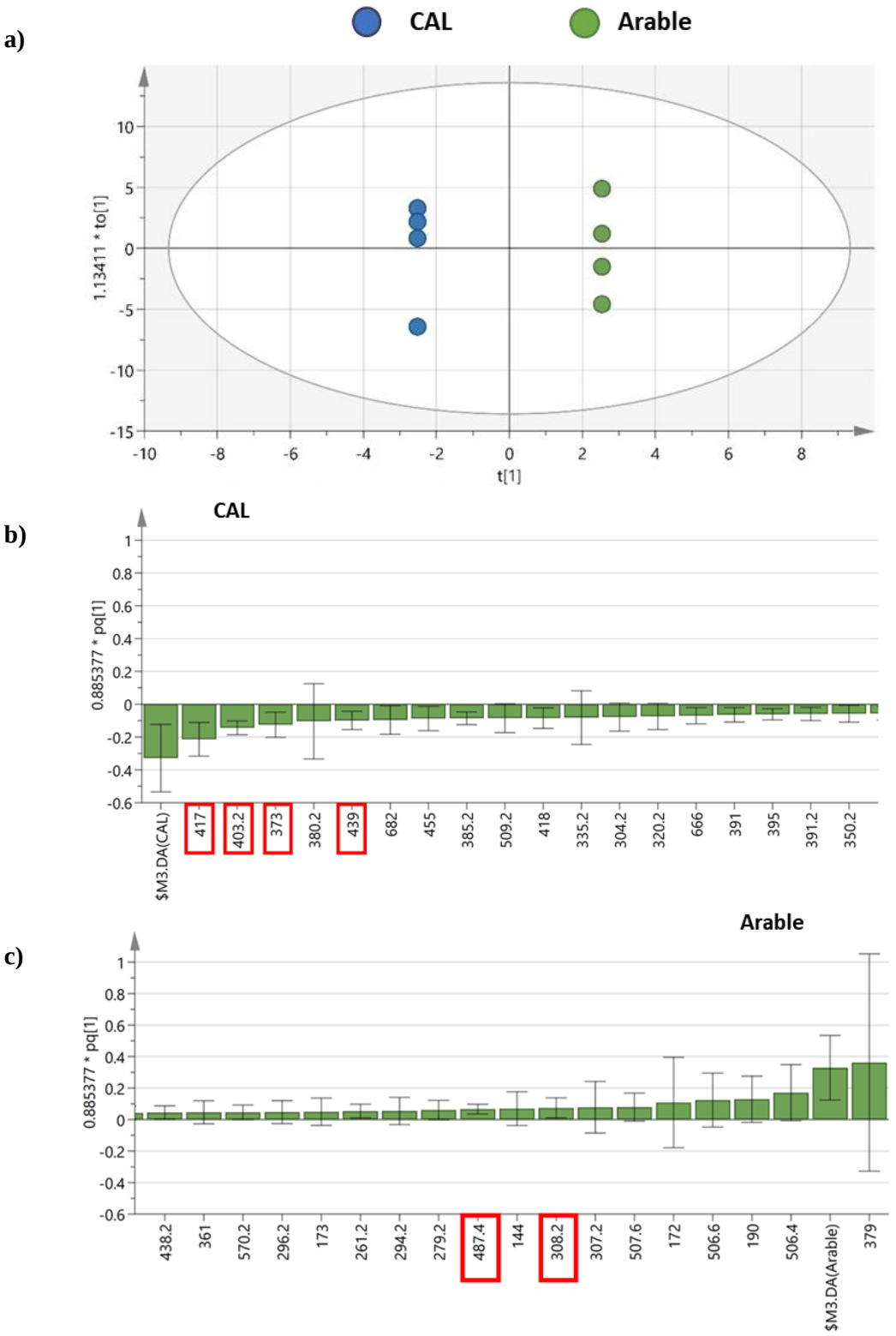

Fig. 4.5. **a)** Supervised multivariate OPLS-DA analysis of the pair-wise comparisons of the metabolite profiles between the Arable and CAL treatments with the corresponding loadings plots demonstrating the mass bins causing the discrimination (highlighted in red) between the associated with the **b)** CAL and **c)** Arable field. Metabolite profiles of the organic layer were analysed in positive ionisation mode. Red boxes around masses show the mass bins selected for putative investigation due to causing separation between treatments. Bars which had error bars larger than the data bars were not chosen as these were unlikely to be statistically different between managements.

4.3.3. Identification of metabolic fingerprints

For each pairwise comparison in the supervised OPLS-DA analyses, up to the top five mass bins (*m/z*) that were shown by the pairwise analyses to be responsible for the separation between treatments in the metabolomics fingerprints in the OPLS-DA plots were selected, not including the bins where the standard error bar surpassed the length of the bar, as these were unlikely to be statistically different between managements. For each bin, several detected masses inside it were putatively identified. Bins could contain more than one compound due to the differences in monoisotopic masses being smaller than the binning size (0.2 Da for positive mode). Table 4.2 shows the overview of the mass bins causing the discrimination between Arable and CAL for the organic, lipophilic layer in positive mode, and the putative identification of the detected masses that they held.

Table 4.2. Discriminant mass bins and their associated detected masses for bins causing discrimination between and associated with the **a)** CAL and **b)** Arable field. These masses are putatively identified, with their accurate masses, name, chemical group and their associated organisms and pathways are displayed. The error in parts per million (ppm) is included to show the difference in variation between detected mass and the weight of the putative compound. Organic, lipophilic layer in positive mode.

a)

Bin	Detected Mass	Accurate Mass	Δppm	Name	Organism	Chemical Group	Pathway
417	416.9722						
	417.0377	417.0371	1	Anastatin B	Plants	Flavanones/flavonoids	Flavonoid biosynthesis
		417.0371	1	Anastatin A	Plants	Flavanones/flavonoids	
		417.0397	4	Epithienamycin F	Fungi/Bacteria	Antibiotics	Carbapenem biosynthesis
	417.0912	417.0945	7	Isorobustin	Plants	Flavanoid	
		417.0945	7	Justicidin A	Plants	Lignans (polyphenol)	Antioxidant role in plant defence
		417.0945	7	Millettosin	Plants	Rotenoid flavonoid (isoflavonoid)	Insecticidal activities
		417.0945	7	Robustin	Plants	isoflavonoid	
		417.0881	7	Propicillin potassium	Fungi	antibacterial, penicillin	
		417.0945	7	12a-Hydroxyisomillettone	Plants	Rotenoid flavonoid (isoflavonoid)	Insecticidal activities
		417.0946	8	Gibberellin A32	Plants	Prenol lipids	Plant development
		417.0946	8	Hydroxyvernolide	Plants	Germacrenes	antimicrobial and insecticidal properties
	416.9706667						
	417.0377	see 2.BSSE.CAL					
	417.0912	see 2.BSSE.CAL					
	416.9722						
	417.0466	417.0443	5	carbenicillin	Fungi	antibacterial	
		417.0397	16	Epithienamycin F	Fungi/Bacteria	Antibiotics	Carbapenem biosynthesis
		417.0371	22	Anastatin A	Plants	Flavanones/flavonoids	
		417.0371	22	Anastatin B	Plants	Flavanones/flavonoids	Flavonoid biosynthesis
		417.0365	24	Ritipenem acoxil hydrate	Fungi/Bacteria	Carbapenem	Penicillin/Peptidoglycan biosynthesis
	416.9155667	416.9247	21	Ferbam		Fungicide	
	416.9811						
	417.0466	417.0443	12	carbenicillin	Fungi	antibacterial	
		417.0397	16	Epithienamycin F	Fungi/Bacteria	Antibiotics	Carbapenem biosynthesis
		417.0371	22	Anastatin A	Plants	Flavanones/flavonoids	
		417.0371	22	Anastatin B	Plants	Flavanones/flavonoids	Flavonoid biosynthesis
		417.0365	24	Ritipenem acoxil hydrate	Fungi/Bacteria	Carbapenem	Penicillin/Peptidoglycan biosynthesis
403.2	403.1208333	403.1184	6	BIBB 515	Fungi/Plants	enzyme	biosynthesis of plant and fungal sterols
		403.1176	7	Calomelanol I	Plants	Flavanone	
		403.1152	13	Robustic acid	Plants	isoflavonoid	
		403.1154	13	Zexbrevin B	Plants	Sesquiterpenoids/Terpenoid	defensive agent/pheremone
		403.1154	13	Gibberellin A55	Plants	Plant growth hormone	Found in common wheat
	403.1647333	403.1628	4	Di-4-coumaroylputrescine	Plants	Alkaloid	Found in Vicia faba (legume bean)
		403.1628	4	Vomicine		Indole alkaloid	
		403.167	5	3-(1,1-Dimethyl-2-propenyl)-8	Roots		
	403.2086333	403.2091	1	[6]-Gingerdiol 3,5-diacetate	Rhizome	Fatty alcohol esters	Found in ginger rhizome
		403.2115	7	Melleolide F	Fungi	Prenol lipids	found in mushrooms
		403.2115	7	Citreoviridin	Fungi	Mycotoxins	
	403.2525333	403.2479	11	Lucidone A	Fungi		
	403.1120333	403.1152	7	Robustic acid	Plants	isoflavonoid	
		403.1152	7	Millettocalyxin B	Plants	Flavones/Flavonols	
		403.1152	7	Multijuginol	Plants	Flavones/Flavonols	
		403.1152	7	7-Prenyloxy-8-methoxy-3',4'-n	Plants		Found in legumes
		403.1152	7	Predurmillone	Plants		Found in leguminous tree
		403.1152	7	Muxiangrin II	Plants	Flavones/Flavonols	
		403.1152	7	Maximaisoflavone C	Plants	Isoflavonoid	
	403.1559333	403.1575	3	Prenyl apiosyl-(1->6)-glucoside	Bacteria/Fungi	Fatty acyl glycosides	Biosurfactants/glycolipids
		403.1575	3	3-Methyl-3-butenyl apiosyl-(1-	Bacteria/Fungi	Fatty acyl glycosides	Biosurfactants/glycolipids
		403.154	4	Ulexone B	Plants	isoflavonoid	
		403.154	4	Tomentolide A	Plants	neoflavonoid	
		403.154	4	cis-and-trans-Inophyllolide	Plants	neoflavonoid	
		403.1599	9	Benzyl O-[arabinofuranosyl-(1-	Plants	glycoside	
	403.1998333	403.2026	6	Carbosulfan		Insecticide	
		403.1963	8	D-Linalool 3-(6''-malonylglucos	Bacteria/Fungi	Fatty acyl glycosides	
		403.2091	22	[6]-Gingerdiol 3,5-diacetate	Rhizome	Fatty alcohol esters	Found in ginger rhizome
	403.2437333						
	403.2876333	403.2843	8	MG(22:6(4Z,7Z,10Z,13Z,16Z,19Z	Bacteria/Fungi/P	monoacylglyceride	Biosurfactants
	403.1091	403.1152	15	Robustic acid	Plants	isoflavonoid	
		403.1152	15	Isoglabrachromene	Plants	Flavanones	

Table 2a. Continued

373	372.9380667	372.9424	11	Profenofos		Insecticide	
		372.9449	18	Menadiol disulfate	Plants	Vitamin	Vitamin K analogue. Found in greens
	372.9901333	372.986	11	Brevifolincarboxylic acid 9-sulf	Plants	Isocoumarin	sweet clover
		372.9956	14	8-Hydroxyquercetagetin	Plants	Flavones/Flavonols	
		372.9956	14	Hibiscetin	Plants	Flavones/Flavonols	
		372.9989	23	2,2'-bithiophenes	Plants	Flavones	
	373.0423	373.0407	4	Proquinazid		Fungicide	Used to control powdery mildew in cereals
		373.0449	7	Dehydrogriseofulvin	Fungi/Bacteria	Antibiotic biosynthesis	
		373.0473	13	19-Hydroxy-8-O-methyltetrang	Fungi/Bacteria	Antibiotic biosynthesis	
		373.0473	13	Sophoracoumestan A	Plants	Coumestan Flavonoids	Common in clover sprouts/beans
		373.032	27	Xanthotoxol arabinoside	Plants	coumarin glycosides	Common in clover sprouts/beans
		373.032	27	Hovenitin I	Plants		Constituent of seeds and fruit of raisin tree
		373.032	27	Amaronol B	Plants	Aurone Flavonoid	
	373.0901	373.0894	1	4-Feruloyl-1,5-quinolactone	Plants	Coumaric acids	Found in the pericarp tissue of wheat and maize
		373.0894	1	3-Feruloyl-1,5-quinolactone	Plants	Coumaric acids	Found in the pericarp tissue of wheat and maize
		373.0909	2	Mitomycin	Bacteria	Antibiotic	Produced by *Streptomyces* actinobacteria
		373.0918	4	7-Hydroxy-3,5,8-trimethoxy-3',	Plants	Flavnoes/Flavonols	
		373.0918	4	5-Hydroxy-3,7,8-trimethoxy-3',	Plants	Flavnoes/Flavonols	
		373.0918	4	Herbacetin 7,4'-dimethyl ether	Plants		metobite in ferns
		373.0918	4	Stemonal	Plants	Rotenoid Flavonoid	Insecticidal activities
	372.9465667	372.9449	4	Menadiol disulfate	Plants	Vitamin	Vitamin K analogue. Found in greens
		372.9424	11	Profenofos		Insecticide	
	372.9986	372.9989	0	Apigenin 7-sulfate	Plants	Flavones	
		372.9965	5	5-(3,4-Diacetoxybut-1-ynyl)-2,	Plants	Fatty acyls	Antifungal properties
		372.9956	7	8-Hydroxyquercetagetin	Plants	Flavones/Flavonols	Found in marigolds
		372.9956	7	Hibiscetin	Plants	Flavones/Flavonols	
		372.9956	7	3,5,6,7,2',3',4'-Heptahydroxyfla	Plants	Flavones/Flavonols	
	373.0507	373.0473	9	19-Hydroxy-8-O-methyltetrang	Bacteria	Tetraphene	Bacterial metabolite
		373.0473	9	Sophoracoumestan A	Plants	Coumestan flavonoids	Common in clover sprouts/beans
		373.0545	10	(S)-4-(1H-imidazol-4-yl)-2-(3-m	Plants	Butanoates	Found in plant oils
		373.0554	12	Norstictic Acid	Lichen	Secondary metabolite	Lichen's form biological soil crusts
		373.0554	12	Repenone	Plants	Rotenoid Flavonoids	Insecticidal activities
	373.0929	373.0918	2	Herbacetin 8-butyrate	Plants	Flavones/Flavonols	
		373.0918	2	Herbacetin 7,4'-dimethyl ether	Plants	Flavones/Flavonols	
		373.0918	2	Melisimplin	Plants	Flavones/Flavonols	
		373.0918	2	Quercetin 3'-isobutyrate	Plants	Flavones/Flavonols	
		373.0918	2	Stemonal	Plants	Rotenoid Flavonoid	Insecticidal activities
		373.0918	2	5-Hydroxy-3,7,8-trimethoxy-3',	Plants	Flavones/Flavonols	
		373.0918	2	Quercetin 4'-isobutyrate	Plants	Flavones/Flavonols	
	372.924						
	373.0507	373.0473	9	19-Hydroxy-8-O-methyltetrang	Bacteria	Tetraphene	Bacterial metabolite
		373.0473	9	Sophoracoumestan A	Plants	Coumestan flavonoids	Common in clover sprouts/beans
		373.0545	10	(S)-4-(1H-imidazol-4-yl)-2-(3-m	Plants	Butanoates	Found in plant oils
		373.0554	12	Norstictic Acid	Lichen	Secondary metabolite	Lichen's form biological soil crusts
		373.0554	12	Repenone	Plants	Rotenoid Flavonoids	Insecticidal activities
	372.9874	372.986	3	Brevifolincarboxylic acid 9-sulf	Plants	Isocoumarins	sweet clover
		372.9956	22	8-Hydroxyquercetagetin	Plants	Flavones/Flavonols	
		372.9956	22	Hibiscetin	Plants	Flavones/Flavonols	
		372.9956	22	3,5,6,7,2',3',4'-Heptahydroxyfla	Plants	Flavones/Flavonols	
		372.9965	24	5-(3,4-Diacetoxybut-1-ynyl)-2,	Plants	Fatty acyls	Antifungal properties
	373.0929	373.0918	2	Herbacetin 8-butyrate	Plants	Flavones/Flavonols	
		373.0918	2	Herbacetin 7,4'-dimethyl ether	Plants	Flavones/Flavonols	
		373.0918	2	Melisimplin	Plants	Flavones/Flavonols	
		373.0918	2	Quercetin 3'-isobutyrate	Plants	Flavones/Flavonols	
		373.0918	2	Stemonal	Plants	Rotenoid Flavonoid	Insecticidal activities
		373.0918	2	5-Hydroxy-3,7,8-trimethoxy-3',	Plants	Flavones/Flavonols	
		373.0918	2	Quercetin 4'-isobutyrate	Plants	Flavones/Flavonols	
	372.9409	372.9424	4	Profenofos		Insecticide	
		372.9449	10	Menadiol disulfate	Plants	Vitamin	Vitamin K analogue. Found in greens
	372.9958	372.9956	0	8-Hydroxyquercetagetin	Plants	Flavones/Flavonols	
		372.9956	0	3,5,6,7,2',3',4'-Heptahydroxyfla	Plants	Flavones/Flavonols	
		372.9956	0	Hibiscetin	Plants	Flavones/Flavonols	
		372.9965	1	5-(3,4-Diacetoxybut-1-ynyl)-2,	Plants	Fatty acyls	Antifungal properties
		372.9989	8	Apigenin 7-sulfate	Plants	Flavones	
		372.986	26	Brevifolincarboxylic acid 9-sulf	Plants	Isocoumarin	sweet clover
	373.0507	373.0473	9	19-Hydroxy-8-O-methyltetrang	Bacteria	Tetraphene	Bacterial metabolite
		373.0473	9	Sophoracoumestan A	Plants	Coumestan flavonoids	Common in clover sprouts/beans
		373.0545	10	(S)-4-(1H-imidazol-4-yl)-2-(3-m	Plants	Butanoates	Found in plant oils
		373.0554	12	Norstictic Acid	Lichen	Secondary metabolite	Lichen's form biological soil crusts
		373.0554	12	Repenone	Plants	Rotenoid Flavonoids	Insecticidal activities

Table 2a. Continued

439	438.9647333						
	439.0197	439.0167	6	Bis(glycerophospho)-glycerol	Plants	Antioxidant	
		439.0281	18	Pyrazolate		Herbicide	
	439.0746	439.079	9	Flavonol 3-O-D-glucoside	Plants	Flavonols	
		439.079	9	Thymusin 6-isobutyrate	Plants	Flavones/Flavonols	
		439.079	9	Isoflavone 7-O-beta-D-glucosi	Plants	glycosyloxyisoflavone	
		439.079	9	Flavonol 3-O-D-galactoside	Plants	Flavonols	
		439.079	9	2-[4-(Acetyloxy)phenyl]-5,6,7,	Plants	Flavones/Flavonols	
		439.079	9	5-Hydroxyflavone	Plants	Flavones/Flavonols	constituent of Medicago sativa - legume
		439.079	9	Flavonol 3-O-D-glycoside	Plants	Glucoside	
	438.9571						
	439.0197	439.0167	6	Bis(glycerophospho)-glycerol	Plants	Antioxidant	
		439.0281	19	Pyrazolate		Herbicide	
	439.0808	439.079	4	Flavonol 3-O-D-glucoside	Plants	Flavonols	
		439.079	4	Flavonol 3-O-D-galactoside	Plants	Flavonols	
		439.079	4	Thymusin 6-isobutyrate	Plants	Flavones/Flavonols	
		439.079	4	Herbacetin 7,4'-dimethyl ethe	Plants	Flavones/Flavonols	
		439.079	4	Isoflavone 7-O-beta-D-glucosi	Plants	glycosyloxyisoflavone	
		439.079	4	alpha-Peltatin	Plants	Lignans	Phenylpropanoid
		439.079	4	5-Hydroxyflavone	Plants	Flavones/Flavonols	constituent of Medicago sativa - legume
		439.079	4	Flavonol 3-O-D-glycoside	Plants	Glucoside	
		439.079	4	2-[4-(Acetyloxy)phenyl]-5,6,7,	Plants	Flavones/Flavonols	
	438.9601667						
	439.0197	439.0167	6	Bis(glycerophospho)-glycerol	Plants	Antioxidant	
		439.0281	19	Pyrazolate		Herbicide	
	439.0869333	439.0894	5	O-Carbamoyl-deacetylcephalo	Fungi	Antibiotic	
		439.0942	16	Lophirone J	Plants	Flavanones	
		439.079	18	Stemonacetal	Plants	Rotenoid flavonoids	
		439.079	18	Mirificoumestan glycol	Plants	Coumestan flavonoids	
		439.079	18	Daunorubicinol aglycone	Bacteria/Fungi	Antibiotics	
		439.0788	18	Calomelanol D	Plants	Flavones/Flavonols	
	439.0227667	439.0281	12	Pyrazolate		Herbicide	
		439.0167	13	Bis(glycerophospho)-glycerol	Plants	Antioxidant	
	439.0839	439.079	11	Flavonol 3-O-beta-D-glucoside	Plants	Flavonol glycoside	
		439.079	11	Flavonol 3-O-D-galactoside	Plants	Flavonols	
		439.079	11	Thymusin 6-isobutyrate	Plants	Flavones/Flavonols	
		439.079	11	Herbacetin 7,4'-dimethyl ethe	Plants	Flavones/Flavonols	
		439.079	11	Isoflavone 7-O-beta-D-glucosi	Plants	glycosyloxyisoflavone	
		439.079	11	alpha-Peltatin	Plants	Lignans	
		439.079	11	5-Hydroxyflavone	Plants	Flavones/Flavonols	
		439.079	11	Flavonol 3-O-D-glycoside	Plants	Glucoside	
		439.079	11	2-[4-(Acetyloxy)phenyl]-5,6,7,	Plants	Flavones/Flavonols	

b)

Bin	Detected Mass	Accurate Mass	Δppm	Name	Organism	Chemical Group	Pathway
308.2	308.1334333	308.1281	17	Cusparine		Alkaloids	Tryptophan derived
		308.1281	17	Rutacridone	Plants/Fungi/Bac	Alkaloids	Shikimate pathway
	308.1756						
	308.2178333						
	308.2639	308.2632	2	Eicosapentaenoic Acid-d5	Fungi	Unsaturated Fatty Acid	
		308.2584	17	R-Dysidazirine	Plants/Fungi/Bac	Sphingolipids (sphingoid bases)	In plasma membrane of cells
	308.1334	308.1281	17	Cusparine		Alkaloids	Tryptophan derived
		308.1281	17	Rutacridone	Plants/Fungi/Bac	Alkaloids	Shikimate pathway
	308.1768667						
	308.2204	308.222	5	Dihydrocapsaicin	Plants	capsaicinoid	Found in peppers
	308.2588	308.2584	1	R-Dysidazirine	Plants/Fungi/Bac	Sphingolipids (sphingoid bases)	In plasma membrane of cells
	308.2972	308.2924	15	Spisulosine	Plants/Fungi/Bac	Sphingolipids (sphingoid bases)	In plasma membrane of cells
	308.1334333	308.1281	17	Cusparine		Alkaloids	Tryptophan derived
		308.1281	17	Rutacridone	Plants/Fungi/Bac	Alkaloids	Shikimate pathway
	308.1769						
	308.2204	308.222	5	Dihydrocapsaicin	Plants	capsaicinoid	Found in peppers
	308.2588	308.2584	1	R-Dysidazirine	Plants/Fungi/Bac	Sphingolipids (sphingoid bases)	In plasma membrane of cells
	308.2972	308.2924	15	Spisulosine	Plants/Fungi/Bac	Sphingolipids (sphingoid bases)	In plasma membrane of cells
	308.1794667						
	308.2229667	308.222	3	Dihydrocapsaicin	Plants	capsaicinoid	Found in peppers
	308.2638667	308.2632	2	Eicosapentaenoic Acid-d5	Fungi	Unsaturated Fatty Acid	
		308.2584	17	R-Dysidazirine	Plants/Fungi/Bac	Sphingolipids (sphingoid bases)	In plasma membrane of cells

4.3.4. Average intensities of % total ion counts of bins

The average intensities of the percentage total ion counts in the putatively identified mass bins were calculated for the top bins causing separation between arable and CAL management treatments (Fig. 4.6), highlighted in red boxes in Figure 4.5b and 4.5c.

Mass bins 403.2 and 439 demonstrate differences between the arable and CAL metabolic fingerprints through significant upregulation of metabolites within these bins under three-year ley management compared to in the conventionally managed arable field (p=0.02, 0.003, respectively; Fig. 4.6). During the putative identification of metabolites, bin 403.2 was described as being associated with legumes, lipid biosurfactants and flavonoids. However, the insecticide "Carbosulfan" was also highlighted in both the BSSW and Hillside fields. Bin 439 also flagged the herbicide "Pyrazolate" in all fields, along with multiple flavonoids and constituents of leguminous plants.

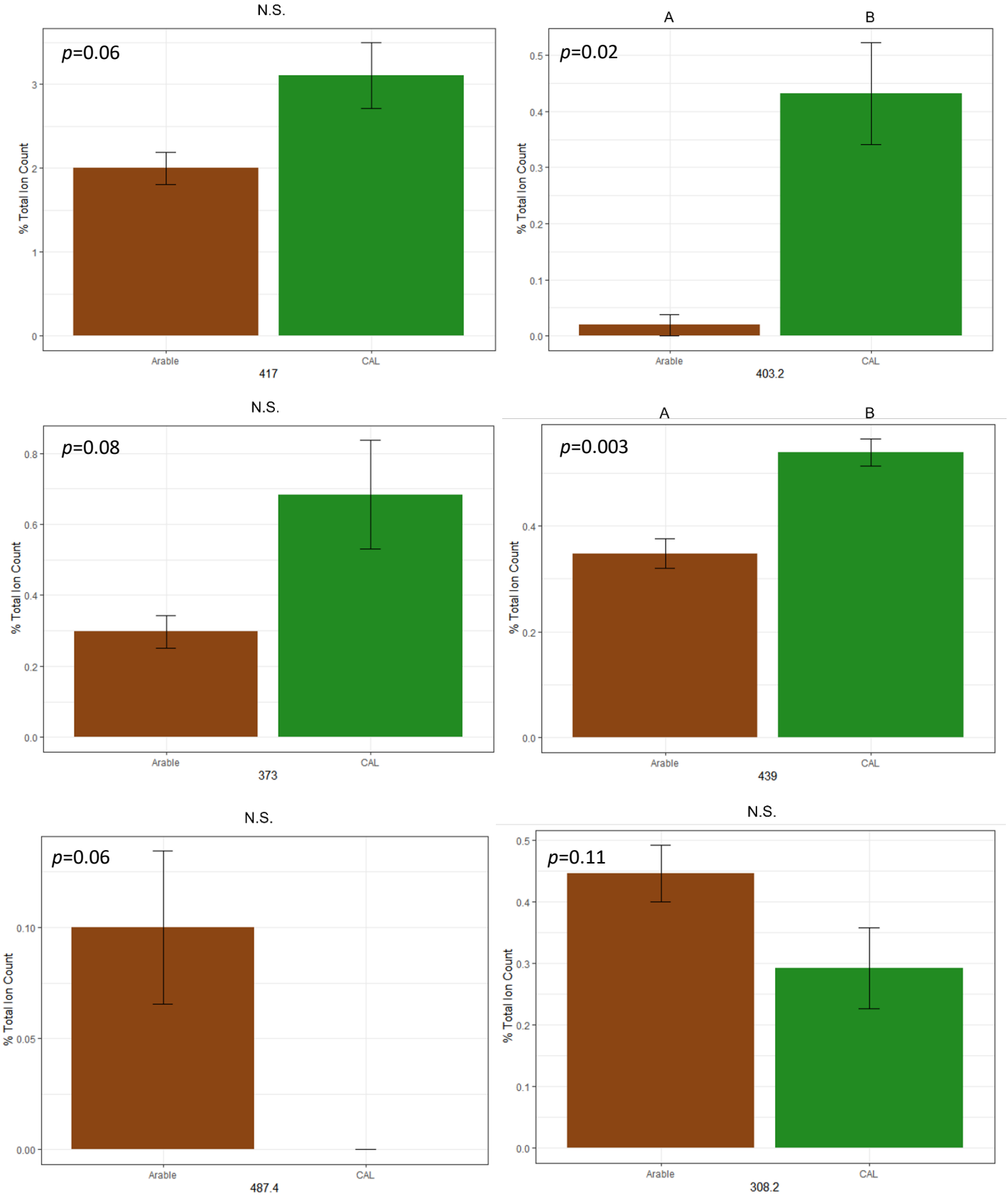

Figure 4.6. Total % ion counts of discriminatory mass (*m/z*) bins of 0.2 Da comparing arable and Connected Arable Ley (CAL) management treatments. Different letters above bars indicate that means are significantly different from each other (Tukey test, $p<0.05$). *P* values for each Tukey test is included for each bar graph.

4.4. Discussion

This study is a starting point for future research on the impact of introducing grass-clover leys into conventionally managed arable rotations on the soil metabolome. With no previous research on this topic, differences in the lipophilic layer using ultra-high-resolution MALDI-MS were focused on, as lipids, mainly due to their hydrophobic properties, are involved in the formation and stability of soil aggregates (Dinel et al., 1991; Paré et al., 1999; Taylor et al., 1999). The results presented in this study show that the main differences between arable soils and those after three years under grass-clover ley were an upregulation of metabolites within the mass bins of m/z 403.2 and 439.

Putative identification of metabolites within mass bin 403.2 suggested a variety of metabolites which could be associated with the changes in land management from arable to ley. Multiple molecules associated with leguminous plants were identified (Table 2a). Although these molecules during the putative identification stage were indicated from previous research as being associated with plants not present in this study system. For example, with the *Vicia faba* (broad bean), the last time a legume crop was grown in the studied fields was vining peas six years earlier in 2013 (See Chapter 1 Table 1.2). These molecules are therefore likely to have come from the leguminous clover plants present within the ley. Although these molecules with legume plant origin are likely to have no impact on soil aggregation, which is the original research question, it is still encouraging to see these molecules that are likely to be the result of the ley being present are being identified, as it is promising evidence that this novel methodology is working correctly.

The several flavonoid molecules which were highlighted multiple times during the putative identification stage are also likely to have originated from the leguminous clover plants present in the ley. Flavonoids are diverse plant secondary metabolites, which were flagged repeatedly from a variety of molecules within the m/z bin 403.2. Flavonoids are involved with a variety of important functions in plants, but in soils, they could be present as signalling molecules emitted by legume plants (for example, clovers present in the ley) to initiate symbioses with rhizobia for the development of root nodules (Reddy and Khandual, 2007).

Lipid biosurfactants were also identified multiple times during the putative identification of the m/z bin 403.2, which could potentially be important in aggregate stability and slaking resistance (Paré et al., 1999). Biosurfactants are known to be produced by bacteria, for example by the gram-positive bacteria *Lactobacilli* (Gomaa, 2013). This bacteria from the genus *Lactobacillus* is commonly found in soils and can stimulate plant growth through the production of auxins and volatile fatty acids (Afanador-Barajas et al., 2021). These bacteria are also sometimes applied to soils as biocontrol agents against pathogenic bacteria (Gajbhiye and Kapadnis, 2016; Lamont et al., 2017). However, no bacteria from the *Lactobacillus* genus were found in Chapter 3. Plant roots also produce mucilages which contain biosurfactants which chemically and physically modify soil properties (Read et al., 2003).

It is surprising that the insecticide 'Carbosulfan' was identified as a potential metabolite upregulated from in the ley compared to arable soils, as any pesticides that were applied to the arable field during the course of the leys were avoided from applying to the leys. However, there is a potential for drift in spray applications to occur over several meters (De Schampheleire et al., 2007), which would make pesticide drift from the conventionally-managed arable field to the ley strips in this experimental design very plausible.

The other m/z bin that was upregulated in the ley soil compared to that under arable rotation was 439. The putative identification stage also flagged many molecules involved in the flavonoid pathway. Another herbicide named 'Pyrazolate' was also identified, which is used for the selective control of annual and perennial grasses and broadleaf weeds.

The only other compound that differed from the m/z 403.2 bin was the presence of lignans, which are low molecular weight polyphenols found in plants, but especially seeds, grains and vegetables. Lignans are one of the major group of polyphenols, alongside phenolic acids, flavonoids and isoflavonoids (Stiller et al., 2021), which are identified regularly in both of the upregulated m/z bins in the ley. Phenolic molecules play an important role in the early stages of aggregate formation, forming cation complexes as a building block for the formation of larger aggregates (Martens, 2000). Phenolic molecules from both plant and microbial sources are also known to increase mean weight diameter of soil aggregates, with the input of plant residues with higher phenolic acid concentrations known to be beneficial for the formation and stabilisation of larger soil aggregates (Martens, 2002).

If the upregulation in soil lignan signal is related to an upregulation of lignan content in plants, this could be a potential indicator of greater AMF abundance in the soils, as AMF inoculation can cause plants to yield more lignans than control, non-inoculated plants (Moraes et al., 2004).

From this novel study using untargeted metabolomics as an initial scan to detect any changes in metabolic soil profiles between soil under arable rotation and grass-clover ley, we were able to detect changes in the soil metabolome between these two different land managements. This study gives a starting point for future research to carry out more targeted analysis on organic molecules of interest that have been identified as being upregulated in ley soils compared to arable. In particular, more research into changes in flavonoids, polyphenols and biosurfactants would be warranted.

In order to further answer the question of the specific roles of individual organic molecules in the formation of soil macroaggregates, it would be of interest to further develop a methodology researching changes in the metabolome between specific aggregate size fractions. This would likely involve wet sieving of fresh soil in the field and flash freezing to avoid any further change to the metabolome. It is likely that some labile compounds would be lost upon wet sieving to separate soil aggregates into different size fractions, although these more labile molecules are probably less likely to be involved in the long-term stability of soil aggregates if they are so transient. One of the reasons in the present work

to focus on lipids was that these would be expected to have a low water-solubility, and so potentially could in future be analysed in wet-sieved soil aggregates that are flash frozen, later cryosectioned, and MALDI imaging used to map the spatial distribution of metabolites from the exterior to interior of aggregates.

4.5. Conclusion

A novel methodology and research has been developed and carried out for the analysis of the impact of the introduction of a three-year grass-clover ley into arable rotation of the lipophilic portion of the soil metabolome. The arable-to-ley conversion resulted in an upregulation of metabolites within m/z bins 403.2 and 439, which relate to upregulation in flavonoids, biosurfactants and lignans. These molecules can all possibly relate to changes in plant cover and microbial communities and related to research on the impact of these organic molecules on the formation of soil macroaggregates. Follow-up studies using more targeted metabolomic approaches must be carried out on these identified organic molecules to further relate their presence to the change in plant or microbial communities or the direct formation or stabilisation of soil aggregates.

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Chapter 5

5. The role of earthworm ecotypes in the formation of macroaggregates and soil organic carbon and nitrogen storage

Abstract

Earthworms are known as ecosystem engineers due to their ability to transform the structure and properties of soil in which they live. Their burrowing and processing of organic matter provides important soil ecosystem services. These include the creation of macropores that improve hydrological functioning by faster infiltration and drainage, and the incorporation of leaf litter, roots and microbial biomass, by ingestion and casting, which contributes to soil aggregation and enhances organic carbon (OC) and water storage. However, effects of earthworms on soil properties in agricultural fields are often assumed rather than proven, as factors that alter earthworm populations, such as changes in land management practices, typically confound resolving causality. Furthermore, the three ecotypes of earthworms: endogeic, epigeic and anecic have very different ecologies and therefore potential different effects on soil properties, but the relative importance of each ecotype in engineering soil structure and functions remain incompletely understood. To address these issues, we conducted a field-based soil monolith study in four conventionally tilled arable fields, in which earthworm populations were either depleted or enriched in parallel with the introduction of a grass-clover ley for 12 months. The effects of total earthworm numbers and each of the three earthworm ecotypes on soil aggregation, and OC and nitrogen (N) storage in different sized water-stable aggregates (WSAs) in surface soil (0-7 cm) were investigated using general linear models. We found that monoliths with greater total numbers of earthworms had greater proportions of WSAs (>2000 μm; p=0.008; R^2=0.12), which was primarily driven by endogeic (p=0.01; R^2=0.11) and epigeic (p=0.011; R^2=0.11) ecotypes, with anecics having no influence in this top 7 cm of the soil (p=0.92; R^2<0.001). The total OC stored within macroaggregates also increased with increasing earthworm numbers (p=0.043; R^2=0.22), driven mainly by epigeics (p=0.03; R^2=0.25), with macroaggregate-associated N mainly driven by endogeics (p=0.04; R^2=0.23). These results demonstrate the importance of endogeic and epigeic ecotypes in the regeneration of topsoil structure and associated OC and N sequestration in arable fields and highlight the potential benefits of changes to agroecosystem land management practices that enable earthworm populations to recover from depletion by conventional intensive arable cultivation and cropping.

5.1. Introduction

Earthworm activity, including feeding, casting and burrowing, alters the soil physically, chemically and biologically through changes in soil structure and nutrient composition (Jégou et al., 2001; Lavelle et al., 1998; Sharma et al., 2017). Because of their ability to modify the soil environment in which they live, earthworms are described as 'ecosystem engineers', a term coined by Jones et al. (1994). The benefits that earthworms bring to soils are both ecologically and economically important, providing agroecosystems with ecosystem services that are important for soil health and sustainability (Blouin et al., 2013), including nutrient cycling (Blanchart, 1999) and improved porosity for root growth, aeration and drainage (Bottinelli et al., 2010). However, despite the importance of earthworms in soils, conventional tillage (CT) involving mouldboard ploughing and harrowing, until recently, has been used to cultivate 60% of arable land in England (Townsend et al., 2016), causing substantial depletion of both their abundance and diversity (Briones and Schmidt, 2017; Crittenden et al., 2014; Edwards and Lofty, 1982).

The importance of earthworms in developing and maintaining soil structure is partly linked to their effects on soil aggregation (Churchman, 2010), and associated soil carbon (C) sequestration (Jastrow, 1996; Six et al., 1998; Stewart et al., 2009, 2008; Tisdall and Oades, 1982; Zhang et al., 2013). Larger aggregates protect soil organic carbon (SOC) from microbial oxidation, increasing carbon storage (Balesdent et al., 2000; Plante and McGill, 2002a), and reducing CO_2 emissions (Lal, 2004c; Stavi and Lal, 2013). Larger aggregates are formed by microaggregates being bound together by a variety of mainly temporary and transient binding agents (Tisdall and Oades, 1982). These include roots and fungal hyphae which enmesh particles (Tisdall and Oades, 1982), and earthworm casts held together by gut and microbial mucilages which act as glues to stick particles together (Zhang et al., 2013), forming more persistent aggregates. Earthworms therefore enhance soil aggregation (Sharma et al., 2017), and also actively draw shoot litter into soils (Fahey et al., 2013), both of these activities contributing to soil C sequestration, and enhancing the structural stability and numbers of larger water-stable aggregates (WSAs) (Ketterings et al., 1997). Soil is ingested into the earthworm's gut, where it is combined with intestinal mucus, containing free polysaccharides, and clays to form new aggregates with rejuvenated microbial activity (Barois et al., 1993; Shipitalo and Protz, 1989; Zhang et al., 2013). Aggregates formed by earthworm casts can be very stable, remaining intact for several years due to the hardening of clay-encased organic debris (Shipitalo and Protz, 1988). This makes aggregates of earthworm origin much more water-stable than fungal-derived aggregates, which are very susceptible to breakage by physical agitation such as tillage (Tisdall and Oades, 1982). A study by Zangerlé et al. (2011) used near infrared (NIR) spectral analysis to determine aggregate origins and found that roots (of two plant species: *Trifolium pratense* and *Plantago lanceolata* L.) and earthworms (*Aporrectodea caliginosa* and *Allolobophora chlorotica* species) can interact to transform up to 42% of soil mass into macroaggregates in only two months, confirming the interaction of these ecosystem engineers to form

macroaggregates can be very fast under favourable conditions, with earthworms producing more aggregates than roots.

The effect that earthworms have on soil properties is species-, soil type- and land use-dependent (Clause et al., 2014; Hedde et al., 2013). Earthworms are categorised into three different ecotypes: anecic, endogeic and epigeic, each having different life strategies and different spatial niches within the soil horizon. Epigeic earthworms live on the soil surface, rarely making burrows, and are considered to have little effect on soil aggregation compared to other earthworm ecotypes (Bossuyt et al., 2006; Shipitalo and Le Bayon, 2004). There are conflicting reports regarding the effects of anecic and endogeic earthworms on soil aggregation. Most studies record increases in the percentages of WSAs for anecic (Buck et al., 2000; Flegel et al., 1998; Hamilton et al., 1988) and endogeic species (Blanchart, 1992; Bossuyt et al., 2005; Buck et al., 2000) due to their burrowing activities and incorporation of organic matter (OM) into the soil. However, some studies found no effect or a decrease in WSAs (Jégou et al., 2001; Schrader and Zhang, 1997; Shuster et al., 2000). A recent mesocosm experiment by Hallam and Hodson (2020) studied the separate effects of the anecic earthworm *Lumbricus terrestris* and the endogeic earthworm *Allolobophora chlorotica* in different depth horizons of soil from two of the same arable fields as the present study and a neighbouring pasture field, and found that both earthworm species significantly increased %WSA in both the upper (0-6.5 cm) and lower (6.5-13 cm) soil layers compared to earthworm-free control mesocosms, correlating with increases in soil water holding capacity (WHC).

Earthworm ecotypes can be split further into sub-groups of ‘compacting’ and ‘de-compacting’ earthworms, which influence soil structure by enhancing soil aggregate turnover through the formation and destruction of new macroaggregates in the upper 15 cm of soil by large and small endogeic earthworms, respectively (Blanchart et al., 1997). Compacting species increase the proportion of large aggregates in the soil, with there being a link between larger earthworms and aggregates due to the creation of larger casts within the earthworm guts. Greater amounts of organic residues in the soil favours stronger and larger macroaggregates (Blanchart et al., 1999), so active incorporation of surface OM deeper into the soil by earthworms, where the OC is more stable can enhance both soil aggregation and carbon sequestration. There is a trade-off between compacting and de-compacting earthworms that work together facilitating a good balance between soil aggregation and water infiltration. Although compacting earthworms are good for macroaggregate production, they increase bulk density, which can have a negative impact upon available water and air capacity (Archer and Smith, 1972), but this can be ameliorated by smaller de-compacting earthworms generating macropore spaces (Blanchart et al., 2004).

Different earthworm ecotypes are affected to different extents by common land management practices. Larger earthworms in the anecic group that may live more than a decade and form deep vertical burrows

are especially impacted by CT which disrupts their burrows and exposes them to maceration and predation (Edwards and Lofty, 1982). The more shallow burrowing, smaller earthworms from the endogeic group are more tolerant, or more rapidly recover, from such disturbance (Edwards and Lofty, 1982; Wyss and Glasstetter, 1992). Conventional tillage is also very detrimental to soil structure, enhancing OM oxidation and loss of macroaggregates, thereby being a major reason why arable farming is a major contributor to soil degradation across England and Wales (Graves et al., 2015).

Alternative tillage practices include reduced, minimum and no-tillage (NT), which all reduce the extent of mechanical physical disturbance to the soil, and often involve shallower soil disturbance (Derpsch et al., 2010; Lal, 2013; Soane et al., 2012). These less invasive practices are less damaging to many soil biota, including earthworms (Edwards and Lofty, 1982) and mycorrhizal fungi, both of which are beneficial in soil aggregation (Six et al., 2002) and C storage (Asmelash et al., 2016; Fernandez and Kennedy, 2015; Wilson et al., 2009; Zhang et al., 2013). With the recovery of earthworm populations, especially of the larger anecic and endogeic species, under less intensive management techniques (Edwards and Lofty, 1982), they may play an important role in regenerative agriculture, leading to improvements in soil properties such as soil structure and nutrient cycling (Chan, 2001; van Capelle et al., 2012).

The 'vermicompost' produced from earthworm processing of OM mineralises nutrients, especially N, and can be added to agricultural soils to improve availability of essential nutrients, and enhance crop productivity whilst also adding OM (Sinha, 2009; Sinha et al., 2010). When combined with changes in land management that are beneficial for earthworm survival and reproduction, such as reduced tillage and the introduction of leys, in which tillage and most chemical inputs cease for several years, these strategies can provide economically viable and environmentally sustainable ways to enhance crop production and improve soil fertility and structure - using biology to regenerate soils.

However, with land management changes that simultaneously introduce new plant species such as grass-clover leys, the extent to which resultant soil changes are driven by the recovery of earthworm populations remains uncertain. Leys give perennial evergreen ground cover in place of seasonal annual arable crops, and those with legumes contribute symbiotically-fixed N and more than double the C inputs via roots compared to wheat roots (McNally et al., 2015; Sun et al., 2018). Furthermore, resolving the specific effects of the different ecotypes and species of earthworms on soil properties under field conditions presents additional challenges, as these ecotypes do not occur as separate populations in nature and likely synergistically and possibly territorially interact with each in ways that would be undetectable in artificial communities with groups separated.

To address these issues, we used a novel field-based soil monolith approach in which earthworm populations from arable fields were collected by removing intact soil blocks, which were manipulated by deep-freezing to deplete their populations, repopulated with earthworms from neighbouring fields,

and then established into a newly sown ley on the same soil type. The effects of the earthworm populations living at the end of the 1-year study was assessed using linear regression models to statistically apportion the involvement of different earthworm ecotypes in the formation of soil macroaggregates and OC and N storage within macroaggregates and the bulk soil. By incubating the monoliths in the ground in arable fields the experiment was conducted under realistic environmental conditions.

5.2. Methods

5.2.1. Field site

Paired grass-clover (*Lolium perenne-Trifolium repens* and *T. pratense*) ley strips (3 m wide, 70 m long and 48 m apart) were previously sown into four fields at Leeds University farm, Tadcaster, England (53° 52' 25.2 N 1° 19' 47.0" W) in May 2015 as part of the NERC-Soil Security Programme SoilBioHedge experiment. These ley strips can be seen from satellite images (Fig. 5.1). The four fields: Copse, Hillside, BSSW and BSSE, had been under continuous arable cropping since 1988, 2009 and 1994, respectively, with annual tillage by mouldboard ploughing and power harrowing. The soils were Cambisols (WRB, 2014), but differ slightly in textural class, with Copse and Hillside being loam and sandy loam, respectively and BSSW and BSSE both being silt loams (Hallam et al., 2020).

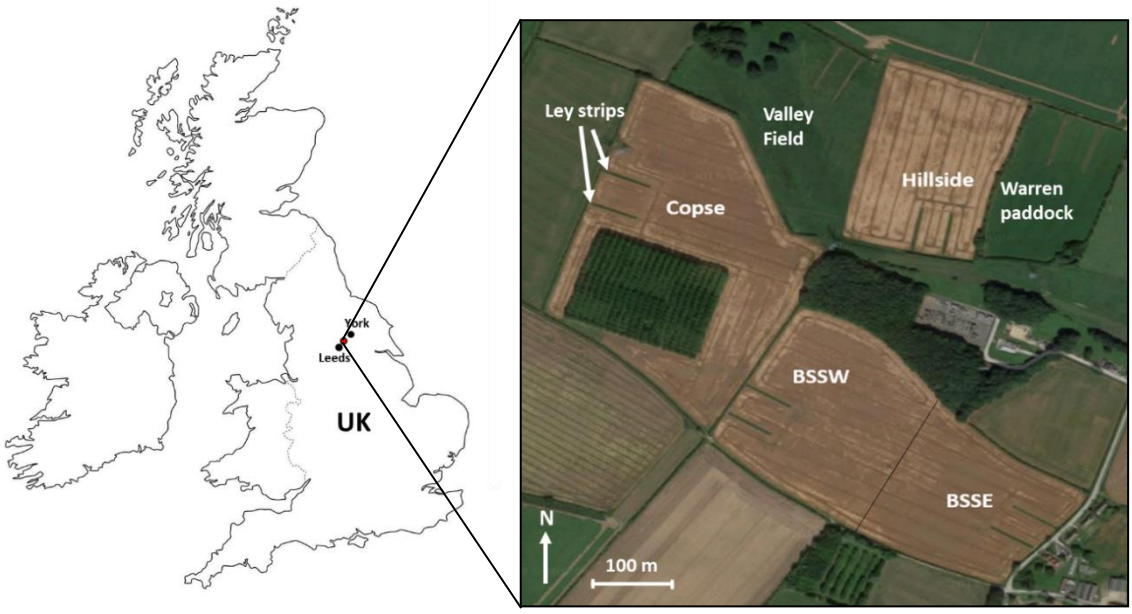

Fig. 5.1. Map of Leeds University Farm, Tadcaster, England, showing the four sampling fields: Copse, Hillside, BSSE and BSSW, and the adjacent pasture fields, Valley field and Warren paddock, used for earthworm community composition control and earthworm collection. The 70 m long ley strips are visible in each field from this Google Earth satellite picture, taken on 17/07/2017.

5.2.2. Monolith treatments

Detailed information on monolith preparation and treatment is described in Hallam et al. (2020). In brief, seven intact soil monoliths were cut to fit in bottomless plastic boxes (22 cm high, 36 cm long, 27 cm wide), from the area of arable field between each pair of ley strips (approximately 70 m from the field margin) in each of the four fields, in March 2017. The monolith extraction procedure was similar to that used by Allaire and Bochove (2006). The monoliths were carefully extruded into an identical plastic box with a bottom in which drainage holes (10 mm diameter on the bottom and 8 mm on the sides) had been drilled and covered with 0.5 mm nylon mesh. One monolith from each field was

immediately placed back into a hole in a ley strip established in the same field from which it was taken, and a 15 cm high barrier of 0.5 mm nylon mesh was placed around the top of the monolith to prevent earthworm migration in and out of it over the duration of the experiment.

The remaining 24 soil monoliths were transferred to a walk-in freezer at the University of Leeds, where they were deep frozen at -20°C for 3 weeks as this treatment has previously reported to be effective at the defaunation of earthworms (Bruckner et al., 1995). The monoliths were then returned to the field and planted into a hole in a ley strip within the same field from which they taken. All 28 monoliths (controls and frozen-thawed) were then sown with a mix of grass seeds and transplanted established red and white clover plants to reflect the plant community found in the ley strips.

Half the defaunated monoliths (n=3 in each field) were repopulated to reach a similar earthworm community to that found in nearby permanent pasture fields that are seasonally grazed by sheep (Warren Paddock and Valley Field, Fig. 5.1), with earthworms collected by excavating and hand-sorting the soil. To ensure earthworm vitality and survival of vulnerable species after the summer and throughout the duration of the experiment, a similar earthworm density and population composition was added to the frozen monoliths with earthworm additions in November 2017, as recommended by Butt (2008). Summary of earthworms added in March and November 2017 to the F+E monolith treatments can be found in Table 5.1, with further details of earthworm collection, culturing and inoculation described in Hallam et al. (2020).

Table 5.1. Total numbers and weights of each added adult earthworm ecotype across all fields in the F+E (Frozen + Earthworm) monolith treatments (n=3 in each field) in March and November 2017. All replicates were supplemented with the same numbers but slightly different weights (Mean ± standard error shown).

Earthworm Ecological Group	Earthworms added on 31st March 2017		Earthworms added on 15th November 2017	
	Number	Weight (g)	Number	Weight (g)
Anecic	2	4.75±0.22	3	10.09±0.51
Endogeic	18	4.93±0.08	17	3.98±0.16
Epigeic	3	0.53±0.03	0	0

The remaining defaunated monoliths (n=3 in each field, n=12 total) were not repopulated with earthworms after freezing and were treated with up to 3 L of allyl isothiocyanate at 0.1 g L^{-1} per monolith in November 2017 (Zaborski, 2003) to bring any earthworms that had managed to survive freezing or recolonize despite the mesh barriers to the soil surface for removal. This was done at the same time as earthworm repopulation in the manipulated monoliths.

The methodology of managing the soil monoliths produced three treatments: Control (C), unfrozen and not inoculated with earthworms to provide the native earthworm populations and control for any effect of freezing on the soil structure and quality, Frozen (F) and not inoculated with earthworms, and Frozen + Earthworms (F+E) where earthworms were added back to the previously frozen monoliths.

5.2.3. Measurements made after monolith removal

In April 2018, after the experimental monoliths had been in the field for ~12 months, they were dug out from the fields and weighed using an engine crane. Up to 3 litres of 0.1 g $litre^{-1}$allyl isothiocyanate per monolith (Zaborski, 2003) was added to the monoliths to aid the emergence of earthworms, which were collected, counted and identified into ecological groups (anecic, endogeic or epigeic), whilst adults were identified further down to species level using the Opal identification key (Jones and Lowe, 2009). See section *5.3.1. Earthworm Collection*, Table 5.2 and Figure 5.3, for a summary of earthworm populations recovered from the monoliths at the end of the experiment in April 2018 and Hallam et al. (2020) for more detailed information including numbers of adults and juveniles and breakdown of weights, numbers, species and fields. The monoliths were then saturated with tap water, allowed to drain under gravity and reweighed to determine field capacity.

In the present study, three intact bulk density soil cores (5 cm deep, 5 cm diameter and 100 cm^3 volume) were taken down to a depth of 7 cm in three places at the surface of the monoliths, avoiding large stones, the sides of the box, and where soil cores had just been removed for other studies by Hallam et al., (2020). All cores were stored in a refrigerator at 4°C for subsequent water-stable aggregate (WSA) fractionation (*section 5.2.4*) and organic carbon (OC) and total nitrogen (N) content of each aggregate size fraction (*section 5.2.6*). After all soil samples requiring undisturbed soil profiles were collected, the soil in the monoliths was emptied out and any remaining earthworms were removed by hand sorting and identified.

5.2.4. Water-stable aggregate fractionation of soil aggregates

A day after collection and storage at 4°C, the fresh soil samples were gently passed through a 2 cm riddle to remove stones, vegetation, roots and any remaining earthworms that had not emerged beforehand, breaking the soil along its natural lines of weakness and homogenising the three subsamples from each monolith. Subsamples of 70 g ±5 g were wet sieved by hand, using a method adapted from Elliott (1986), using a series of four sieves to obtain five aggregate size fractions following Puerta et al.

(2018): large macroaggregates (>2000 μm), medium macroaggregates (1000-2000 μm), small macroaggregates (250-1000 μm), large microaggregates (53-250 μm) and small microaggregates and disaggregated soil (<53 μm). The remaining soil was stored at 4°C for a repeat of the wet-sieving procedure two and four weeks after initial collection. This information will determine if cold storage prevents microbial activity enough to prevent the degradation of soil aggregates over a short period of time. If so, this will enable the processing of larger experiments where sieving is not able to be completed within a week after sample collection.

Soil was submerged on the 2000 μm sieve in water, 15 mm above the sieve mesh, for 5 minutes and moved vertically back and forth 50 times over a period of 1 minute and 30 seconds. Any floating material and stones were removed and aggregates on the sieve were washed into a pre-weighed aluminium dish. This process was repeated on the remaining soil using a 1000 μm sieve (40 times over 1 minute 10 seconds), a 250 μm sieve (30 times over 1 minute 20 seconds) and a 53 μm sieve (10 times over as long as needed). All fractions were dried in a 105°C oven for 48 hours and weighed.

To calculate the relative contribution of each aggregate fraction to the total sample, the following equation was used: $\frac{fraction\ weight\ (g)}{total\ sample\ weight\ (g)} x100$, total sample weight being the weights of all five fractions in one sample.

The percentage of the largest macroaggregates (>2000 μm) and smallest microaggregates (<53 μm) as a proportion of the total bulk soil was used to determine how the distribution of aggregate size classes changes after storage of up to 4 weeks at 4°C (Fig. 5.2). Over the period of 4 weeks, there is no significant change in the proportion of >2000 μm (p = 0.081) or <53 μm (p = 0.87) aggregates. Therefore, for the rest of this study, the results gained from water-stable aggregate fractionation will be pooled for greater replication. However, as seen in Figure 5.2, the soil in the BSSW field seems very degraded, with a consistently significant higher proportion of small microaggregates and a significantly lower proportion of large macroaggregates compared to all other fields (p <0.001). This trend has been seen before in BSSW compared to the other fields, although the causes behind it is unknown. As a precaution, the analyses in the results section of this project will be carried out excluding the BSSW data.

5.2.5. Inorganic C removal from dried aggregates

Each oven-dried aggregate fraction was homogenised individually by mortar and pestle into a visibly fine powder and 90 mg += 5 mg was weighed into Eppendorf tubes. To each tube, 500 μl 6M HCl was added, which was stirred using a blunt needle and left for 30 minutes. More HCl was added in increments of 100 μl and stirred until the sample stopped effervescing. Samples were left for 24 hours in a fume hood to settle. The supernatant was then pipetted off and samples put in the oven at 105°C for 24 hours to evaporate the remaining HCl.

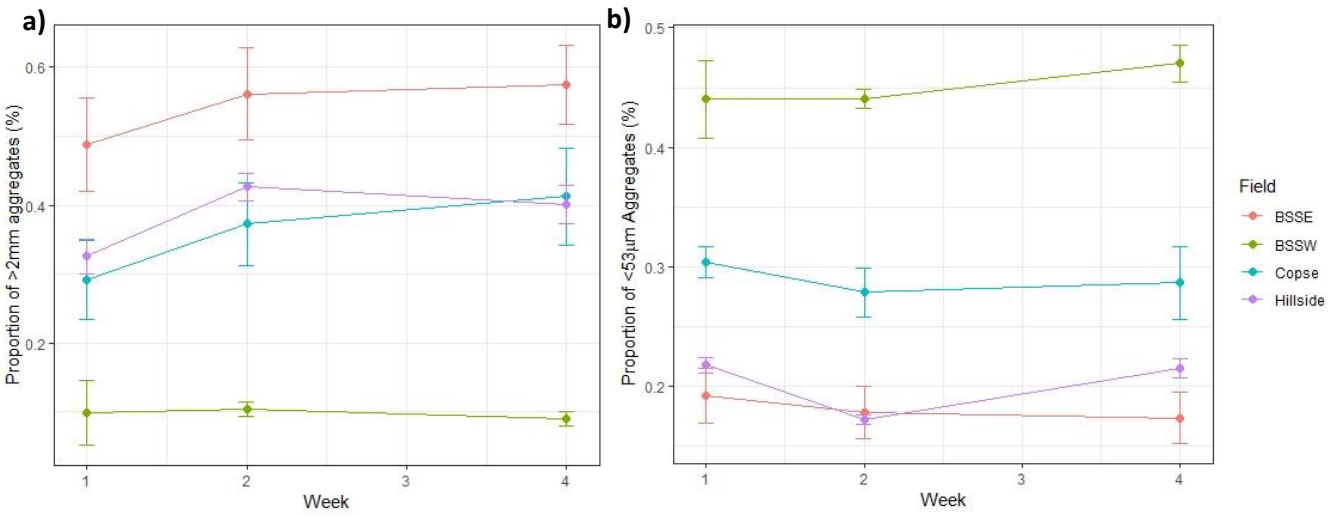

Fig. 5.2. The percentage of **a)** >2000 µm macroaggregates and **b)** <53 µm microaggregates as a proportion of the total bulk soil determined by water-stable aggregate sieving after being stored at 4°C for 1, 2 and 4 weeks.

5.2.6. Total C, organic C and total N analysis of dried aggregates

Sub samples of 30-50 mg of the acid-stripped soil were weighed into tin boats for analysis of OC and total N percentage through dry combustion in a CN analyser (Vario EL Cube, Hanau, Germany). 3-8 mg acetanilide was used as a standard.

To calculate the contribution of each aggregate fraction to the overall C and N content of the total sample, the following equation was used: $\frac{fraction\ weight\ (g)}{total\ sample\ weight\ (g)}\ x\ Concentration\ of Nutrient\ (\%)$

5.2.7. Statistical analyses

The water-stable aggregate distribution data and concentrations of OC and total N were analysed by 2-way ANOVA followed by a Tukey multiple comparison test. The common dependent variables are treatment (F+E, F or control), aggregate size fraction (commonly focused on >2000 µm or <53 µm) and earthworm number or weight. The common independent variable is the proportion of a certain aggregate size fraction. If there was no significant difference between field sites, fields were merged.

As part of the regular management of the arable fields within which the experimental monoliths were located, a selective herbicide (ASTROKerb®, MAPP 16184, Corteva Agriscience™, Cambridge UK) was applied in November 2017. This drifted onto the ley strips in the field 'Hillside', killing the grass in replicate three of the F+E and F treatment, and is implicated in having an adverse effect on the earthworm population, being reported to be of "moderate toxicity to earthworms" (Corteva Agriscience™, 2014). For this reason, data from these samples were removed from the statistical analysis of this dataset.

5.3. Results

5.3.1. Earthworm collection

Table 5.2 shows the total numbers and weights of earthworms in each ecological group in each field recovered from the Control, F, and F+E monolith treatments in April 2018, whilst Fig. 5.3 shows the mean numbers of earthworms from the different ecotypes collected across all fields.

The monolith treatments had a significant effect on total earthworm populations (one-way ANOVA, $p<0.001$), with significantly greater numbers of earthworms collected from the F+E monoliths compared to both the F (frozen only; $p<0.001$) and control ($p=0.02$) treatments. The earthworm population size found in the control treatment monoliths was in between the F+E and F treatments.

However, the monolith treatments (Control, F, F+E) had varying impacts on the different earthworm ecotypes, with numbers of anecic and endogeics being affected by changes in monolith treatment ($p<0.001$), but not epigeics ($p=0.90$). Numbers of anecic earthworms did not significantly differ between the F+E and control treatments ($p=0.13$) but were significantly lower in the F treatment ($p=0.003$, $p<0.001$, respectively), whereas numbers of endogeics in both the F and F+E treatments were significantly lower ($p=0.01$), and higher ($p<0.001$) than the numbers found in the control monoliths, respectively.

Figure 5.3b reports the same data of Figure 5.3a but extrapolated to average number of earthworms per square meter rather than per monolith. This allows the comparison of the earthworm numbers found in this research to previous published work, which tend to use the unit 'earthworms m^2'.

Table 5.2. Total recovered earthworm numbers and weights for each field replicate at the end of the experiment in April 2018. F= Frozen monolith only, F+E = Frozen monolith with earthworm addition. BSSW field is greyed out due to this field being omitted from later analyses due to degraded soil.

Field	Earthworm Ecological Group	Total Earthworms recovered in April 2018					
		Control		F		F+E	
		Number	Weight (g)	Number	Weight (g)	Number	Weight (g)
BSSE	Anecic	11	7.6592	2	1.8837	23	26.6266
	Endogeic	12	3.3319	6	1.3998	84	16.7117
	Epigeic	12	1.9677	27	4.5874	21	2.6981
BSSW	Anecic	19	21.8319	12	5.0583	41	44.6409
	Endogeic	20	5.1358	6	1.8211	76	18.33333
	Epigeic	6	0.89857	49	9.5281	35	5.1734
Copse	Anecic	15	10.587	5	8.5252	24	31.655
	Endogeic	5	1.1328	0	0	79	17.3276
	Epigeic	1	0.259	9	1.4418	18	3.5452
Hillside	Anecic	6	11.3594	8	14.6506	11	17.5971
	Endogeic	13	3.8414	2	1.0146	64	14.6839
	Epigeic	1	0.1536	15	0.8538	7	0.9073
Average	Anecic	12.75	12.86	6.75	7.53	24.75	30.13
	Endogeic	12.50	3.36	3.5	1.06	75.75	16.76
	Epigeic	5	1.04	25	4.1	20.25	3.08

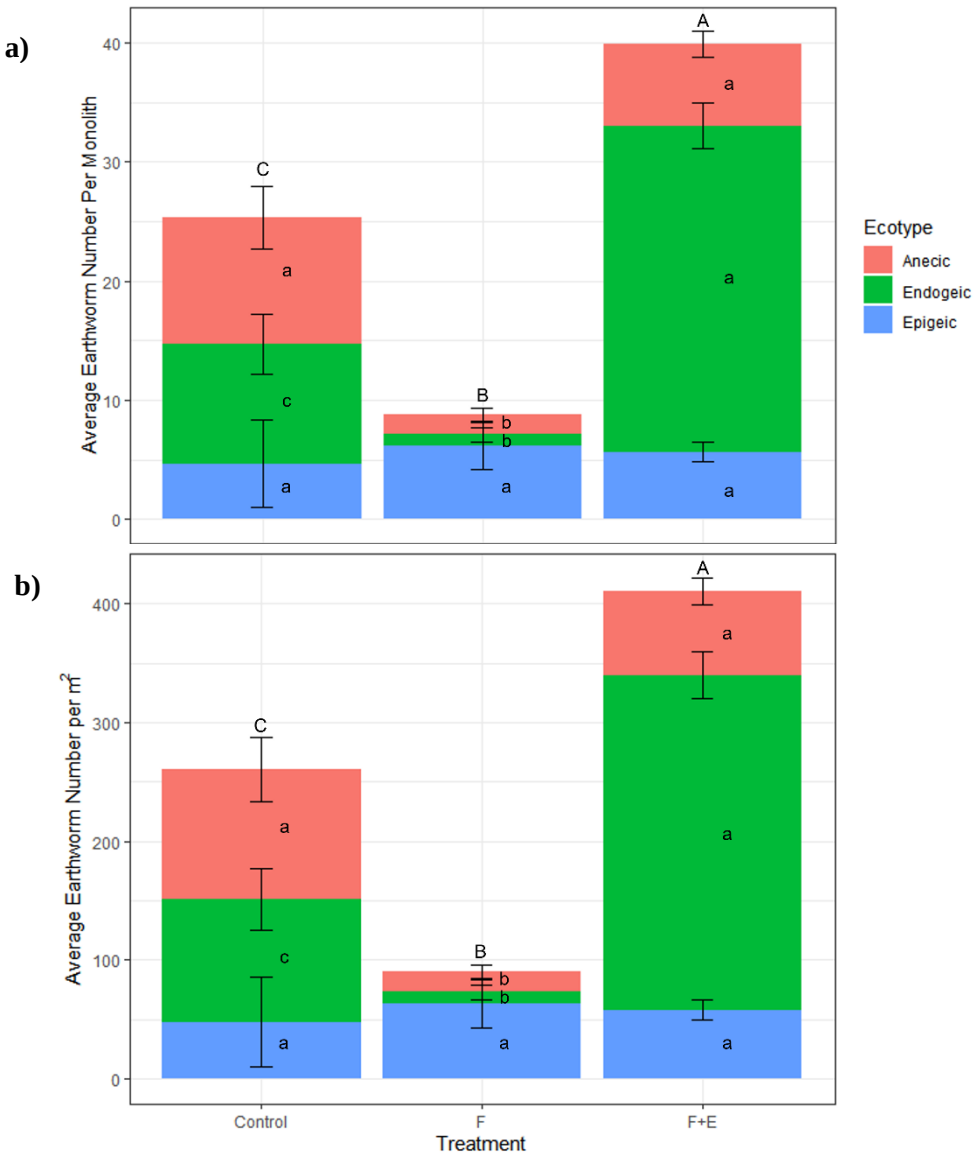

Fig. 5.3. Average earthworm population numbers a) per monolith and b) per m^{-2} separated control, F (frozen) and F+E (frozen + earthworms) treatments and showing the population composition by earthworm ecotype. Different capital letters above bars indicate the total number of earthworms in each treatment is significantly different from each other (Tukey test, $p<0.05$). Different lower-case letters indicate numbers of that ecotype of earthworm are significantly different to that ecotype number in a different treatment (Tukey test $p<0.05$). Error bars represent standard error of the mean (Control n = 3, F and F+E n=9).

5.3.2. Soil water-stable aggregate size distribution by treatment

There was a significant interaction between the effect of monolith treatment on the proportion of soil in each aggregate size fraction (Two-way ANOVA, p=0.022), however, there was only a significant difference between the treatments (control, F and F+E) on the proportion of soil in the 1000-2000 µm fraction Figure 5.4; p=0.01) , with proportionally more 1000-2000 µm aggregates in the control monoliths (6.07%) compared to the F+E-treated (3.89%) monoliths (Tukey test, p=0.007).

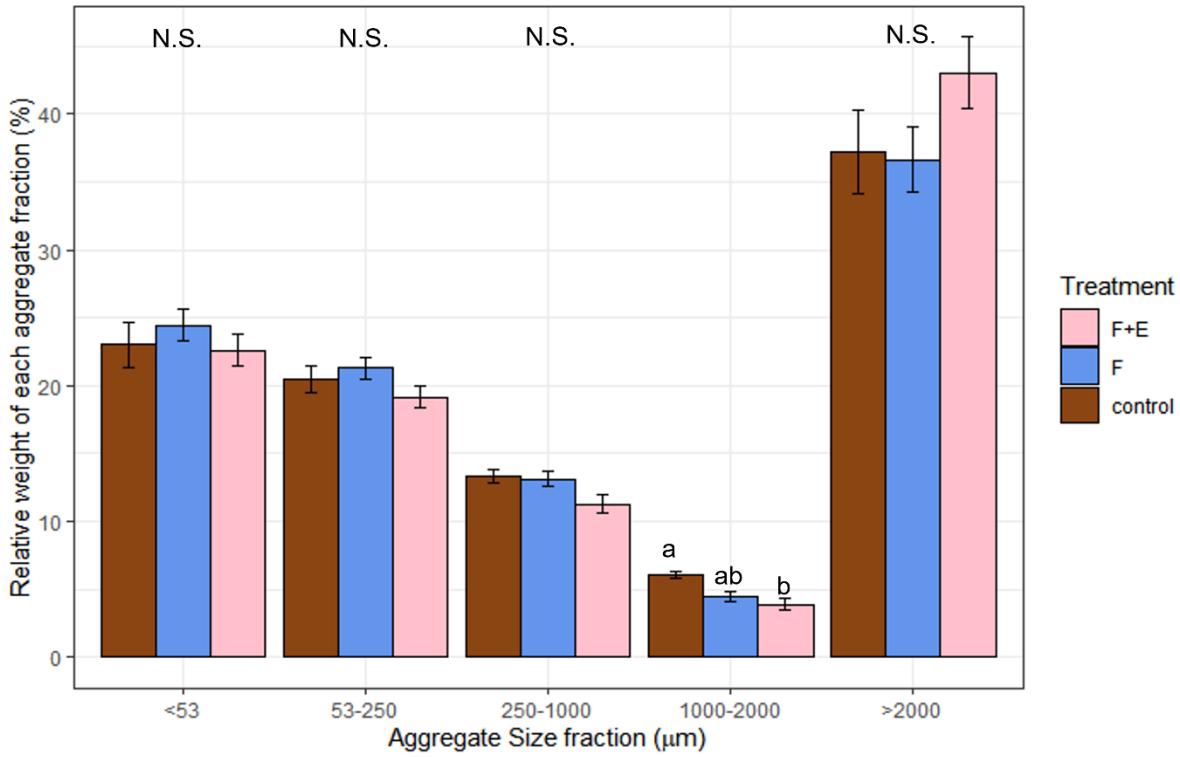

Fig. 5.4. The relative proportion of each aggregate fraction as a proportion of the total soil sample weight, separated by different treatments: control, frozen only (F) and frozen + earthworms (F+E).

The lack of difference between the monolith treatments in the other aggregate size fractions could be potentially because of earthworm numbers and weights being either similar or overlapping between some F+E and F treatments. Although the experiment was initially set up as a replicated experiment with separate treatments, in reality, the monolith replicates are different to each other due to either earthworm or cocoon survival during the freezing stage or earthworm escape or recolonisation during the experiment. Consequently, within-treatment monoliths have a range of earthworm numbers and weights, meaning the treatment groups weren't entirely distinct from one another. Therefore, to combat this, a retrospective analysis using linear regression was used to consider the effect of actual earthworm numbers collected at the end of the experiment on changes in WSA distribution and other analysis of soil properties, rather than the monolith treatments as explanatory variables.

5.3.3. The influence of earthworm numbers on proportion of water-stable macroaggregates

Increasing the total number of earthworms significantly increased the proportion of macroaggregates >2000 μm in the soil monoliths (regression, d.f. = 1, 55, F=7.46, R^2=0.12, p=0.008; Fig. 5.5). It can be seen from the scatter plots that the data points group vertically in multiples of three. This is because soil samples from the same monolith were analysed for water-stable aggregate distribution at three different time points: at one, two and four weeks after sample collection and storage at 4°C. They are used as replicates due to no difference in aggregate size distribution between different numbers of weeks in cold storage (see *section 5.2.4*). Data in Figure 5.5a is coloured by week, allowing the effect of differing storage lengths before WSA fractionation on proportion of soil in the >2000 μm fraction to be visualised. It shows that samples sieved only one week after storage regularly have lower proportions of soil weight in the >2000 μm aggregate fraction compared to the other sampling weeks, although this was shown not to be significant from earlier analysis (*Section 5.2.4, Fig. 5.2*). Figure 5.5b shows the same data as 5a, but colour coded by monolith treatment (control, F, F+E) to enable visualisation of how earthworm numbers were distributed across the different treatment types.

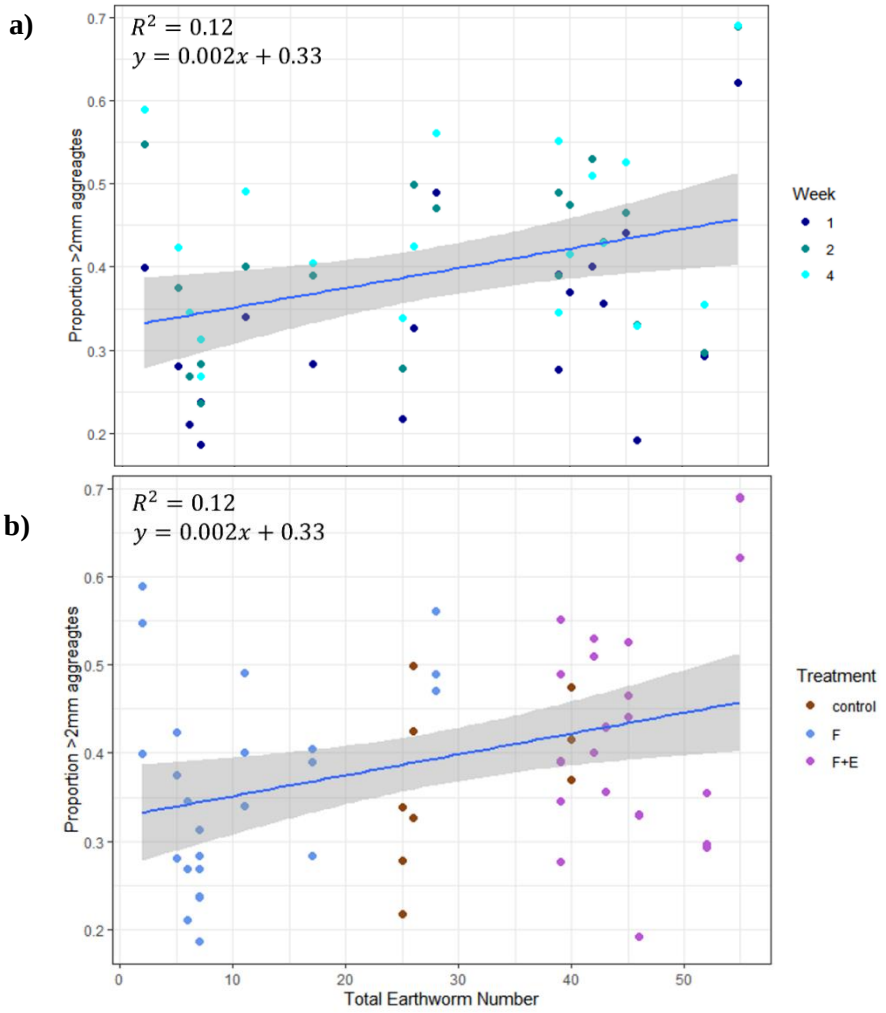

Fig. 5.5. The effect of the total number of earthworms, including all ecotypes, on the percentage weight of soil within the >2000 µm aggregate fraction. A line of the linear regression model is fitted onto the graph (equation of the line *y = 0.002 x +0.33*). Colours of scatter plot points are separated by **a)** week, showing the effect of cold storage on the >2000 µm aggregate fraction or by **b)** monolith treatment, showing how the total numbers of earthworms are distributed between the three different monolith treatments (control, F, F+E). Shaded area around the line of regression indicates a 95% confidence interval.

To determine if any particular earthworm ecotype was associated with increased macroaggregation, due to their different ecologies and activities on different part of the soil profile, lines of regression were plotted for each earthworm ecotype (anecic, endogeic and epigeic) against the proportion of aggregates >2000 µm in the soil (Figure 5.6). Increasing numbers of Anecic earthworms had almost no effect on the proportion of >2000 µm in the bulk soil (d.f.=1,55, F=0.011, *p*=0.92, Figure 5.6a). However, the addition of Endogeic (d.f.=1,55, F=7.01, *p*=0.01, Figure 5.6b) and Epigeic earthworms (d.f.=1,55, F=6.88, *p*=0.011, Figure 5.6c) was significantly related to the increase of macroaggregates.

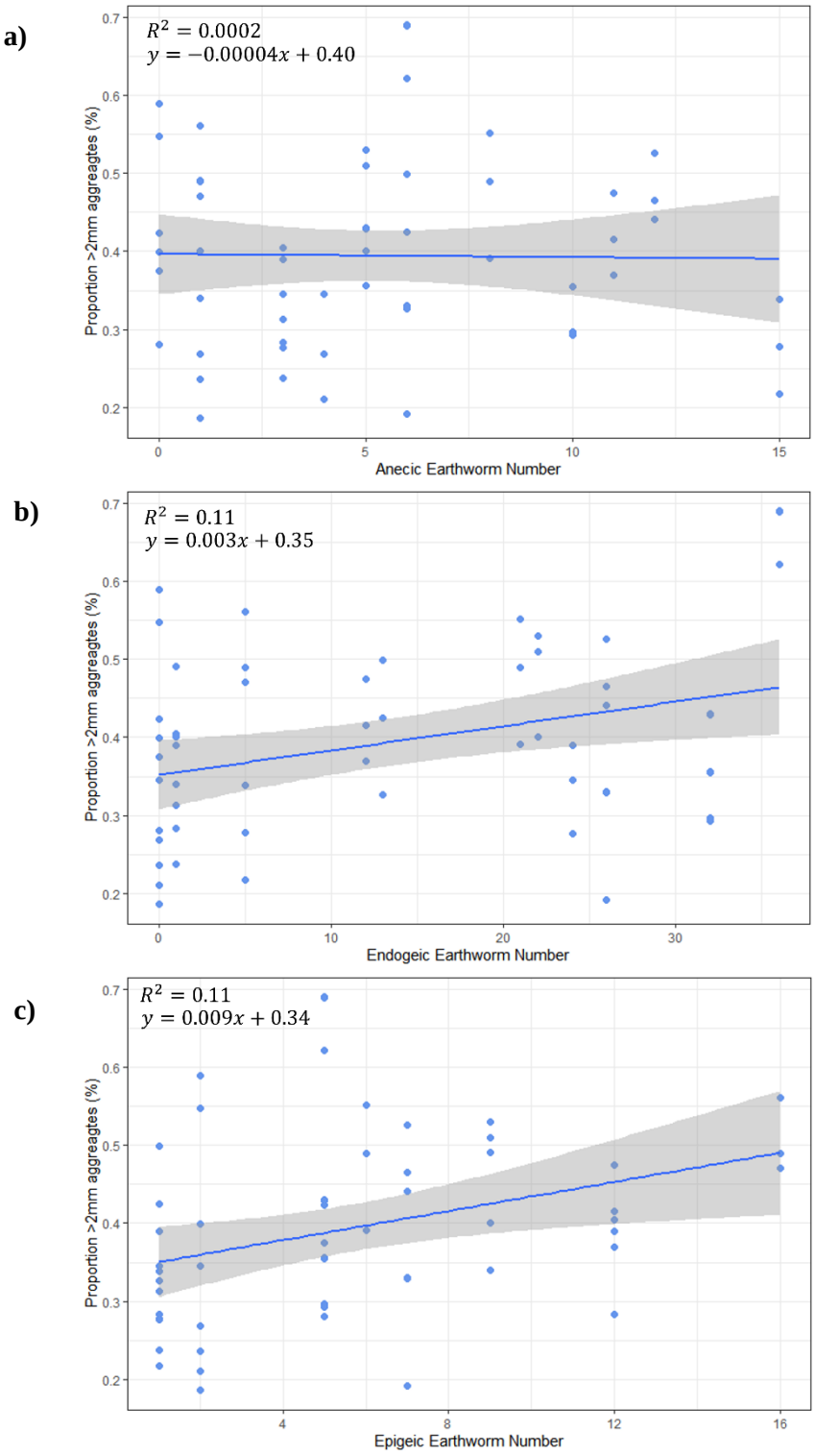

Fig. 5.6. Relationship between the proportion of >2000 µm aggregates in the bulk soil and **a)** anecic, **b)** endogeic and **c)** epigeic earthworm numbers, showing the fitted line of the linear regression model, ($y = 0.40 - 0.00004x, y = 0.35 + 0.003x, y = 0.34 + 0.009x$, respectively).

5.3.4. Organic carbon storage by aggregate size

There was no significant effect of earthworm treatments in the monoliths on total SOC in bulk soil (one-way ANOVA, p=0.76), with both F and F+E soils having a total SOC concentration of around 1.71% and control monoliths having a slightly lower value (mean = 1.66%). However, apportioning OC by aggregate size fractions revealed that the, F+E-treated monoliths had significantly greater OC stored within the >2000 μm macroaggregates (Tukey test, p=0.029) but significantly less OC stored within the <53 μm microaggregates (Tukey test, p=0.002) compared to the F-treated monoliths (Fig. 5.7a). Nitrogen storage follows a similar trend, where there is no significant difference in total N of bulk soil between monolith treatments (one-way ANOVA, p=0.98), but there is a significantly greater proportion of this N stored within the >2000 μm macroaggregate fraction of the F+E compared to the F monoliths (Tukey test, p=0.008). There are no significant differences in amounts of N in any of the aggregate size fractions <1000 μm across the three monolith treatments (one-way ANOVA, p>0.05; Fig. 5.7b).

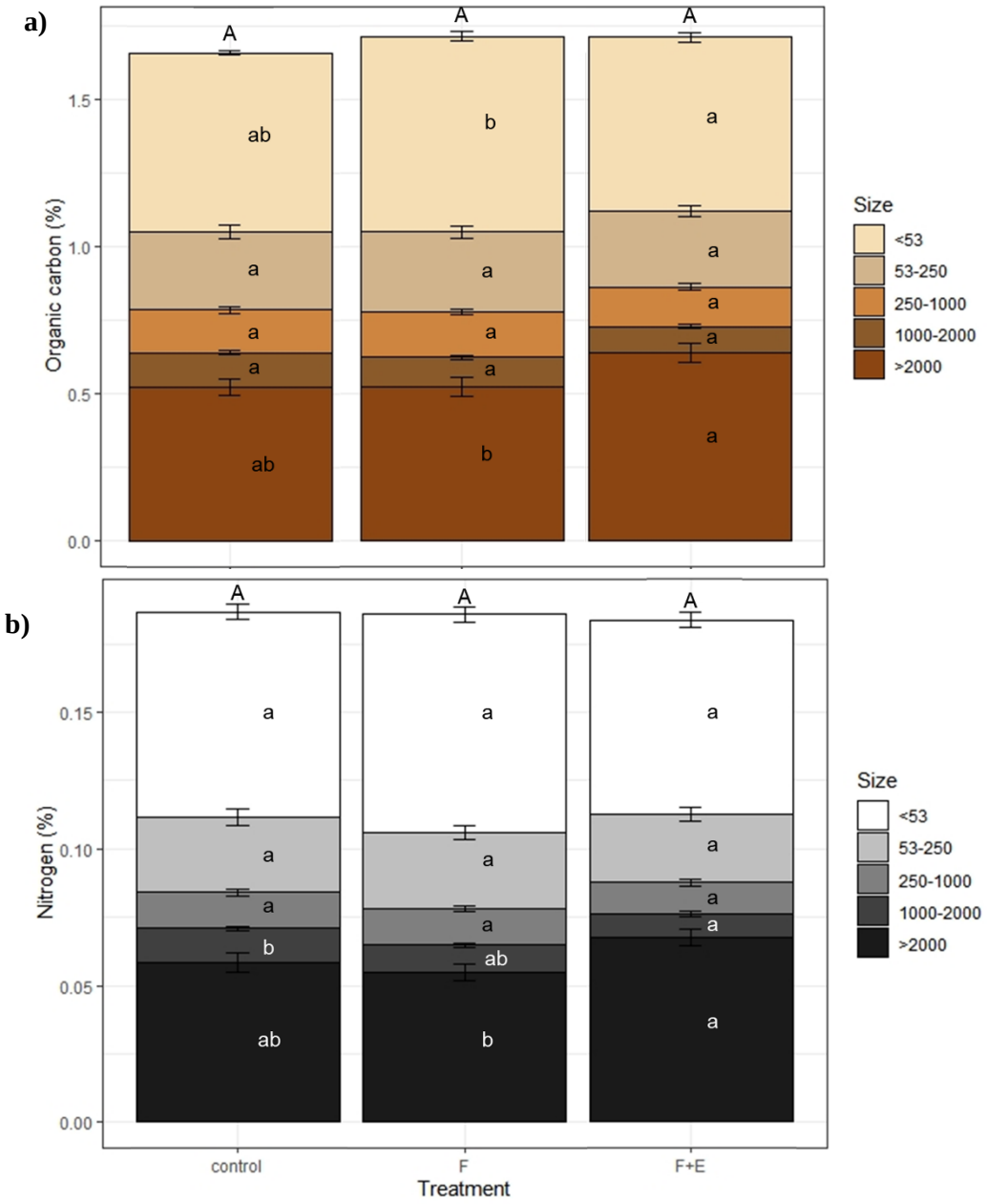

Fig. 5.7. The effect of monolith treatment (F, F+E and control) on **a)** OC and **b)** N storage within five different aggregate size fractions. Aggregate size fractions of the same size with different lowercase letter codes denote significant differences between F, F+E or control means (Tukey test, $p<0.05$) and bars with different capital letters above bars indicate a significantly different total SOC or N concentration between monolith treatments (Tukey test, $p<0.05$). Bars represent standard error.

5.3.5. *Effects of earthworm numbers on organic carbon storage*

The relationships between total earthworm numbers and numbers of earthworm ecotypes on OC and N storage was investigated to better understand their contributions to soil properties (Fig. 5.5b). With an increasing total number of earthworms, the relative contribution of the >2000 µm fraction to the bulk SOC increases (R^2=0.22; d.f.=1,17, F=4.78, p =0.043; Fig. 5.8a). The contribution of the individual earthworm ecotypes differs. Increasing the number of anecic and endogeic earthworms does not increase the contribution of the >2000 µm macroaggregate fraction to the bulk SOC% (p=0.91, p=0.067, Fig. 5.8b,c, respectively). However, the amount of OC stored in the macroaggregate fraction does increase with increases in numbers of epigeics (R^2=0.25; p=0.03; Fig. 5.8d). Numbers of endogeic and epigeic earthworms explain the trend in OC storage within macroaggregates the most (R^2=0.18, 0.25, respectively), whilst anecics do not (R^2<0.001).

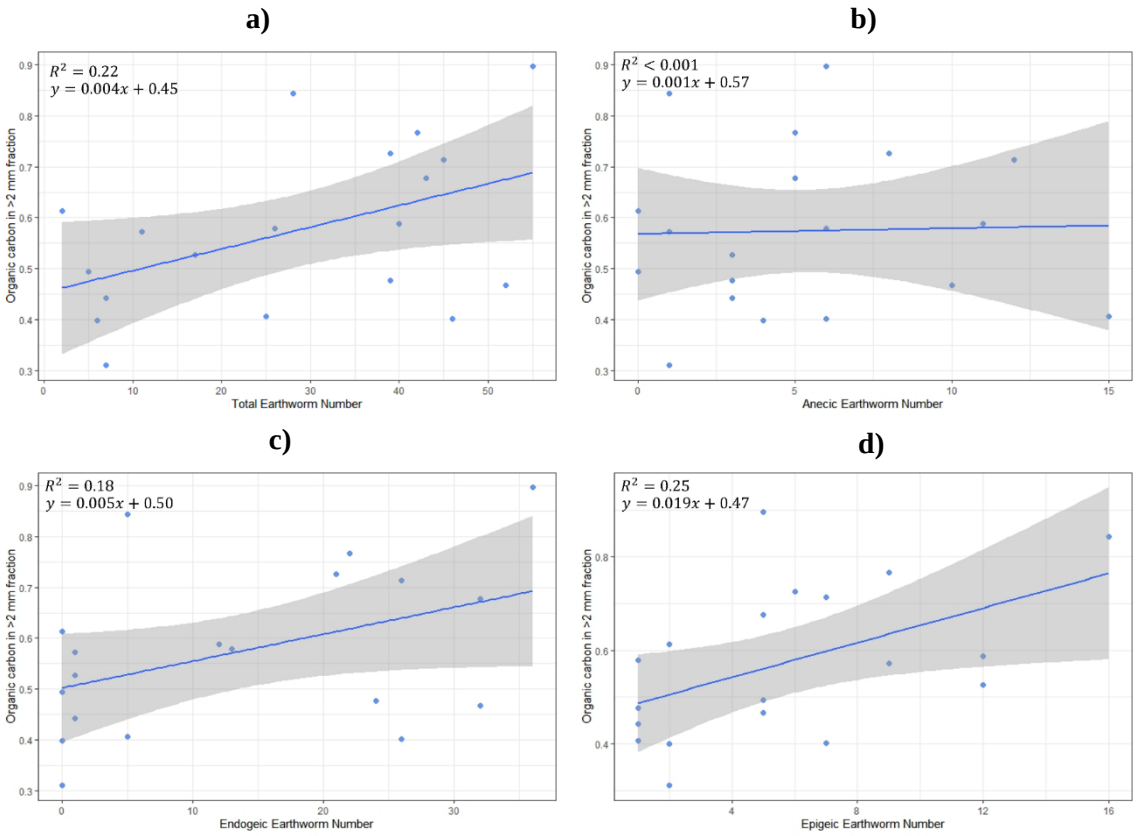

Figure 5.8. The relationship between the number of **a)** total, **b)** anecic, **c)** endogeic and **d)** epigeic earthworm numbers and the contribution of the >2000 µm aggregate fraction to the total organic carbon % of the bulk soil, showing the fitted line of the linear regression model, equation of the line and multiple R^2 value. The shaded area around the line of regression shows the 95% confidence interval.

5.3.6. Nitrogen Storage Distribution

Similar to the trend seen in the OC storage, increasing the total number of earthworms also increases the storage of bulk N within the >2000 µm fraction (R^2=0.23; d.f.=1,17, F=5.19, p=0.036; Fig. 5.9a). Also, increasing the number of anecic earthworms does not increase the contribution of the >2000 µm aggregate fraction to the overall N content of the soil (R^2=0.002; d.f.=1,17, F=0.03, p=0.86; Fig. 5.9b). In contrast to the patterns seen in SOC storage, endogeic earthworms have the greatest influence upon N storage within macroaggregates (R^2=0.23; p=0.04; Fig. 5.9c), whilst epigeics are not influential (R^2=0.15; p=0.1061; Fig. 5.9d).

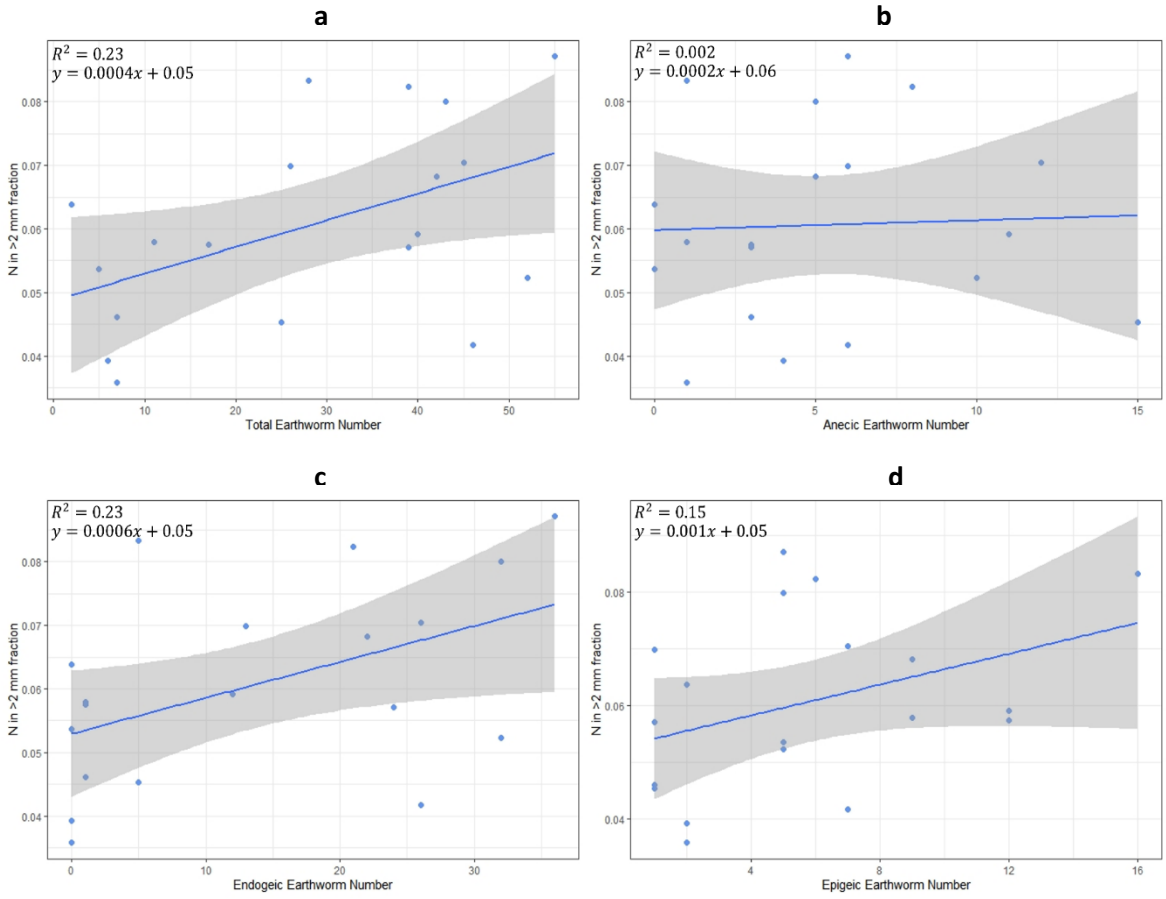

Figure 5.9. The relationship between the number of **a)** total, **b)** anecic, **c)** endogeic and **d)** epigeic earthworm numbers and the contribution of the >2000 µm aggregate fraction to the total nitrogen % of the bulk soil, showing the fitted line of the linear regression model, equation of the line and multiple R^2 value. The shaded area around the line of regression shows the 95% confidence interval.

5.4. Discussion

This study builds on recent research by Hallam et al. (2020) which determined the roles of earthworm ecotypes and a variety of earthworm species on soil physico-hydraulic and chemical properties, including effects on the proportion of WSAs >250 μm. In this study, we build on this knowledge using the same monolith treatment experiment to investigate changes in the distribution of more detailed aggregate size fractions, plus, in addition to conventional measurements of earthworm influence on total soil organic matter (SOM) and N, in this study, we determine how the storage of OC and N are distributed throughout the different aggregate size fractions.

The F+E treatments were successful in enriching earthworm numbers compared to F and control treatments (Fig. 5.3). This was mainly driven by significant increases in endogeic and anecic earthworms (Fig. 5.3), ecotypes which are most abundant in the grassy margins of the arable fields studied in the present study, and in adjacent permanent pastures (Holden et al., 2019). In contrast, a study by Prendergast-Miller et al. (2021) on the same arable fields found endogeics to be the most abundant, followed by epigeics, but annual earthworm numbers per m^2 found the field margins were 619 ± 355 from 2015-2017, overlapping with the density of 410 ± 39 m^2 in this study for the F+E treatment. The majority of earthworms recovered from the F treatment were epigeics, which are known to have high dispersal rates (Chatelain and Mathieu, 2017), making it likely that these earthworms either evaded the nylon mesh barriers surrounding the monoliths during the experiment, or they tolerated the freezing temperatures of the defaunation stage. The latter is more likely due to their greater cold resistance compared to anecic and endogeic ecotypes (Meshcheryakova and Berman, 2014).

Hallam et al. (2020) measured the effect of the same monolith treatments (control, F and F+E) on the percentage of WSA >250 μm, which significantly increased in the F+E treatment compared to the F monoliths (70 ± 3% vs 60 ± 3%). However, in this study we see no significant difference between the F+E and F treatments in any of the fractions above 250 μm diameter (Fig. 5.4). However, we do see a significant increase in percentage of macroaggregates in the >2000 μm fraction after using absolute earthworm numbers as an explanatory variable in a regression analysis in preference to monolith treatments (Fig. 5.5). WSAs are a key determinant of soil structure and quality (Churchman, 2010), allowing for higher water-holding capacity (Hallam and Hodson, 2020; Zibilske and Bradford, 2007). Hallam and Hodson (2020) found that both the addition of the anecic earthworm *Lumbricus terrestris* and the endogeic earthworm *Allolobophora chlorotica* increased the percentage of WSAs >250 μm in the upper 6.5 cm of soil to 74 ± 7% and 74 ± 11 %, respectively compared to 53 ± 9% in the earthworm-free controls, with *L. terrestris* having a weaker effect in the lower 6.5-13 cm of soil where casts are less abundant, increasing %WSA to only 67 ± 7%. However, much higher earthworm densities (2 *L. terrestris* or 8 *A. chlorotica* per 16x13 cm mesocosm) were used in this experiment due to the short 40-day time scale. In this study, we see no correlation between an increase in anecic earthworm numbers and % macroaggregates >2000 μm when sampling to a similar soil depth as Hallam and Hodson (2020)

(6.5 vs. 7 cm in this study). The behaviours of anecic earthworms in producing vertical burrows may be more important for the role of water infiltration during heavy rainfall events than in the process of soil macroaggregate formation (Andriuzzi et al., 2015; Pitkänen and Nuutinen, 1998), which are becoming more critical as a result of climate change increasing the frequency and intensity of extreme rainfall events (Groisman et al., 2005; IPCC, 2019).

We do, however, see a significant relationship between the numbers of endogeics and % macroaggregates >2000 μm (p = 0.01), reflecting results from previous studies which have linked endogeic earthworms and increasing %WSAs (Blanchart, 1992; Bossuyt et al., 2005; Buck et al., 2000). Their involvement is likely due to the burrowing activities of endogeics incorporating OM into the soil, which is known to play a key role in promoting soil aggregation and stability (Li et al., 2021). In this study we also find that epigeics are correlated to increasing the percentage of >2000 μm aggregates (p=0.011). This is surprising due to their preference to live on the soil surface and rarely make burrows, which has been previously attributed to their lesser effect on soil aggregation compared to the other earthworm ecotypes (Bossuyt et al., 2006; Shipitalo and Le Bayon, 2004).

The non-significant differences in bulk SOC between the F and F+E treatments (Fig. 5.7a) is not surprising, as any increases in bulk SOC over this relatively short timescale are often small compared to the existing stocks, masking any differences between treatments. For example, most short-term studies investigating the introduction of leys on SOC stocks, which are known to increase SOC through increased OM inputs (Wiesmeier et al., 2019) often only find non-statistically significant differences in bulk SOC (Gosling et al., 2017; Puerta et al., 2018), with studies spanning multiple decades needed to detect these differences (Jarvis et al., 2017; Johnston et al., 2017; Kirk and Bellamy, 2010; Poeplau and Don, 2015; Prade et al., 2017).

Puerta et al. (2018) did detect a significant preferential accumulation of OC within large macroaggregates (>2000 μm) after arable soil had been put under ley for two years but did not consider changes in earthworm populations from arable to ley conversion. An increase of OC within this fraction is important as macroaggregate structures provide protection of OC in mineral soils (Aoyama et al., 1999; Mikha and Rice, 2004; Puget et al., 2000; Yu et al., 2015; Zhou and Pan, 2007). Macroaggregate-associated OC is better protected against microbial degradation (Balesdent et al., 2000; Plante and McGill, 2002a) by physical protection of microaggregate-bound OC further encased in macroaggregates (Bronick and Lal, 2005; Devine et al., 2014). With layers being built to form larger soil aggregates, older OC is trapped within (Santos et al., 1997), preventing its release into the atmosphere (Lal, 2004a; Stavi and Lal, 2013) and preventing further contribution to the already high global agricultural anthropogenic greenhouse gas emissions (IPCC, 2007). Therefore, it is important to investigate whether any changes in the distribution of OC storage throughout the different aggregate size fractions is occurring.

We do find a preferential storage of OC within macroaggregates >2000 μm in this study (Fig. 5.7a). This development has previously been seen in soils under no-till management (Messiga et al., 2011; Wright and Hons, 2005), which is also experienced during the experimental period where the monoliths are buried within the grass-clover ley strips. A similar trend is seen in the N storage distribution, where bulk N does not increase from F to F+E treatment but is preferentially stored within the largest macroaggregates (Fig. 5.7b). A study by Fahey et al., (2013) tested the difference between leaf litter C and N incorporation into SOM pools in communities dominated by the anecic earthworm *L. terrestris* and the epi-endogeic species *L. rubellus*, to try and quantity the effects of both earthworm species on these processes. In communities dominated by both earthworm species, labelled N and C was found to be enriched in macroaggregates (>250 μm) and microaggregates (53-250 μm). However, plots with no earthworms, had the highest ^{13}C and ^{15}N enrichment in the silt and clay fraction, which is less likely to result in long-term stabilisation. This is partially reflected in this present study, as the preferential storage of OC within macroaggregates >2000 μm (p=0.043; Fig. 5.8a) and N (p=0.036; Fig. 5.9a) is correlated with an increase in total number of earthworms but is only associated with greater numbers of epigeic and endogeic ecotypes, and not anecics.

Earthworms are widely recognised as beneficial for rebuilding soil structure and quality, with this study highlighting the particular importance of endogeic and epigeic earthworms in grass-clover leys in forming macroaggregates and storing OC and N within these structures, potentially contributing to OC sequestration in arable to ley conversions. However, current agricultural land management practices must change, as common approaches, such as tillage (Briones and Schmidt, 2017; Crittenden et al., 2014; Edwards and Lofty, 1982) and herbicide application (Gaupp-Berghausen et al., 2015; Van Hoesel et al., 2017; Zaller et al., 2014) are detrimental to earthworm abundance, diversity, activity and reproduction.

A collection of published papers produced from the same ley strips sown into the same conventionally-tilled arable fields as this present study develop a resounding argument for the improvement of soil aggregation and related soil functioning by leys, including improved drought and flood resilience, SOC sequestration and earthworm populations (Berdeni et al., 2021; Hallam et al., 2020; Prendergast-Miller et al., 2021). However, with the multitude of variables that change with the introduction of a grass-clover ley, this study helps to distinguish the importance of earthworm population recovery within leys for soil aggregation and OC sequestration. Here, we are able to detect the specific effects of earthworm and their individual ecotypes on top of the other likely beneficial effects that are associated with the introduction leys, including perennial roots and their associated mycorrhizal fungal hyphae which enmesh soil aggregates together (Tisdall and Oades, 1982). This study, after only one year under ley, also provides strong support that changes in soil structure precedes changes in SOC accumulation into macroaggregates which can be detected using this methodology of detecting where bulk SOC is stored throughout the WSA size fractions. It is interesting to compare to Chapter 2, where the same

methodology was used to detect changes in SOC distribution, as more than double the amount of macroaggregate-associated (>2000μm) OC was found in the three-year ley compared to that found here after a one-year ley.

The introduction of leys to improve the prevalence of earthworms would need to be used alongside a less intensive form of agriculture when the field is put back into arable rotation after the ley to avoid depleting their abundance, beneficial activities (Briones and Schmidt, 2017; Crittenden et al., 2014; Wyss and Glasstetter, 1992), and further detrimental effects on SOC stocks and soil structure, leading to disaggregation, slumping and compaction (Chyba et al., 2014; Pires et al., 2017; Haghighi et al., 2020). Even though anecics are especially sensitive to CT compared to smaller, more shallow burrowing endogeics (Edwards and Lofty, 1982; Wyss and Glasstetter, 1992), which we found are especially important for macroaggregation and macroaggregate-associated N, anecics are beneficial for their alternative ecosystem service provision for their role in soil hydrological functioning (Andriuzzi et al., 2015; Pitkänen and Nuutinen, 1998). Alternatively, less disruptive tillage systems, such as conservation or no-tillage, are needed to support the populations of both earthworms (van Capelle et al., 2012) and soil microbes (Badagliacca et al., 2018a, 2018b; Cookson et al., 2008; Helgason et al., 2009; Zuber and Villamil, 2016) including arbuscular mycorrhizal fungi (Lu et al., 2018), which also play a key role as biological agents in the formation and stabilisation of soil aggregates (Johan Six et al., 2002; Tisdall and Oades, 1982) and form key interactions with each other (Brown, 1995). Briones and Schmidt (2017) demonstrated that NT and conservation agriculture significantly increases earthworm abundance (137% and 127%, respectively) and biomass (196% and 101%, respectively) compared to when soil is managed under CT.

Although, there are consequences to NT management, such as the increase in prevalence of certain pathogens that originate from crop residues, for example *Fusarium graminearum,* the predominant cause of the major cereal disease Fusarium Head Blight, which can cause serious impacts upon grain quality and yield (Del Ponte et al., 2017; Singh et al., 2016; Smith, 2007; Sutton, 2009). However, recent work published by Jorge-Escudero *et al.* (2021) evidences that these pathogens can be substantially reduced to undetectable levels by anecic and epigeic earthworms by reducing straw coverage on the soil surface. Earthworms are also successful at combatting soil-borne plant diseases and have the potential to be used as a biocontrol agent (Lagerlöf et al., 2020). Therefore, building up active populations of litter-feeding earthworms during the ley phase of arable rotations may work well in tandem with no-till cropping to facilitate a transition to a lower intensity system with less disease burden and other enhanced soil ecosystem services which earthworms can help provide (Plaas et al., 2019).

Finally, it must be noted that although we see significant correlations between total earthworm numbers and the abundance of >2000 μm macroaggregates and OC and N stored within this fraction, driven by

endogeic and epigeic earthworm ecotypes, R^2 values in all significant correlations indicate that changes in total and ecotype earthworm numbers do not fully explain the variation seen in the datasets. This means that other factors are involved in the processes we measured, which likely involve the other biological, physical and chemical aggregating agents described by Tisdall and Oades (1982), such as plant roots and fungal hyphae which are enmesh macroaggregates and bacteria mucilages and polysaccharides which act as glues to build microaggregates up into macroaggregates.

5.5. Conclusion

This experiment determined how WSA distribution and OC and N storage within macroaggregates altered when long-term arable fields under CT are converted under ley with a supplemented and depleted earthworm population. The findings from this study not only confirm the importance of enhancing earthworm numbers for better soil aggregation, but also their ability to better store OC and N within the important protective macroaggregate structures, and the particular importance of endogeic and epigeic ecotypes in these processes. Although there are other factors and aggregating agents involved in these processes, there is a clear importance to alter agricultural land management practices to promote earthworm abundance and improve earthworm ecotype diversity to enhance the functional diversity in soil ecosystem services which can be provided by these invaluable ecosystem engineers.

Acknowledgements

I am thankful for the generosity of Jamal Hallam and his supervisor Mark Hodson to allow the sampling of his earthworm manipulation monoliths which were integral for this study, and for the help of Jonathan Leake and Thorunn Helgason for their help with preparation of this chapter.

Chapter 6

6. General Discussion

6.1. Introductory remarks

In this study, I have conducted a wide-ranging investigation into the impacts on key soil physical, chemical and biological properties of incorporating grass-clover leys into conventionally managed, ploughed arable rotations that are typical of arable land in Eastern England and Scotland, making this research applicable to farming systems across much fertile land throughout the UK. This thesis benefited from the use of the 'SoilBioHedge' experimental leys funded by the NERC Soil Security Programme based at The University of Leeds Farm in Tadcaster, UK (53°52'25.2"N 1°19'47.0"W). This project involved the sowing of mown, but not fertilised grass-clover ley strips into conventionally ploughed fields that had been under arable rotation for decades. This 'space for time' experimental approach allowed the comparison of multiple soil variables sampled at the same time between arable and three-year ley soils across four replicate conventionally managed arable fields during the course of my PhD, which would not have been possible if the leys were established at a whole-field scale. This space-for time approach allowed me to sample soil on the same day in the same year, enabling the comparison of soil variables such as metabolomes, microbiomes and even soil aggregates that could vary seasonally and inter-annually depending on preceding weather conditions.

Chapter 2 assessed the effect of introducing the grass-clover ley for three years on the water-stable aggregate (WSA) size distribution and the distribution of organic carbon (OC) and nitrogen (N) stored throughout these size fractions and the bulk soil. Chapter 3 assessed the impacts of introducing the three-year grass-clover ley on the soil microbial community structure, through sequencing of ribosomal RNA of both fungal and bacterial communities. In Chapter 4, the effect of introducing the three-year grass-clover ley on the soil metabolome was assessed, determining whether organic molecules which are known to be involved in the formation of soil macroaggregates increase under ley. Chapter 5 used a monolith-based field experiment approach of arable-to-ley conversion for one year, where earthworm populations were physically manipulated to determine the effect of overall earthworm numbers and individual earthworm ecotypes on soil aggregation and OC and N storage within macroaggregates.

Here, in this general discussion, I will synthesise the key findings across these experimental chapters, focusing on the soil parameters which change after the introduction of a grass-clover ley into an arable rotation, and how this is likely to increase the formation of soil macroaggregates and macroaggregate-associated OC and N, as presented in Chapter 2. I also discuss the implications of this thesis for policy, especially in terms of rewarding farmers for sustainable farming practices via the new post-Brexit

Environmental Land Management Scheme (ELMS; Defra, 2021a), which will replace the Common Agricultural Policy (CAP) subsidies from the European Union (EU).

6.2. Summary of findings

6.2.1. Grass-clover leys improve soil aggregation and carbon storage

Chapter 1 reviewed the current knowledge of the effect of intensive farming practices on soil properties and functions together with consideration of the potential restorative effects of leys being reintroduced into arable rotations. Throughout this thesis, an emphasis has been placed on the potential positive benefits of introducing grass-clover leys into arable rotations to enable soil to recover structurally and nutritionally from intensive arable cropping, involving short rotations of profitable cereal crops, often established by annual ploughing and harrowing (Townsend et al., 2016; Wezel et al., 2014).

The research presented in this thesis has contributed to advancing knowledge about the mechanisms and rates by which arable-to-ley conversion can regenerate soil properties and functions and has particularly emphasised the importance of living roots and soil organisms in these processes (Fig. 6.1).

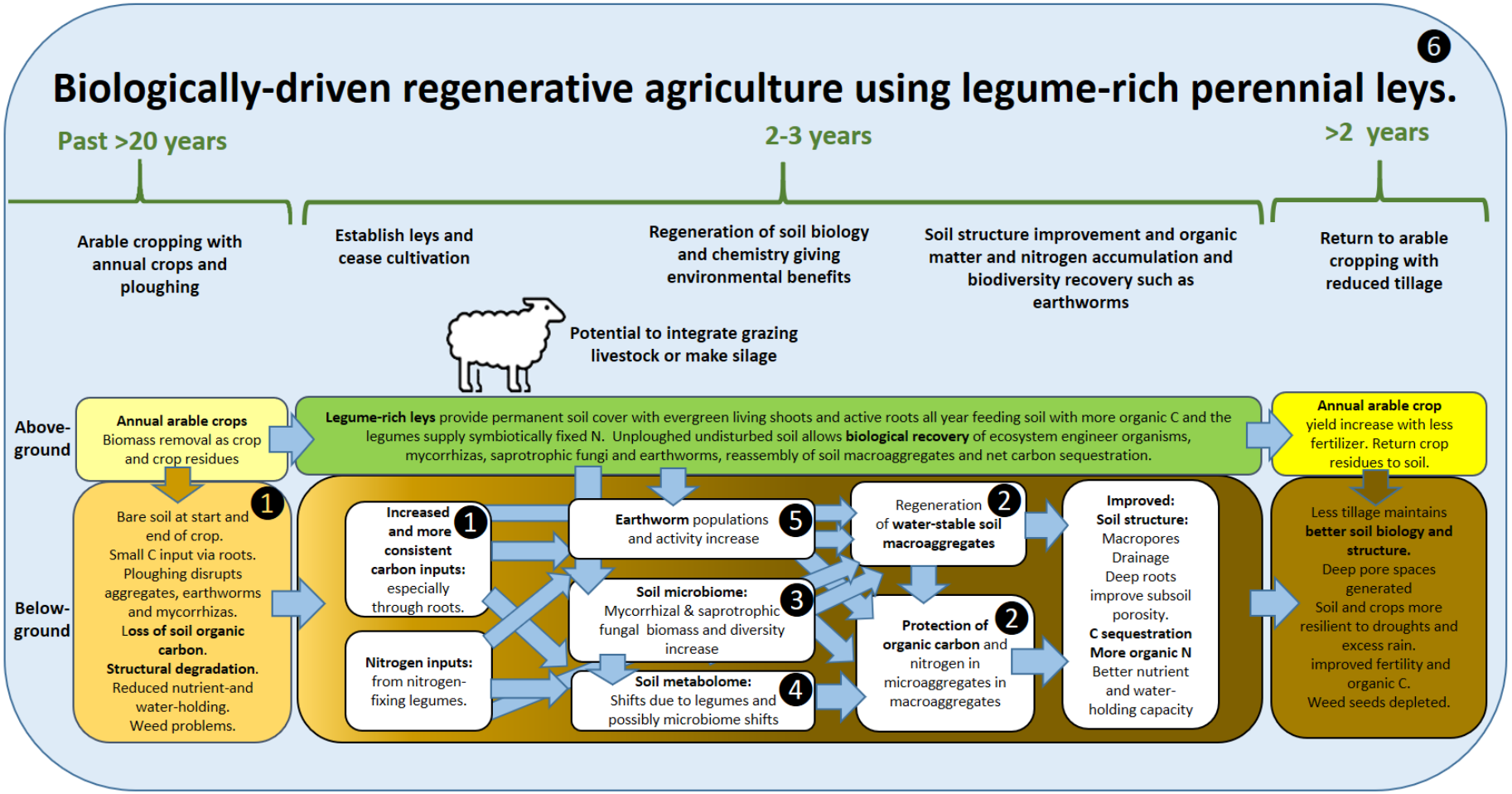

Fig. 6.1. Overview of the effects of reintegration of clover-rich leys after long-term arable annual cropping with ploughing and harrowing for many decades, as has been common in lowland farming in Eastern England and Scotland. The numbered components indicate the chapter numbers in this thesis that review and present new information on the specific components of the system. Arrows show drivers and changes as a result of the change in land use and management.

In Chapter 2, I revealed that the introduction of a three-year grass-clover ley into arable fields which had been cropped annually for decades, significantly increased the proportions of the largest macroaggregates (>2000 μm) 5.4-fold, recovering to similar abundances as that seen in adjacent hedgerow soils. This substantial increase in proportion of macroaggregates is likely to equate to better functioning soils with better structure and macropores to support better hydrological functioning and crop growth (Hallam et al., 2020). Even more importantly, the total amount of OC stored within macroaggregates increased significantly from 2.0 Mg ha^{-1} in the arable soils to 9.6 Mg ha^{-1} in the leys (p=0.02), which no longer differed significantly from the 12.1 Mg ha^{-1} under hedgerows (p=0.48). This equates to a three-times faster rate of soil organic carbon (SOC) accumulation in this specific pool than in the bulk soil, at 2.53 Mg C ha^{-1} yr^{-1}. This macroaggregate-associated OC is more likely to be protected from microbial decomposition (Balesdent et al., 2000; Plante and McGill, 2002a) and subsequent release into the atmosphere, therefore sequestration into these structures helps to reduce agricultural anthropogenic CO_2 emissions. The rate of SOC sequestration in the bulk soil over the three years of ley equated to 2.3 Mg C ha^{-1}, equalling a yearly increase of 0.77 Mg ha^{-1} yr^{-1} or 2.83 Mg CO_2 ha^{-1} yr^{-1}. Although this increase was not statistically significant it is highly likely that it is real effect, especially as it is attributable to the accumulation of SOC in macroaggregates, which was highly statistically significant. If the increase in SOC in the top 7 cm of soil we found over 3 years was repeated by arable-to-ley conversion over the 4.5 million ha used to grow UK crops, this would equate to 12.7 million tonnes CO_2 per year which is 4% of UK emissions of 326 million tonnes C in 2020 (Department for Business, Energy and Industrial Strategy, 2021).

Clearly not all land would be put into ley at the same time, and the amounts of C sequestered in the magnesium and calcium rich Aberford series soil (Cranfield University, 2021) near Tadcaster in the present study may be greater than on some other soil types. Furthermore, without studying the profile depth distribution of SOC it is not possible with certainty to equate the increases seen in surface topsoil to net increases in sequestration (Sun et al., 2014), although in the present study this is likely to be a whole-profile increase. In the five-year study of effects of no-tillage arable on SOC at the Scottish Crop Research Institute, Sun et al. (2014), found SOC in the top 10 cm of arable soil increased from 30 kg m^3 in the conventional ploughed treatment to over 42 kg m^3 in a no-till treatment. However, the total SOC to 55 cm depth did not differ between the treatments, partly due to ploughing apparently burying organic matter (OM) deeper in the soil where it may be more stable. An important difference between arable-to-ley conversion and adopting no-tillage arable is that leys add more OC to the soil, especially through roots (McNally et al., 2015). Although, the additional C does appear to preferentially accumulate in the top 6 cm of the soil in the first two years of ley (Puerta et al., 2018), we would expect these effects to extend progressively deeper into the soil, with time. Reassembly of C-storing WSA >2000 μm has been shown to happen first at the top of the topsoil and takes much longer to regenerate deeper into the profile, as first shown by Greacen (1958) in arable to pasture conversion in Australia.

In the present study, N sequestration and storage distribution throughout the different aggregate size fractions paralleled that seen for SOC, and macroaggregate-bound N increased after arable soil had been under ley for three years. The 0.18 Mg ha^{-1} increase in total N that was seen from the arable-to-ley conversion equates to a rise of 180 kg ha^{-1} over the three years, which is greater than the yearly average N fertiliser applications in England, at 143 kg N ha^{-1} for 2019/20 (Defra, 2021d). This soil fertility building potentially can lead to significant reductions in fertiliser demand, at least for the first crop grown after the ley (McKenna et al., 2018b). As environmental pollution from manufacturing and use of nitrogen fertiliser accounts for 43% of the greenhouse gas emissions associated with the production of a loaf of bread in the UK (Goucher et al., 2017), this fertility increase will have substantial public benefits.

The earthworm manipulation experiment in Chapter 5 evidenced that greater numbers of earthworms equated to greater proportions of macroaggregates >2000 μm, which was mostly driven by endogeic and epigeic ecotypes. The amount of macroaggregate-associated OC also increased with increasing total earthworm numbers, but was especially driven by epigeics, and macroaggregate-associated N being driven by endogeics. These results are based on correlations as the earthworm treatments showed some convergence, with some of the defaunated frozen monoliths apparently either experiencing earthworm colonisation from the surrounding ley strips, or survival of earthworms or their cocoons despite deep-freezing, as discussed by Hallam et al. (2020). It is important to recognize that the earthworm populations in the mesocosm study with new ley up to 1 year old by the end of the experiment were low and still recovering relative to the population densities seen after 2 years (Prendergast-Miller et al., 2021) and after 3 years (Llanos, 2021), as shown in Fig. 6.2.

This suggests that the mesocosm study underestimates the importance of earthworms in soil aggregation, and longer-term studies over 3 years of manipulating earthworm populations would be needed to better resolve their overall contributions to soil aggregation and soil organic C sequestration in typical 3-year leys. Such studies are difficult to conduct because of the need to have treatments in which earthworms are eliminated without changing other soil variables, where recolonization is then prevented for 3 years.

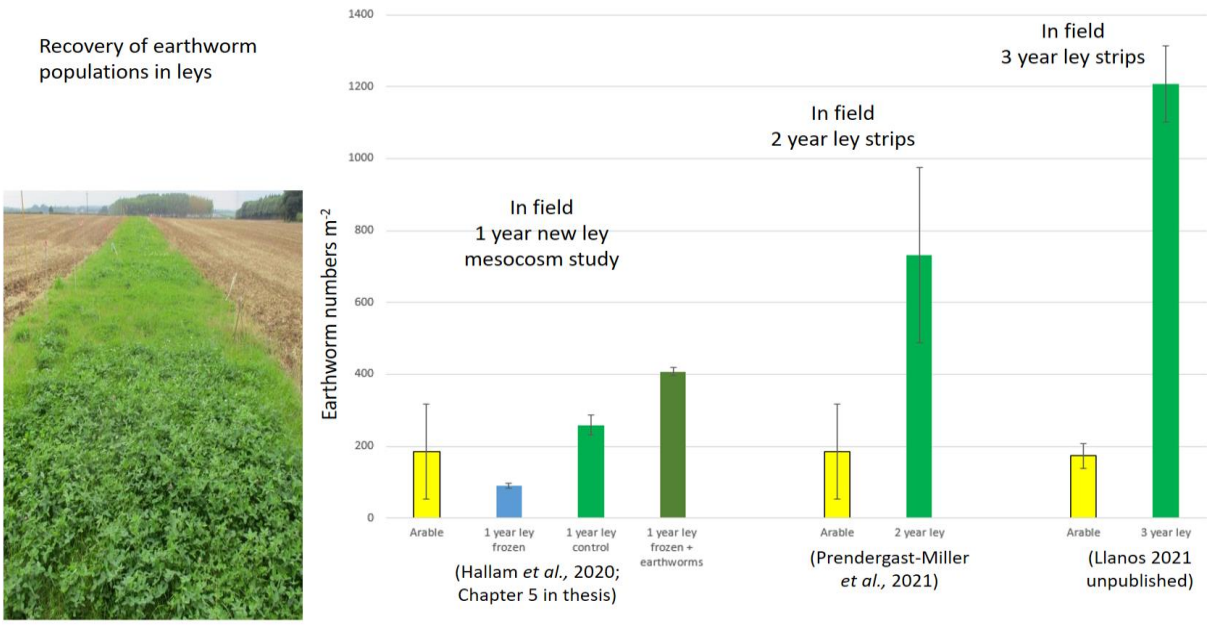

Fig. 6.2 Earthworm population increases over 3 years in arable-to-ley conversion in the four study fields at Leeds University farm, suggesting positive feed-back between improvements in soil quality and earthworm population growth.

6.2.2. Changes in microbial communities and organic molecules under ley

In Chapter 3, it was identified that the introduction of a three-year ley into a conventionally managed arable rotation causes significant changes in active soil fungal communities, with a greater relative abundance of fungi in the Ascomycota phylum but a reduced abundance of those in the Basidiomycota and Mortierellomycota phyla. Ley soils had a substantially greater relative abundance of fungi within the Glomeromycota phylum, associated with most, but not all, of the arbuscular mycorrhizal fungi (AMF), causing a significant increase in the amount of Glomeromycotean species present compared to both arable and hedge soils. Hedge soils had a very distinct community in the Glomeromycota phylum, dominated by species within the *Claroideoglomus* genus. These results suggest that the temporary cessation of ploughing, fertiliser, fungicides and herbicide application under ley is beneficial for total fungal and AMF abundance and diversity. Although, we saw no strong convergence of the ley fungal communities towards those found under the adjoining hedgerows, which were not the refugia for high microbial diversity that we might expect, there was some recruitment of *Claroideoglomus* in the ley, but it was not clear that this had originated from hedge soil refugia. In contrast to fungi, bacterial communities were not significantly affected the introduction of grass-clover ley into arable soils.

In the absence of clear evidence that the ley recruited fungi from the field margin hedgerow soils, it seems likely that the increased diversity seen, for example in Glomeromycota, arose from activating depleted populations of these fungi within the arable field itself. This is likely due to the effects of root exudates and symbiotic C supply to these fungi from the plants then enabling these fungi to grow in

biomass and reproduce. It is possible that some movement of mycorrhiza propagules takes place into the leys through vectoring by earthworms (Gange, 1993), and the fallow strip and stainless steel mesh barriers at the field margin of the UAL ley strips, that extended 5 m away from the leys were designed to prevent this. It is assumed the absence of consistent effects of this barrier compared to the CAL strips that were continuous to the field margin indicates no hedge to field vectoring. However, it was not possible to verify that the barriers and fallow strip were actually effective or were evaded and ineffective, although this seems unlikely.

A novel approach was used in Chapter 4 to determine the impact of introducing grass-clover leys into arable rotations on the soil metabolome, which has the ability to identify organic molecules which are associated with different functional groups of fungi and previously associated with the formation and stability of soil macroaggregates (Fig. 6.1). Three years after the introduction of a ley, a significant upregulation was seen in the mass-to-charge ratio (m/z) bins 403.2 and 439. After putative identification of metabolites, these bins were associated with increases in biosurfactants, lignans and flavonoid pathways. Surprisingly, herbicides were also flagged as potential metabolites within these m/z bins, despite no deliberate applications of herbicide occurring in the grass-clover ley strips for at least three years before sampling occurred. This raises questions about herbicide drift from the juxtaposed arable parts of the fields, and the possible increase of recalcitrance of these synthetic chemicals in the better aggregated ley soils in which OC compounds were accumulating.

6.3. Consequences for policy

This research is very timely, given the inclusion of financial incentives for grass or herbal leys as part of Defra's recent sustainable farming incentive (Defra, 2021g) to encourage C-friendly farming through improved management of soils. The English government's new farming policies following the launch of the 25-year Environment Plan is seeking to reward farmers for delivering public goods and benefits by sustainably managing soils, and diversifying rotations (Defra, 2018). Introducing grass or herbal leys into arable rotations for at least one year in three-year farm agreements to improve the additions of OM and overall soil health are being supported by subsidy payments. For example, a Wiltshire mixed farm has been paid £309 ha^{-1} to grow diverse herbal leys, and the farmer has found the herbage quality so good that he saved £7000 on concreate feeds for his sheep and lambs (Balsom, 2021). The post-Brexit replacement of the EU-funded CAP, ELMS, will look to help achieve Defra's 25-year Environment Plan goals of net zero C emissions, with the associated goal of sustainably managing all of England's soils by the year 2030. Further research is needed to determine whether the new combinations of grasses, legumes and forbs, including herbal species with potentially medicinal properties, consistently out-perform the traditional simple grass-clover leys with respect to effects on soil structure, C and N sequestration, earthworm, microbial and other important kinds of biodiversity, livestock production and

reduced greenhouse gas emissions. There is emerging evidence of many of these benefits (Cummins et al., 2021; Grace et al., 2019b, 2019a).

However, in order to evaluate the benefits of different land management practices to achieve the new policy goals, a simple, cost-effective method of measuring short-term improvements in soil health and changes in OC is needed. In Chapter 2, we suggest a methodology of measuring short-term changes in soil structure and quality in order to know whether a new land management strategy is working on a certain area of land, and how much to reward farmers for improved soil health. A new methodology is needed, especially, because previous methods of measuring bulk SOC alone is not sensitive enough to detect changes in SOC sequestration over short-term studies. For example, studies by Gosling et al. (2017) and Puerta et al. (2018), found no significant increase in bulk SOC after two-three year leys are introduced into arable rotations, and only multi-decadal studies have the ability to reveal these changes (Jarvis et al., 2017; Johnston et al., 2017; Poeplau and Don, 2015; Prade et al., 2017).

In Chapter 2, we suggest that the measurement of OC within macroaggregates, especially within the largest WSAs (>2000 μm), compared to bulk SOC provides a robust early indicator of improvements in soil quality, enabling early detection of statistically significant and functionally important short-term changes in soil structure and quality. Observations of increasing OC and N within the largest macroaggregates are a clear indicator that the soil biology, chemistry and structure is regenerating under leys, further establishing the importance of rewarding farmers for their incorporation into arable rotations to make them a financially viable option.

Conventional tillage involving ploughing and harrowing (Briones and Schmidt, 2017; Crittenden et al., 2014; Edwards and Lofty, 1982) and the application of herbicides (Gaupp-Berghausen et al., 2015; Van Hoesel et al., 2017; Zaller et al., 2014) cause substantial depletion to earthworm abundance, diversity, activity and reproduction. It is therefore not surprising that the introduction of a ley period, which involves the cessation of both of these activities, is beneficial to the recovery of earthworm populations (Fig. 6.2). This has been previously shown by Prendergast-Miller et al. (2021) and Llanos (2021) who found substantial increases in earthworm population abundance two and three years after the implementation of ley, respectively, using the same experimental field site. Even though earthworm numbers significantly increased from arable to two-year ley conversion (Prendergast-Miller et al., 2021), the research provided by Llanos (2021) found that earthworm numbers double again from the second to third year of ley. The evidence provided in Chapter 5 provides substantial evidence of the impact of increasing these earthworm populations under ley specifically for enhancing the formation of macroaggregates and the stabilisation of macroaggregate-associated OC and N. This is further justification for the rewarding of farmers for implementing grass-clover leys into arable rotation, but even more so for maintaining these leys for a period of three years to allow the soil biology to regenerate to its full potential.

Figure 6.3 summarises how the introduction of a grass-clover ley can aid the restoration of soil health within arable rotations. The detrimental effects of putting fields back into conventionally tilled arable rotation after the ley period could be ameliorated by the introduction of a conservation tillage or no-till approach instead.

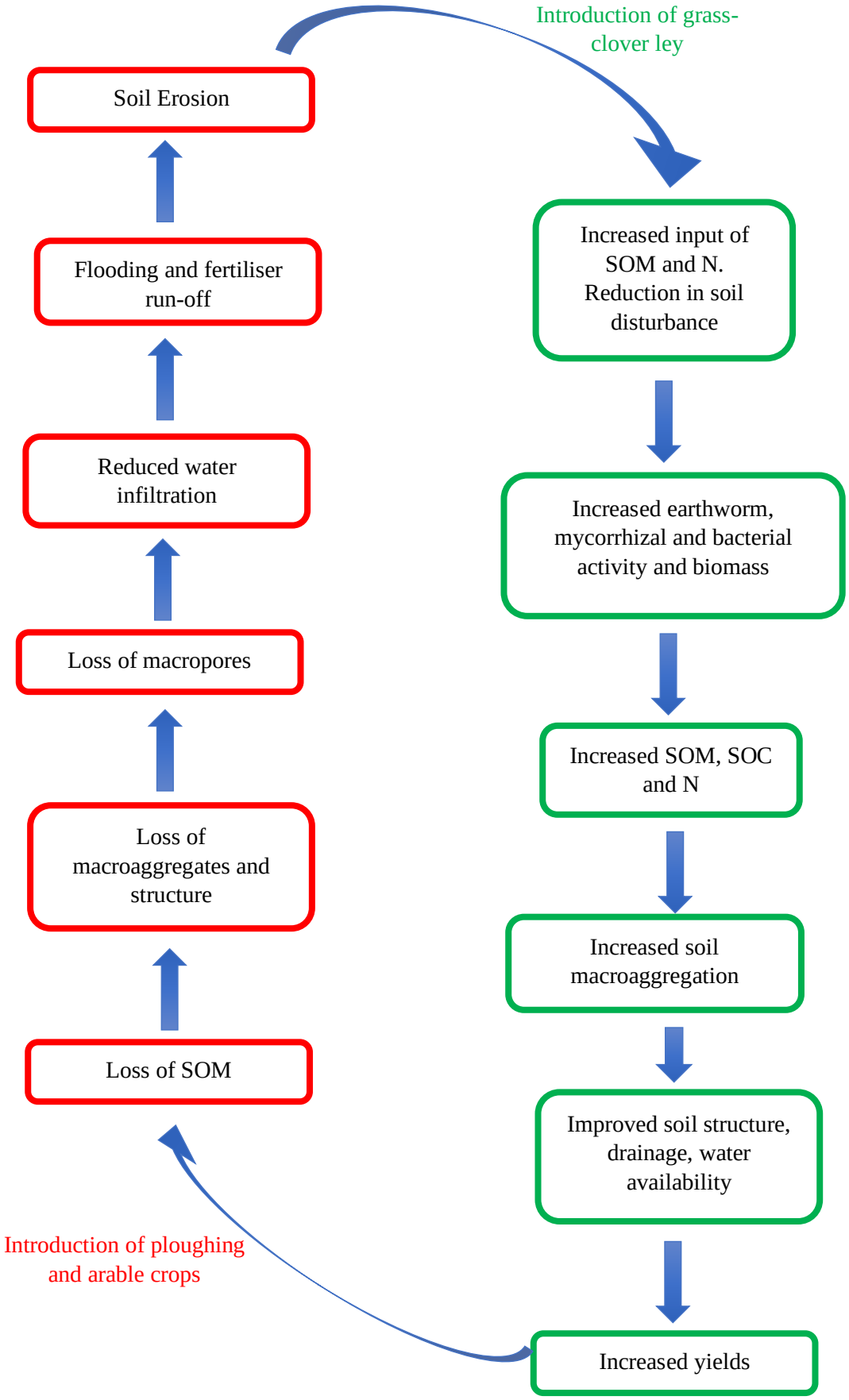

Fig 6.3. A summary diagram of the positive and negative feedback mechanisms during the introduction of grass-clover leys into a conventionally managed arable rotation.

6.4. Contributions to research and avenues for future research

The research presented in this thesis provides a significant contribution to current research on soil aggregation and OC stabilisation, but also on the research behind the role of introducing grass-clover leys into arable rotation on improving soil structure and quality (Fig. 6.1).

In Chapter 2, we provide substantial evidence for the ability of short-term leys to enhance macroaggregate-associated SOC, where previously it had been concluded that arable to ley conversion is a poor candidate for meeting C sequestration targets in Europe (Gosling et al., 2017). This was due to measurements of bulk SOC typically not detecting statistically significant increases, due to the amount of SOC accumulated over the ley period being small compared to the existing stocks (Gosling et al., 2017; Puerta et al., 2018). The methodology we propose in Chapter 2 for the monitoring of spatial variability of SOC storage within different aggregate size fractions for the short-term monitoring of soil health now needs to be trialled more widely. It needs to be evaluated on differing soil types throughout the UK and World for its suitability for assessing soil quality changes not only in short-term arable-to-ley conversions, but also in changes from conventional to reduced tillage, the use of cover cropping, and additions of composts and more crop residues to soils, where bulk SOC changes may be small.

The information from Chapter 3 also prevents new information, as although there are multiple studies on changing from conventional to no-till farming (Badagliacca et al., 2018a, 2018b; Cookson et al., 2008; Helgason et al., 2009; Zuber and Villamil, 2016), there is previously very limited research into the effect of changing from arable to ley affects below-ground microbiota. I found only one previous study of similar to my work, a 25-year study in Norway by Chen et al., (2020). This reported that mixed farming production systems, involving a mixture of ploughing and harrowing a variety of crops in rotation with various lengths of grass-clover leys, have increased microbial biomass including fungal and bacterial gene copies, compared to rotations with crops only. There are also some studies into the impacts of grasslands on microbial communities compared to arable rotations (Zelles et al., 1995), but these are known to have different microbial communities than those that develop under mixed legume and grass cover (Zhou et al., 2017).

From the present study, I have produced a metagenome dataset which could be explored further, as well as providing some important insights into fungal, including mycorrhizal funga,l diversity, changes in which in response to the introduction of leys into arable field soils are poorly researched. In addition, my studies of the hedgerow soil microbiome, which appears distinctive but has previously received very little attention apart from a community-level PCA analysis reported by Holden et al. (2019), is of considerable importance since there are over 0.5 million km of hedges and 0.2 million km of field margins with relict lines of trees around fields in the UK (Countryside Survey, 2007). Amongst the most important species in these hedgerows is hawthorn (mostly *Crataegus monogyna,* locally with *Crataegus laevigata*). The first report of mycorrhizal communities in *Crataegus monogyna* roots was

published in October this year from a study in Belgium, and showed that it can form both arbuscular mycorrhiza and ectomycorrhiza (Boeraeve et al., 2021). The arbuscular mycorrhizal fungi associated with its roots were dominated by *Glomus*, followed by *Claroideoglomus* (Boeraeve et al., 2021), the dominant fungus I found in hedgerow soil. Interestingly, ectomycorrhizal colonization of *C. monogyna* appeared to be rare where the plant grew in grassland or other settings without other ectomycorrhizal hosts, so it is likely it will be mainly arbuscular mycorrhizal in arable field hedges. It was the dominant species in the fields studied in the present work accounting for 60% of the length of the sampled hedges in the same fields (Holden et al., 2019).

Chapter 4 presents novel information to the field, as currently, to our knowledge, there is no study on using matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) to determine changes in the soil metabolome after arable soil has been put under ley. This study provides a basis for much future research with more targeted metabolomic approaches to identify changes in the prevalence of specific organic molecules that have been identified in this study, including biosurfactants, lignans and flavonoid molecules. The MALDI-MS method deployed in the present study was based on extracting compounds and spotting these onto target-plates with an ionization matrix. This was a proof-of-concept study that could in future be extended to use of MALDI-MS imaging (Walker, 2021), on cryosectioned soil aggregates to investigate the spatial locations of metabolites in relation to soil aggregate structures from the exterior to interior of previously intact aggregates.

Chapter 5 also contributes significantly to present research, as effects of earthworms on soil properties in previous studies are often assumed rather than proven experimentally, as changes in land management practices alter many variables. However, through the use of the earthworm manipulation experiment, which was previously designed to investigate the effects of earthworms on soil physio-hydraulic and chemical properties, now published by Hallam et al. (2020), correlations between numbers of total earthworms and individual ecotypes have been made between the formation of soil macroaggregates and macroaggregate-associated OC and N. As these are correlations, not causations, this opens a path for more research on earthworm manipulation studies on these important soil properties, confirmed also by Chapter 2, to further certify the impacts of earthworms on soil aggregation and C storage.

Figure 6.4 revisits the previous knowledge about soil macroaggregate formation presented in Chapter 1, adding information from the contributions to research that this thesis has provided.

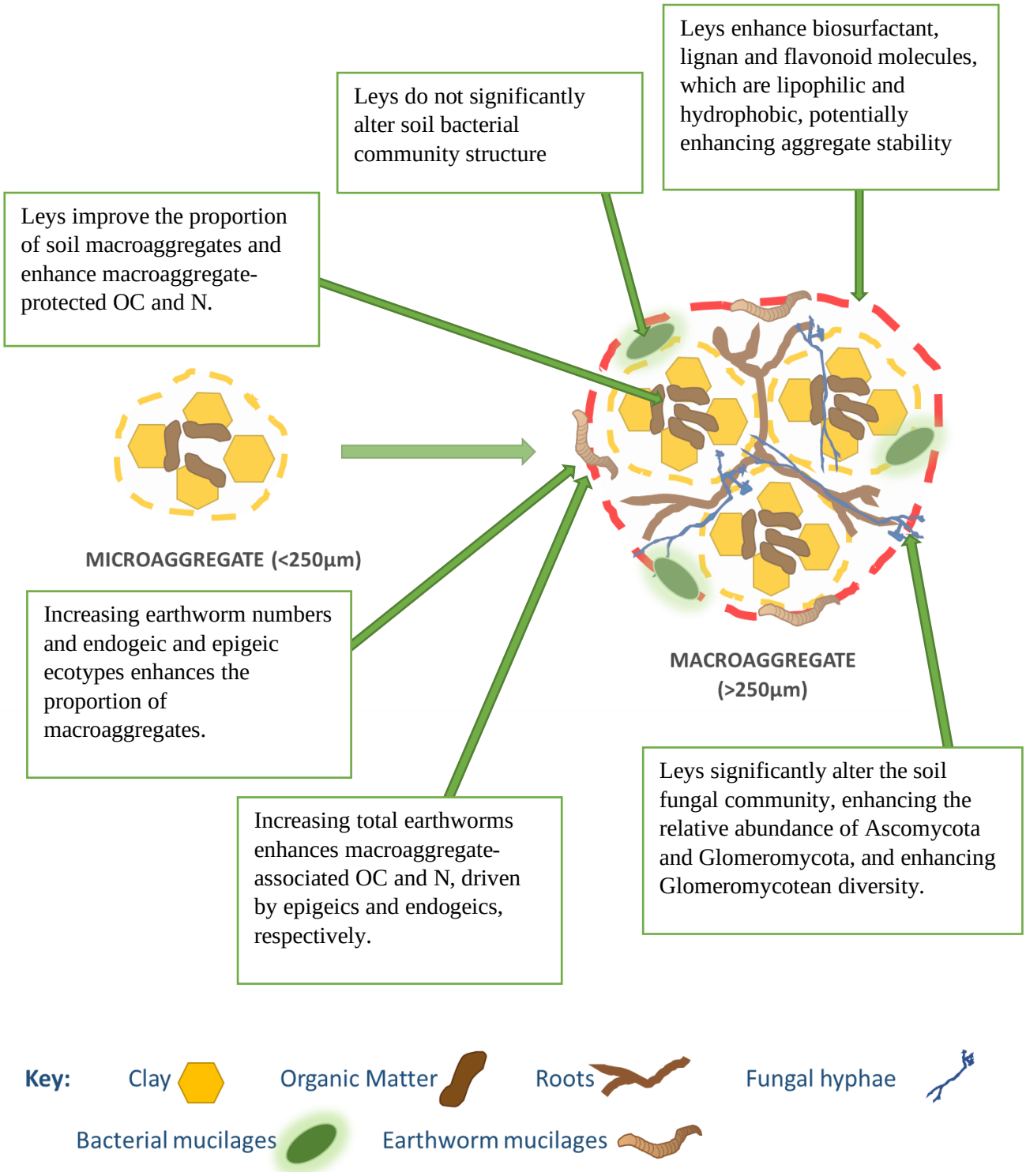

Fig. 6.4. A summary of the previous knowledge on soil macroaggregation and the contributions of this thesis to research.

6.5. Conclusions

In this thesis, I have provided a suite of evidence on the positive impacts that the introduction of grass-clover leys into arable rotations bring to soil structure, through enhanced formation of soil macroaggregates, and soil quality, through enhanced macroaggregate-associated OC and N. I have also added to the current mechanistic knowledge on how grass-clover leys enhance soil structure and quality, through the investigation of changes in soil microbial communities and organic molecules. The results provided in this thesis provides further evidence and justification for the reward of farmers for the incorporation of three-year grass-clover leys into arable rotations for the biological, chemical and physical enhancement of soil health. The introduction of leys will bring a more regenerative approach into agriculture, leaning towards lower input, more sustainable farming which will be more likely to preserve soil and the invaluable ecosystem services it provides, for many generations to come.

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