Can soil microbial diversity mitigate water stress and maintain crop yields in agricultural systems?

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Abstract

Intensive conventional agriculture allows us to increase crop yields in line with global demand, but it puts future yields and food security at a disadvantage due to the increased vulnerability to changes in the environment that intensive systems are less able to withstand. Arbuscular mycorrhizal fungi (AMF) are a key target for increasing agricultural sustainability as they moderate aspects of ecosystem functioning including plant productivity, nutrient cycling, soil structural maintenance, water relations and pathogen regulation. Restoring AMF functioning could be of importance to restoring degraded soil properties to confer climate change resistance and resilience. The main aims of this thesis were (a) to investigate how various agricultural management practices impact the community composition and diversity of AMF, and (b) to explore the causal pathways of AMF diversity and community composition in conferring benefits to soil health and functioning through crop yields and water stress mitigation.

Overall, the thesis considered AMF communities against whole-community functional phenotypes and soil health under contrasting agricultural management practices. This included AMF inoculation of soils, the inclusion of grass-clover leys in arable rotations and contrasting tillage intensities. The thesis increases our knowledge of the link between management practices, soil health and crop yields that can be used to inform management choices in real-world agricultural situations. This is particularly important for grass-clover leys which are a relatively understudied management practice. From the findings of this thesis, it is recommended that future studies employ a reductionist approach to assessing AMF function under variable situations using a combination of targeted mechanistic experiments and larger holistic experiments. These experiments would assess individual AMF functions under different environmental contexts that will provide a trait-based framework of individual function and compliment them with larger scale community experiments that can expand upon the mechanistic knowledge gathered to begin to predict community-level function.

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Author's Declaration

I declare that the thesis is a presentation of original work and I am the sole author. This work has not previously been presented for an award at this, or any other University. All sources are acknowledged as References. My PhD has benefited from the contribution of others to experiment set-up and data collection, who are acknowledged below.

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1. General Introduction

1.1. Climate Change and Food Security

The intensification of food production through the use of conventional agricultural practices (i.e., deep tillage, increasingly high mineral fertilizer, herbicide and pesticide inputs) has been vital to propel the rapid yield gains seen through the *'Green Revolution'* of the 1950s that have supported global development (Tilman *et al.* 2002). Global cereal yields have never been higher and must continue to increase to meet growing demands though the incremental gains of yield seen year-on-year have been gradually declining and we are now facing a plateau in the yields of global staple crops (Knight *et al.* 2012; Ray *et al.* 2012; Fischer *et al.* 2014). Climate change is further expected to compromise the capacity for reliable increases in food production as changing environmental conditions and weather patterns will lead to both the increased incidence and severity of flooding and drought during key crop development periods (Ekström *et al.* 2005; Fowler *et al.* 2005; Bates *et al*. 2008; Murphy *et al.* 2010). The projected unpredictability of precipitation events will affect food security as yields are expected to fluctuate drastically due to these environmental changes (Porter and Semenov 2005; Schmidhuber and Tubiello 2007; Fuss *et al.* 2015; Ray *et al.* 2015). This is evident across Europe where wheat yields have been estimated to decrease 33 – 50 % due to the extreme expected variations in temperature and precipitation (Thaler *et al.* 2012; Mäkinen *et al.* 2018). This is a large vulnerability considering that wheat is Europe's most important cereal crop, accounting for 128.99 million tonnes of production in 2018, or 43.71 % of all European cereal yields (Eurostat 2018).

Agricultural yields are particularly vulnerable to these environmental perturbations due to the long-term negative effects of intensive practices on soil functions and its biodiversity concerning the key groups that can contribute to drought and flood prevention in soils and crop tolerance (e.g., de Vries *et al.* 2012a; de Vries *et al.* 2012b; Cole *et al.* 2019). Intensive agricultural practices degrade important soil properties such as soil organic matter (SOM), soil organic carbon (SOC) and structure which affect the infiltration and drainage capacity of soils, water holding capacity and fertility (Tiessen, Cuevas, and Chacon 1994; Guber *et al.* 2003; Lipiec *et al.* 2007)). This renders agricultural soils more vulnerable to compaction and erosion (Soane

and van Ouwerkerk 1995) making water more likely to pool within the surface layers of the soils under heavy precipitation events thus leading to potential flood events due to poor drainage (Schilling *et al.* 2014). Soils are also less able to retain the water inputs from precipitation which increases vulnerability to water scarcity caused by drought events compromising yields (Bot and Benites 2005). In conjunction to this, tillage and fertilizer additions further damage key ecosystem engineers such as arbuscular mycorrhizal fungi (AMF, Helgason *et al.* 1998; Oehl *et al.* 2003; Hijri *et al.* 2006; Lu *et al.* 2018), which moderate aspects of ecosystem functioning including plant productivity, nutrient cycling, soil structural maintenance, water relations and pathogen regulation (Rillig 2004; Cavagnaro *et al.* 2006; Wehner *et al.* 2010). While intensive conventional agriculture allows us to increase crop yields in line with global demand, it puts potential future yields and food security at a disadvantage due to the increased vulnerability to changes in the environment. Restoring AMF functioning could therefore be of great importance to restoring degraded soil properties and conferring future climate change resistance and resilience advantages (Thirkell *et al.* 2017)

1.2. Soil Health and Function

1.2.1. What is Soil Health?

Soil health is "The capacity of a living soil to function, within natural or managed ecosystem boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and promote animal and plant health." (Doran 2002). Simply, it can be thought of as the net sum of ecosystem functions. This is often used interchangeably with the concept of soil quality though the latter often focuses on a soil's fitness for a specific use (i.e., primary productivity for crop production, Doran and Parkin 1994; Karlen *et al.* 1997, Lehmann *et al.* 2020) rather than as a holistic overview of the soils as a living, dynamic medium (Doran 2002). Soil health is not a static property but one that changes over time. The properties contributing to it in natural or balanced ecosystems typically exist in an equilibrium based on innate soil properties such as soil texture, bedrock type, climate, and vegetation cover (Jobbágy and Jackson 2000; Luo *et al.* 2017).

Soil health in agricultural fields cannot be directly assessed and is instead monitored through several indicators that reflect various ecosystem functions. These indicators are informative individually but can also be combined into a unified soil health index, SHI (also referred to as a soil quality index, SQI). The choice of indicators often depends on the function or functions that are being monitored including plant productivity, water quality, human health and climate mitigation (Lehmann *et al*. 2020). This can be used in on-farm decision making and policy guidance through enabling an easy comparison of management practices (Karlen and Stott 1994; Andrews *et al.* 2002; Karlen *et al.* 1994; Glover *et al.* 2000; Hussain *et al.* 1999; Masto *et al.* 2008; Laishram *et al.* 2012). Past studies concerning soil health and quality have omitted the assessment of AMF and focused primarily on easily measurable chemical, physical and hydrological properties, or biological components such as earthworm abundance and diversity (e.g., Chapman *et al.* 2018 and references therein). Owing to the importance of AMF to ecosystem functions (Rillig 2004) there is however increasing recognition of their assessment as an important aspect of soil health assessment (Abbott 2014; Mahdi *et al.* 2017). Lehmann *et al.* (2020) also highlighted that there is a need to develop methodologies to increase the ease at which the presence and activity of all biotic groups (e.g., fauna, microbial biomass, soil respiration, N mineralization) can be observed in general, along with other traditionally difficult-to-measure parameters related to soil water-relations such as aggregation, water storage and infiltration rates

SOM and SOC are often considered a key indicators of soil health and function through their intrinsic link to soil fertility (Tiessen *et al.* 1994), and the structural properties of soils which contribute to hydrological functioning through a circular feedback loop (Blanco-Canqui and Benjamin 2015). In tandem with the physical entanglement of soil particles by plant roots and fungal hyphae, SOC is fundamental to the formation of soil aggregates by acting as a key binding agent that holds particles together (Tisdall and Oades 1982). SOC further contributes to maintaining the stability of soil aggregates i.e., the resistance of the aggregate to stress such as mechanical stress and compression. Soil aggregates can be operationally defined as microaggregates (20-250μm) and macroaggregates (>250), the arrangement of which is often considered as an indicator of soil structure (Tisdall and Oades 1982; Díaz-Zorita *et al.* 2002). The size and stability of macroaggregates is important to water

relations as the maintenance of greater aggregation in soils increases the number of large pores (macropores) and the connectiveness between them (Mangalassery *et al.* 2013; Guo *et al.* 2020). Greater proportions of macropores and connectiveness facilitates the lateral and vertical water and nutrient flow through the soil (often measured through saturated hydraulic conductivity and infiltration rates, (Soracco *et al.* 2019). Pore architectural benefits from increase macroaggregates therefore result in better receptivity, retention and release of water, or water holding capacity (Vervoort and Cattle 2003). Increasing aggregate stability also decreases the susceptibility of soils to compaction and erosion (Nunes *et al.* 2015; Nunes *et al.* 2019). Compaction is measured by indicators such as bulk density, which reflects the underlying pore space. As particles aggregate, small micropores within them are formed that can be unique habitats from the surrounding bulk soil creating niche space for diverse microorganisms (Bach *et al.* 2018). Some of these micropores are too small for even microbes to enter or have very little oxygen within them thus occluding SOC from microbial mineralization and stabilizing it in the long-term to increase carbon sequestration (Tisdall and Oades 1982; Six *et al.* 2000). Due to the intricate link between SOC, soil structure and water relations, maintaining high levels of soil health through management may therefore be incredibly important in mitigating the risk of flood events occurring in soils by allowing adequate drainage (Schilling *et al.* 2014) and increasing the resilience of soils to drying out during droughts (Bot and Benites 2005), both of which may benefit the potential yields of crops under these conditions.

This is a generally simplified 'model' of the interactions between various soil health indicators and their contribution to water and carbon maintenance and fertility. There are variations in this relationship and the intrinsic capacity of all these functions based on soil properties such as soil type and climate (e.g., (Samuel-Rosa *et al.* 2013; Viscarra Rossel *et al.* 2019). This general conceptual model of interactions informs subsequent chapters.

1.2.2. Managing Soil Health in Agricultural Systems

Intensive agricultural practices disrupt the natural equilibrium that maintains soil health and functioning through altering the balance of inputs and outputs affecting ecosystem scale processes (Ito 2018; Luo and Weng 2011; Peck 1990). Management decisions can therefore be made to enhance soil health through the consideration of multiple soil functions, or lead to degradation of health through focusing only on one such as short-term crop productivity (Doran 2002).

Conventional intensive tillage practices including mouldboard plough, disrupt soil structure, breaking apart aggregates and thereby affecting pore space. Through meta-analysis across a range of tillage practices Nunes *et al.* (2020) demonstrated that tillage of soil generally resulted in lower aggregate size and slightly increased bulk densities, which could be indicative of long-term compaction. Tillage has been further shown to negatively affect water drainage and surface run-off, increasing flood and erosion risk (Dick *et al.* 1989). The disruption of aggregates increases the mineralization of soil organic matter by microbes through the exposure of previously protected carbon and the increased aeration of soils (Paustian *et al.* 2000; Plante and McGill 2002; White and Rice 2009). This is exacerbated by synthetic inorganic fertilizer additions which can further accelerate the decomposition (Khan *et al.* 2007; Mulvaney *et al.* 2009), and the removal of crop residues after harvest for bioenergy, feedstock etc., which diminishes the organic matter returned to soils after each crop season (Ngwira *et al.* 2012). Long-term conventional tillage results in considerable SOC loss over time, which can be as high as 400 kg C ha^{-1} yr^{-1} under winter wheat cropping systems (Heenan *et al.* 1995; Persson *et al.* 2008). Through the complex interactions between SOC and soil structure, intensive tillage can reduce the water holding capacity of soils by as much as 26 % relative to no-till agriculture (Govindasamy *et al.* 2020).

Despite the widescale degradation of soil health, worldwide cereal yields have never been historically as high as they are now (Fischer *et al.* 2014), which may be due to the continued high application rates of inorganic fertilizer seen since the *'Green Revolution'*. In the UK N fertilizer additions have remained consistent over the past 30 years, with typical application rates between 137-146 kg N ha^{-1} between 2015 and 2019 (DEFRA 2020a). Continuous fertilizer additions in this range have been shown to confer ever increasing benefits to crop yields over time, thereby offsetting any of

the potential negative effects that may be seen on yields due to intensive management and the cascading effects of this on soil health and fertility over the same period (Persson *et al.* 2008). This is not however an economically or ecologically viable strategy. Fertilizer use efficiency of crop plants has been falling over time (Tilman *et al*. 2002), and less than 50 % of fertilizer applied is taken up by crops (Smil 2000; Cassman 2002; Yan *et al.* 2020). The low capacity of agricultural soils to receive and retain water (e.g., Dixit *et al.* 2019; Li *et al.* 2019) leaves unabsorbed fertilizer vulnerable to leaching into groundwater and surrounding water bodies, or lost through soil erosion and surface run-off (Liu *et al.* 2010; Smith *et al.* 2015). This can cause damage to surrounding ecosystems through the eutrophication of surface water (Matson *et al*. 1997; Carpenter *et al.* 1998; Withers *et al.* 2014), and through nitrate leaching in to human water supplies can be particularly detrimental to human health (Ward *et al.* 2018).

In recognition of the environmental costs of intensive agriculture and the food security threat of climate change there is a growing impetus to manage agriculture more sustainably through decreasing the intensity of disturbance, or number of inputs while maintaining consistent yield increases. This can be referred to as sustainable intensification, where practices are adopted which maintain a balance between sufficiently high crop yields and the environment (Godfray *et al.* 2010; Pretty and Bharucha 2014; Bender *et al.* 2016). Sustainable agricultural management relies on strategies which can **(a)** conserve soil organic matter **(b)** minimize soil erosion **(c)** Balance crop production and the environment **(d)** better use renewable resources available within the system itself (i.e., use the natural capital of soil biodiversity to regulate ecosystem services) (Doran 2002). This is supported by initiatives such as the '4 per 1000' initiative to increase carbon sequestration in the world's agricultural soils, and the UN sustainable development objectives for Zero Hunger, Life on Land, and Climate Action (UN 2015).

1.3. The AMF Symbiosis

1.3.1. Symbiosis Overview

Around 70 % of all terrestrial plant species form arbuscular mycorrhizal (AM) symbioses, including most staple cereal crops such as wheat, rice, maize and sorghum (Smith and Read 2008). The fungi underpinning this symbiosis belong to the sub-phylum *Glomeromycotina* within the *Mucuromycota* phylum (Schußler *et al.* 2001; Spatafora *et al.* 2017). AMF exist at the interface of the plant and soil environment. the fungus creates an intra-radical mycelium (IRM) within the root environment composed of hyphae and specialised structures called arbuscules and vesicles through which nutrients can be transferred between the host plant and the fungus (Gutjahr and Parniske 2013). An extraradical mycelium (ERM) of hyphae also extends into the surrounding bulk soil environment, increasing the surface area for nutrient and water acquisition of the host plant (Smith and Smith 2011). AMF are often considered obligate symbiotrophs, relying entirely on the host plant for the carbon that they require to thrive (Bago *et al.* 2000), for which a reciprocal transfer of soil-derived water and nutrients to the plant host is expected. Through this reciprocal transfer AMF can be principally beneficial in agriculture for their role in promoting the growth of host plants through increased nutrient uptake. AMF have been shown to transfer important macronutrients such as phosphorus P (Ezawa *et al.* 2002), Nitrogen N (Hodge *et al.* 2001; Thirkell *et al.* 2016) and a suite of other macro and micronutrients to the host plant (Behie and Bidochka 2014, and references therein). As in any resource-exchange situation however, the relative scale of resources moving in either direction can be context-dependent and the symbiosis can exist on a spectrum of symbiotroph-neutral-pathotroph depending on soil resource conditions (e.g., the trade-balance model, Johnson *et al.* 1997; Johnson 2010; Johnson *et al.* 2015), host plant-fungus compatibility,(Hetrick *et al.* 1993; Kahiluoto *et al.* 2001; Hoeksema *et al.* 2010; Thirkell *et al.* 2020), and atmospheric CO_2 concentrations (Thirkell *et al.* 2020).

1.3.2. Contribution to Soil Health and Water Stress Response

Beyond the direct nutritional benefit to host plants AMF are key ecosystem engineers that can moderate soil structure and aggregate stability (Rillig and Mummey 2006), affecting carbon cycling, water relations and gas exchange (Rillig 2004). The hyphae composing the ERM can increase the stability of soils through the physical entanglement of soil particles (Tisdall and Oades 1982) and the exudation of a suite of soil binding proteins currently considered under a wide umbrella term of Glomalin-Related Soil Proteins (GSRP, (Wright and Upadhyaya 1998; Rillig *et al.* 2001; 2015; Ji *et al.* 2019). They may also increase hydrophobicity through the superficial hyphal colonization of aggregate surfaces (Rillig *et al.* 2010). GSRP is a recalcitrant form of soil organic matter (6-42 years residence time, Treseder and Allen 2000; Steinberg and Rillig 2003; Rillig 2004), with potential capacity to store carbon in soils long-term. AMF may further indirectly moderate soil structure through symbiosis-induced changes in the host plant root biomass, architecture and exudates (Norman *et al.* 1996). Increasing SOC, aggregate size and stability and pore space allows soils to hold more water and have higher infiltration rates in to the soil (Acín-Carrera *et al.* 2013) which are beneficial to drought and flood stress prevention.

AMF also stimulate a range of host-physiological responses that increase their capacity to respond to flood and drought stress (Augé 2001). AMF improve the stomatal conductance and sustained photosynthesis in droughted and flooded plants through moderating phytohormone concentrations within the plant. Drought and flood stressed plants both produce abscisic acid (ABA) which mediates transpiration rates through inducing stomatal closure, root hydraulic conductivity and aquaporin expression (Ouledali *et al.* 2019). Due to this photosynthesis decreases under water stress conditions leading to potential yield declines in stressed plants. AMF colonization reduces the concentration of ABA found in the leaves, allowing stomata to stay open and photosynthesis to continue for longer periods during water stress (de Ollas and Dodd 2016; Ouledali *et al.* 2019). AMF also reduce osmotic stress in the cells of host plants by stimulating the build-up of osmotic regulators including sugars, proline and free amino acids which allow cells to remain turgid in the absence of water (Augé 2001; Bárzana *et al.* 2014). The ERM extending in to the soil can also benefit host plants through increased nutrient and water uptake under stress conditions (Augé 2001). The decreased diameter of AMF hyphae relative to plant

roots particularly allows them to access water that would otherwise be inaccessible in smaller pore spaces as drought causes shrinkage to the soil (Whitmore and Whalley 2009).

Drought can further damage plants through reactive oxygen species (ROS e.g., oxygen radicles, hydrogen peroxide H_2O_2, hydroxide ions HO etc.), which cause oxidate damage to proteins, DNA and lipids (Apel and Hirt 2004). ROS production from vital cell organelles such as chloroplasts, mitochondria and peroxisomes are elevated under drought conditions through the disruption of metabolic processes (Ding *et al.* 2010; Miller *et al.* 2010). This can lead to necrosis and cell death (Morgan, Kim, and Liu 2008), thus exacerbating the other issues caused by the water deficit. AMF may protect hosts from this oxidative damage through either the avoidance of ROS generation through the transfer of water taken up by fungal hyphae to the plant host, or through the increased activity of defence enzymes that interact with and neutralize various ROS such as peroxidase (POD, Roldán *et al.,* 2008; Sofo *et al.,* 2005; Zarik *et al.,* 2016)

1.3.3. AMF in Conventional Agriculture

Intensive deep tillage damages AM fungal communities through disrupting the ERM hyphal network spread throughout the soil. This results in reduced spore abundance and ERM hyphal length density in the soil (Oehl and Koch 2018), and further negatively affects the ability of AM fungi to colonize plant roots from the soil (Jasper *et al.* 1989; Goss and De Varennes 2002; Jansa *et al.* 2006). Tillage disturbance subsequently shifts AM fungal community composition and diversity, often being associated with a decrease in richness and evenness (Jansa *et al.* 2003; Oehl and Koch 2018). This is likely due to species variation in life history traits and colonization strategies across species influencing the recovery of AM fungal communities after tillage disturbance. For example, through meta-analysis van der Heyde *et al.* (2017) showed that AM fungi from the *Gigasporaceae* family were the lineage most negatively affected by disturbance pressures. *Gigasporaceae* have a high investment in to ERM hyphal production (Jakobsen *et al.* 1992; Hart and Reader 2002b; Maherali and Klironomos 2007), and are far less effective at colonizing plants from root fragments than they are from hyphae and spores (Biermann and Linderman 1983; Abbott *et al.* 1992; Klironomos and Hart 2002), making their persistence more vulnerable to tillage disturbance. This has also been confirmed through molecular

analyses, where *Scutellospora* (a genus within *Gigasporaceae*) was found to have a strongly reduced presence in the roots of Maize grown in ploughed soils (Jansa *et al.* 2003).

The reduction of hyphal length density in soils and reduced capacity to regenerate it through key hyphal generating community members such as *Gigasporaceae* may therefore impact the long-term capacity of AM fungal communities to contribute to soil stabilization, SOC accumulation, nutrient uptake, and water regulation (Tisdall and Oades 1982; Jakobsen *et al.* 1992; Augé 2001; Rillig and Mummey 2006; Maherali and Klironomos 2007; Thonar *et al*. 2011). Lu *et al.* 2018) showed that the *Glomus* genera abundance was negatively affected by tillage, and through principal components analysis strongly associated with soil aggregation, though this study was in an incredibly species poor soil where only *Glomus* and *Septoglomus* genera were observed. While they did not consider tillage treatments, using single-species inocula Ji *et al.* (2019) were also able to show that *Gigaspora margarita* demonstrated higher levels of *GRSP* than *Glomus mosseae* under both ambient and drought soil conditions, and through this increased the dispersive energy (and therefore stability) of water stable macroaggregates.

Further to affecting the capacity of AM fungi to maintain soil structure and water relations, tillage poses an extreme selection pressure on AM fungal communities that may directly affect the placement of the symbiosis on the pathogen-neutral-symbiont spectrum. It has been hypothesised that this regular extreme disturbance may select for rapidly growing and sporulating species of AM fungi (i.e., r-selection for disturbance tolerance (Pianka 1970; van der Heyde *et al.* 2017)), which prioritise their own short term growth and proliferation at the expense of nutrient transfer to their host (Johnson *et al.* 1992, Verbruggen and Kiers 2010). AM fungal communities which are present in conventional agricultural systems may therefore be less symbiotic in nature than those from systems with less disturbance, affecting crop nutrition and yields under both ambient and water stress scenarios.

Fertilization has also been shown to affect AM fungal community composition and diversity (Hu *et al.* 2019), and select for less mutualistic mycorrhizas (Johnson 1993). The trade-balance model posits that the relative availability of soil N and P govern the extent to which host plants may benefit from the symbiosis, with the maximum benefit expected under sufficiently high N concentrations and limiting P

concentrations. Under these conditions neither plant photosynthesis (i.e., carbon transfer capacity) or AM fungal growth (i.e., hyphal extension and nutrient transfer capacity) will be limited by N, allowing the transfer of P to proceed maximally (Johnson 2010). The conventional agricultural practice of synthetic fertilizer addition may therefore limit the beneficial capacity of AM fungi. While there has been a downward trend in P fertilization since its height in the 1980s, P fertilizer additions have stayed around 26-30 kg P ha^{-1} in tillage crops over the same period (DEFRA 2020a). Indeed, AM fungi have been shown to be more beneficial under low P conditions (Hoeksema *et al.* 2010), and long-term P fertilization at rates of 45 kg P ha^{-1} in agricultural soils has been shown to reduce the benefit of AM fungi to host plant nutrition and growth (Kahiluoto *et al.* 2001). There is also such a thing as too much nitrogen. Synthetic fertilizer additions of 150 kg N ha^{-1} (similar to those seen in conventional UK agriculture) can depress the abundance of AM fungi in soils (Albizua *et al.* 2015).

1.3.4. Biodiversity and Ecosystem Function

Managing the diversity of AMF and community composition is as importance as managing their abundance to maximize the benefits or mitigate trade-offs in optimizing agricultural crop production and resilience (Rillig *et al.* 2016). AMF are not a homogenous functional group but a group that contains great diversity in functional traits concerning life history strategies, intra-root structures and hyphal development (Hart and Reader 2002b; Varela-Cervero *et al.* 2016b) which can be leveraged for synergy and niche space optimization to maximise ecosystem multifunctionality (Jansa *et al.* 2008; Powell and Rillig 2018).

At the ecosystem level there is a strong asymptotic relationship between increasing above and belowground biodiversity and food web complexity to ecosystem functioning through increasing the number of novel functional groups, optimization of niche space use, and eventually reaching high levels of functional redundancy (de Vries *et al.* 2013; Wagg *et al.* 2014; Bradford *et al.* 2014; Allan *et al.* 2015; Lefcheck *et al.* 2015; Soliveres *et al.* 2016). This concept may be further extended to the biodiversity observed at lower hierarchical levels within an ecosystem where there is still considerable variation in function such as the fungal kingdom (Frac *et al.* 2018), and the AMF guild within this kingdom (Powell and Rillig 2018). Studies of artificially created AMF communities containing varying levels of species richness have shown

that host-plant productivity significantly increases as community richness does up to an optimum of 8 species (van der Heijden *et al.* 1998; Vogelsang, Reynolds, and Bever 2006; Maherali and Klironomos 2007). There is no evidence that community richness plays a role in soil functional properties such as water maintenance.

The role of AMF species variation in plant nutrition and biomass accumulation has been previously explored using single species profiling (Maherali and Klironomos 2007; Thonar *et al.* 2011; Gosling *et al.* 2016), though this has not been well considered within more complex naturally assembling 'field-relevant' communities. For example, Haskell (2017) assessed the importance of community composition and the contribution of specific members to P and Si acquisition. Further to this there is very little evidence directly linking AMF species and their hyphal distribution or host plant physiological response to non-nutritional functions such as soil aggregation and water stress tolerance of host plants. Through single-species inocula Ruiz-Lozano *et al.* (1995) demonstrated inter-specific variation within the *Glomus* genus for conferring drought resistance through contrasting effects on host-plant CO_2 exchange rate, water use efficiency, transpiration rate and a suite of other plant physiological responses. Ji *et al.* (2019) were also able to show that *Gigaspora margarita* demonstrated higher levels of glomalin related soil proteins (GSRP) than *Glomus mosseae* under both ambient and drought soil conditions, and through this increased the dispersive energy (and therefore stability) of water stable macroaggregates. Further to this, Lu *et al.* (2018) recently showed evidence of reduced species richness and altered community composition between conventional and no-tillage managed farms and were able to link soil aggregate composition to the relative abundance of AMF belonging to the *Glomus* and *Septoglomus* genera through principal components analysis. This research notwithstanding, there is still however a large gap in our understanding of the causal mechanisms of species richness, identity, and overall community composition to these aspects of ecosystem functioning due to a lack of studies of this nature.

1.3.5. Monitoring AMF Biodiversity through Molecular Methods

AMF are a key target for their potential capacity to bridge yields gaps between intensive and sustainable agriculture, and increase ecosystem functioning (Thirkell *et al.* 2017). Achieving a better understanding of the functional capabilities of naturally assembling AMF communities under various management practices will allow us to

make informed agricultural management decisions that will maximise the functional benefits of agro-ecosystems in sustainable agriculture to deliver maximum yields while minimizing their environmental impact and ensuring the resilience of yields to the future challenges that climate change will bring.

AMF present many challenges to the characterization of species from field samples. AMF were traditionally identified from spore morphology, though genetic advances have shown that phylogenetically distant species can have similar morphologies (e.g., species within the *Glomus* and *Paraglomus* genera, Morton and Redecker 2001), and single species can be dimorphic (e.g., *Glomus dimorphicum* (Boyetchko and Tewari 1986). The assessment of spores is also now known to be an inappropriate method to assess the AMF species that may be functionally beneficial in roots, as different species are known to be more represented in either the root or bulk soil compartment (Varela-Cervero *et al.* 2015). AMF hyphae are less useful than spores for morphological AMF identification, allowing only identification down to the family level under most circumstances (Merryweather and Fitter 1998), and in some lineages not staining at all (Morton and Redecker 2001). Genetic sequencing has now become the standard approach to characterizing AMF from environmental samples (Redecker *et al.* 2003; Gorzelak *et al.* 2012; Hart *et al.* 2015).

DNA sequencing of amplicons is frequently employed to conduct taxonomic profiling of AMF (Gorzelak *et al.* 2012). Amplicons are fragments of DNA from a marker gene region, amplified to great quantities through Polymerase Chain Reaction (PCR). Regions within the ribosomal RNA (rRNA) genes are commonly used for phylogenetic comparisons between fungi. This is due in part to the functional importance of the gene to life, meaning it is exposed to similar selection pressures across all eukaryotic life and is highly preserved allowing universal primers to be designed (Hillis and Dixon 1991; Moore and Steitz 2002). For AMF categorization the most popular marker region within this locus is the is the small subunit rRNA gene encoding region (SSU rRNA), though the internal transcribed spacer region (ITS) can also be used, and is regarded as the universal barcode marker for fungi (Schoch *et al.* 2012). One reason for the lack of popularity of the ITS region in AMF studies however is that many primer pairs designed for amplifying the ITS region have poor amplification for AMF (Tedersoo *et al.* 2015; Tedersoo *et al.* 2018) and therefore require large sequencing depths (number of DNA sequences) to detect AMF. The ITS region has also been

shown to have sub-standard discrimination of AMF clades in comparison to the SSU region (Stockinger *et al.*, 2010; Thiéry *et al.* 2012; 2016). Despite this, the two marker regions can report comparable estimates, potentially since most samples are dominated by AMF within the *Glomeraceae* family which are well amplified for both regions (Berruti *et al.* 2017; Lekberg *et al.* 2018).

Much like how the shift from morphological to genetic identification revolutionized our understanding of AMF ecology, there have also been significant advances within the discipline of molecular characterization. High throughput sequencing resulting in potentially millions of DNA reads through platforms such as Illumina *MiSeq* and *HiSeq* has become increasingly more accessible and affordable to users to assess both the diversity of, and fine grained community composition of microbes including AMF (Öpik *et al.* 2009; Caporaso *et al.* 2012; Gohl *et al.* 2016). This sequencing technology was touted as the '*Next-Generation*' of sequencing at the time of inception, though is now considered '*Second-Generation'* sequencing as technologies have further developed. *'Third-Generation*' sequencing technologies are now being delivered by companies such as Oxford Nanopore Technologies and Pacific Biosciences (Rhoads and Au 2015; Bayega *et al.* 2018). DNA sequencing read length is constrained under current *'Second-Generation'* sequencing technology to approximately 250-300 bp, while *'Third-Generation'* sequencing technologies can sequence longer lengths of DNA (over 1000 bp). This may eventually be used to resolve the gap in AMF classification ability between ITS and SSU marker regions through the ability to sequence both as part of one long read, though the technologies are still relatively juvenile and require further development and optimization (Tedersoo *et al.* 2018). Illumina *MiSeq* sequencing was used for the two chapters in this thesis which employ genetic sequencing and will be subsequently described in detail.

Illumina *MiSeq* and *HiSeq* use a flow-cell based '*sequencing-by-synthesis*' approach using fluorescently labelled deoxyribonucleotide triphosphates (dNTPs, otherwise known as the base units which constitute DNA- adenine, guanine, cytosine, and thymine) which give out bursts of light as they incorporate into a synthesized DNA strand that can then be recorded to determine the base. Flow cells are glass slides containing channels (/lanes) coated with two types of oligos (short nucleotide sequences) that serve as anchors for the sequencing-DNA to bind with.

Samples are prepared for amplicon sequencing through a two-step process (Kozich *et al.*, 2013, summarized in *Figure 1.1*). The initial PCRs are used to amplify the marker region of interest. This may be done through a single PCR to amplify the region, or a nested approach wherein a longer fragment is initially amplified followed by a shorter fragment nested within this locus. The nested PCR approach is commonly used in the assessment of fungi (where PCR using eukaryotic primers may also amplify the majority plant DNA found in samples) to increase the specificity of amplification to the target group, and ensure the amplification of low-abundance sequences (Dumbrell *et al.* 2011). For Illumina sequencing a specialized sequencing primer and 12 random bp sequences are added to either end of the amplicon fragment during this first step. In the second step another PCR is performed to add 6bp sample barcodes, and adaptor regions complementary to one of the two DNA oligos present on the flow-cell at either end of the strand.

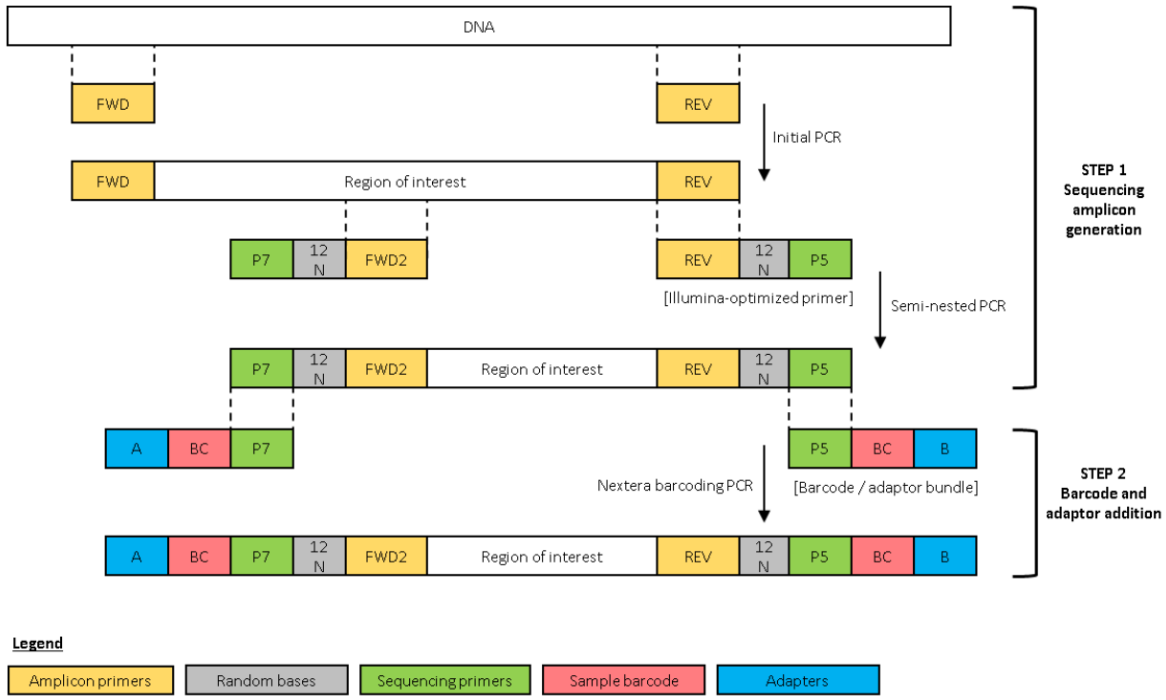

Figure 1.1. Overview of the two-step process used to generate DNA amplicons for Illumina *MiSeq* sequencing as per Kozich *et al.* (2013). This overview is representative of the semi-nested PCR approach used throughout this thesis used for the amplification of DNA from the SSU and ITS rRNA regions. Full PCR protocols used are described in Chapter 2 *Table 2.2*.

Before sequencing of the amplicon DNA can commence, each single strand of DNA from the sample is amplified to great numbers in clusters through '*bridge-amplification'*. In this process the adaptor region of the DNA amplicon hybridizes with the complementary oligo anchor on the flow cell, and a DNA polymerase synthesizes a compliment of this strand from the oligo anchor. The DNA hybrid is then denatured, and the original strand washed away. The end of the anchored strand folds over for the second adaptor region to bind with its alternate complementary oligo anchor (i.e., creates a *bridge* between the two oligo anchors) from which another complementary strand is created in the reverse direction. Denaturing occurs once again, and the two strands separate but are not washed away this time since they are both attached to oligo anchors. The strands continue to fold over to create bridges from which further amplification can be carried out several more times. At the end of this, all reverse direction strands are cleaved and washed off the flow cell so that only forward strands of the same clonal sequence within each cluster remain. Sequencing can now be carried out.

Fluorescently labelled dNTPs are incorporated through the synthesis of complementary DNA strands to those remaining anchored to the flow cell from the sequencing primers. As dNTPs are incorporated a fluorescent signal is emitted, the wavelength and intensity of which can be used to determine which dNTP was incorporated. This is carried out simultaneously across all DNA clusters, which contain large amounts of DNA to create a strong enough signal to be detected. The identity of each cluster is determined from the 12 random bases incorporated into the beginning of each sequence (Which is unique to each original DNA amplicon from which clonal clusters were created).

1.4. Thesis Aims and Hypotheses

The central question presented in this thesis is "Can soil microbial diversity mitigate water stress and maintain crop yields in agricultural systems?". This was approached through the lens of AMF microbial diversity and community composition, as they are a key target for their potential capacity to bridge yields gaps between intensive and sustainable agriculture, and increase ecosystem functioning (Thirkell *et al.* 2017). It is important that we look beyond just the presence of AMF within agricultural systems, but also to the individual species and communities that assemble in response to various management approaches. AMF demonstrate a wide range of life history traits and morphological variation that could inform their capacity to contribute to agriculturally relevant processes including plant productivity, soil structural maintenance, carbon sequestration and water relations (Rillig 2004). This makes their diversity of key importance to maintaining soil health, and ensuring that agricultural ecosystems can have maximal ecosystem multifunctionality and resilience to future climate change scenarios involving drought and flood (Powell and Rillig 2018). Despite this, there is very little evidence beyond presence/absence studies that directly links AMF species or naturally assembling consortia across agricultural management approaches to non-nutritional functions such as soil aggregation and water stress tolerance of host plants (e.g., Ruiz-Lozano *et al.* 1995; Lu *et al.* 2018; Ji *et al.* 2019). This leaves a gap in our understanding of the causal mechanisms of species richness, identity, and overall composition to these aspects of ecosystem functioning.

Achieving a better understanding of the functional capabilities of naturally assembling AMF communities under various management practices will allow us to make informed agricultural management decisions to maximise the functional benefits of agro-ecosystems in sustainable agriculture. Through this agro-ecosystems can be shifted to deliver maximum yields while minimizing their environmental impact and ensuring the resilience of yields to the future challenges that climate change will bring.

The specific objectives of this thesis were:

1. To investigate how various agricultural management practices impact the community composition and diversity of AMF.
2. To explore the causal pathways of AMF diversity and community composition in conferring benefits to soil health and functioning through crop yields and water stress mitigation.

Through a combination of experimental approaches at different scales and levels of complexity and across two study systems containing contrasting land uses and agricultural practices, the following main hypothesis was investigated:

1. Agricultural management practices will be a key determinant of AMF communities:
    a. Adopting AMF inoculation of soils will increase the richness and abundance of AMF and alter communities under minimum tillage agriculture, thus increasing the functional potential of the symbiosis to positively impact host crop performance in-field. (*Chapter 2*).
    b. Higher agricultural land use intensity (e.g., grassland vs arable, low vs high intensity tillage, grass-clover crop rotation vs continuous cropping) will have a greater negative impact on AMF community diversity and composition. (*Chapter 3, 4*).
2. AMF community properties will be significantly associated with soil health properties (e.g., SOC, bulk density, hydrology, SHI) and the functional outputs of crop yield and water maintenance under ambient and water stress scenarios (*Chapter 2, 3, 4*).

2. Assessing the effect of arbuscular mycorrhizal fungal inoculum and wheat cultivar (*Triticum aestivum*) on plant growth response and fungal community composition in-field

2.1. Introduction

Since the 'Green Revolution' of the 1950s wheat yields have steadily increased. This has been supported by advances in plant breeding and management practices such as the increased mechanization of tillage and increases in fertilizer and pesticide additions (Foley *et al.*, 2005). These practices have yielded great short-term gains, but resulted in negative environmental impacts, land degradation and soil health declines in the long-term. (Matson *et al.* 1997; Doran 2002; Tilman *et al.* 2002; Foley *et al.* 2005). As these impacts become more apparent and climate change adds uncertainty to the food production required to feed an ever-growing population, we are observing a paradigm shift in the conversation around food security and sustainability through initiatives such as the United Nations Sustainable Development Goals for Zero Hunger, Life on Land, and Climate Action (UN 2015)

A particular focus of government initiatives in reshaping agricultural management practices is through the monitoring and regulation of fertilizer additions. This is informed by the environmental consequences of run-off and leaching from high input systems (Robertson and Vitousek 2009; Goucher *et al.* 2017), and the increasingly high energetic costs associated with fertilizer manufacture and depletion of natural resources (Cordell, Drangert, and White 2009). Through the EU Nitrate Directive (91/676/EEC), member states are required to identify areas in which groundwater nitrate concentrations exceed 50 mg L^{-1}, or that are at risk of nitrate contamination. These areas are known as Nitrogen Vulnerable Zones (NVZs) and cover approximately 55 % of land within the UK. Within NVZs the amount of both organic and inorganic fertilizer along with the timing of additions is limited in comparison to arable land in other areas of the country. To maintain yields within NVZs that cannot have the same levels of input, it may therefore be necessary to consider innovative

alternative solutions to ensure that crops can continue to access nutrients at a level that maintains the commercial viability of the crop and wider food security.

AMF addition to soils may be a sustainable solution to this issue in mitigating the need for nutrient additions (Rillig *et al.* 2016; Thirkell *et al.* 2017). AMF form symbiotic relations with a majority of modern land plants including many important staple food crops across the world such as winter wheat (Brundrett and Tedersoo 2018). The adoption of modern conventional arable practices have however resulted in a low abundance and diversity of AMF in agricultural systems (Helgason *et al.,* 1998, Fan *et al.,* 2020). AMF are obligate symbiotriophs belonging to the sub-phylum Glomeromycotina within the Mucuromycota phylum (Schußler *et al.,* 2001; Spatafora *et al.,* 2017). AMF colonize host plant roots, within which they form structures called vesicles and arbuscules, and from which they extend a dense extraradical mycelium into the surrounding soil. Through this interaction they deliver many ecosystem services beneficial to food production and sustainability (Rillig 2004; Thirkell *et al.* 2017). AMF are principally beneficial in agricultural food production through their role in promoting host plant growth by increasing the uptake of water and inorganic nutrients including phosphorus (Ezawa *et al.* 2002) and nitrogen (Hodge 2000; Thirkell *et al.* 2016). AMF also interact with the wider fungal and bacterial community and are particularly beneficial in fostering systemic resistance to fungal pathogens (Harrier and Watson 2004). With a focus on maintaining AMF functioning in systems, farmers may also be encouraged to adopt management practices that benefit long-term biological activity within the soil system such as no- or low-tillage, intercropping regimes and conservation agriculture. This would confer additional benefits of AMF as key ecosystem engineers to soil health through soil stabilization, water and nutrient retention and carbon sequestration (Cameron 2010; Cavagnaro *et al.* 2015; Lehmann *et al.* 2016).

The use of AMF inoculum in-field is well studied (See the meta-analyses by Lekberg and Koide 2005; Pellegrino *et al.* 2015; Zhang *et al.* 2019 and studies referenced therein) and generally positively affects nutrient acquisition and crop yield, It is only with the advent of high throughput sequencing technologies that the fate of species added within inoculants, and the effect of this on the in-field microbial community can be monitored (Antunes *et al.* 2009; Mummey *et al.* 2009; Koch *et al.* 2011; Janoušková *et al.* 2017). AMF can inhabit different niches in space and time, and

perform different functions through variation in their life history strategies and morphology (Hart and Reader 2002b; Varela-Cervero *et al.* 2015; 2016a; Weber *et al.* 2019). This can confer synergistic effects in co-colonization (Koide 2000; Jansa *et al.* 2008). Further to this increasing the richness seen in AMF communities has been associated with increased nutrient uptake and productivity in host plants (van der Heijden *et al*. 1998; Maherali and Klironomos 2007). It therefore stands to reason that would be beneficial for agricultural sites to have robust and diverse compliments of AMF present, though we still understand little about the value of this in 'naturally-assembling' communities under contrasting management. Very few studies have actively assessed the effect of inoculation on AMF community composition (Antunes *et al.* 2009; Haskell 2017; Janoušková *et al.* 2017; Elliott *et al.* 2020), showing variable effects on both communities and their functional phenotypes that requires further exploration under a greater range of scenarios.

Many factors may determine the effectiveness of an AMF inoculation. This can include the form and intensity of tillage, fertilizer and pesticide applications, the inoculum potential of the added inoculum, underlying soil edaphic properties, and host-crop dependency on mycorrhizal associations (Köhl, Lukasiewicz, and Van der Heijden 2016; Verbruggen *et al.* 2013). Wheat cultivars have been shown to exhibit a range of responses to AMF inoculation. The percentage of root length colonization (RLC) in inoculated plants can range from 8 to 71 % RLC in modern cultivars, and from 6-71 % RLC in landrace cultivars, which demonstrate a with a range of positive, neutral, and negative nutrient uptake and growth responses (Azcon and Ocampo 1981; Hetrick *et al.* 1993; Hetrick *et al.* 1996; Elliott *et al.* 2020; Garcia De Leon *et al.* 2020; Thirkell *et al.* 2020). The variable capacity for wheat to form AMF associations means that to maximize the functional benefits of AMF to nutrient uptake and production we must consider the impact of inoculum addition across numerous candidate wheat cultivars. This will allow us to increase food security through ensuring that only cultivars which are known to benefit from AMF interactions will be inoculated, while those which function better without AMF will be spared. Few studies have documented wheat cultivar associated AMF microbiomes under field conditions, though from the limited evidence it is apparent that there is a host-fungus compatibility not only controlling colonization rates but also structuring community assembly (Aguilera *et al.* 2014; Mao *et al.* 2014).

In this study the inoculum growth response and rhizosphere fungal communities of five wheat cultivars was assessed over a two-year field trial. Wheat was grown with a multi-species commercial mycorrhizal inoculum (Root Grow, Plant Works). The field site was in North Yorkshire, UK. This region represents a suitable study system for a farm that could benefit from AMF contribution to nutrient uptake and plant growth through its presence within an NVZ, requiring innovative solutions to nutrient acquisition and maintenance of crop yields under reduced fertilizer inputs. The main aim of this study was to determine how the AMF inoculum performed in-field with regards to plant biomass, and whether this performance was driven by inoculum-mediated shifts in AMF community composition. The general fungal community was also assessed to both place the AMF community within a wider context to assess whether inoculum increased their relative abundance, and to explore whether AMF community changes impacted the general fungal community through shifts in pathogen abundances that may contribute to yield changes. The following questions were addressed: **(a)** Does a commercial multi-species AMF inoculum result in changes to wheat agronomic properties in-field? **(b)** Does inoculation alter fungal rhizosphere communities in-field? **(c)** Do wheat cultivars differ in their responsiveness to AMF inoculation? **(d)** Do rhizosphere fungal communities differ between wheat cultivars? It was hypothesised that the introduction of an AMF inoculum would increase plant biomass accumulation through increasing the mycorrhizal potential of AMF, and their relative abundance in the roots of plants, or through adding novel beneficial AMF that may be absent from the system. Based on previous research showing wheat cultivar-specific differences in mycorrhizal receptivity and function it was also expected that wheat cultivars would respond differently to the inoculum. Finally, for the same reason, it was expected that wheat cultivars would harbour distinct general and AMF communities.

2.2. Methods

2.2.1. Site Description and Experimental Set-Up

The field experiment was conducted at Leeds University Farm Research Unit (FRU) in North Yorkshire, UK (53° 52' 30.3'' N, 1° 19' 15.2'') over two seasons covering the 2014/15 and 2015/16 growing seasons. The site was sown with five cultivars of winter wheat over this period (*T. aestivum L. cv*. 'Avalon', 'Cadenza', 'Robigus', 'Holdfast', 'Mercato', RAGT Seeds, Cambridgeshire, UK). Wheat cultivar year of introduction, country of origin and expected mycorrhizal capacity are listed in *Table 2.1*.

Table 2.1. Summary of the five winter wheat cultivars grown in this experiment. Data is from the 'Genetic Resources Information System for Wheat and Triticale (CIMMYT)': http://wheatpedigree.net/

Cultivar	Year of Registration	Origin	Parentage
Avalon	1980	UK	Maris-Ploughman x Bilbo
Cadenza	1992	UK	Axona x Tonic
Robigus	2005	UK	Z836 x 1366 (PUTCH)
Holdfast	1936	UK	Yeoman x White-Fife
Mercato	2005	France	Unknown

A mycorrhizal inoculum was added to half of all plots, with the remaining half acting as a non-inoculated control. Wheat cultivars and inoculum treatments were planted and applied in a random block design over three blocks within the field- A, B and C. Inoculum treatments were randomly assigned to plots within sets of two rows inside each block (*Figure 2.1*). Two plots were unable to be included in the live strip of block C and were sown in the spare strip to the side of the experimental blocks. Individual plots measured 1.2 m x 1.7 m (2.02 m^2), with a buffer zone of 0.5 between plots.

Figure 2.1. Field plan of experimental plots. X, Y position co-ordinates represent the distance two the centre of each plot from the northwest corner of the experimental site.

The mycorrhizal inoculum used was "Rootgrow Professional" (PlantWorks Ltd. Kent, UK). This is a granular formulation containing propagules of spores, hyphal and root fragments. The AMF species claimed to be included within this inoculum are *Funneliformis mosseae* (also known as *Glomus mosseae*), *F. geosporum* (also known as *G. geosporum*), *Claroideoglomus claroideum*, *Glomus microaggregatum* and *Rhizophagus irregularis*. These species designations were searched against the MaarjAM database to find the VTs commonly assigned to each species hypothesis. These are cautiously assigned, as they are by name and not by sequence identity (*Table 2.2*). Few genetic sequences are available for *G. microaggregatum*, none of which are SSU sequence based as is required for VT assignment. It is likely that no type material of *G. microaggregatum* has been sequenced as they are commonly found growing within the spores of other AMF species (Wang *et al.*, 2009).

Table 2.2. Potential AMF species within the mycorrhizal inoculum ("Rootgrow" by PlantWorks Ltd) matched by name to fungal VTs within the MaarjAM database.

Inoculum species	MaarjAM VTs	Accession
F. mosseae[1]	VTX00067 [TYPE]	AJ206438
F. geosporum[1 2 3]	VTX00065	ESA02502
C. claroideum [3]	VTX00193	AJ276080
G. microaggregatum[4]	NA	NA
R. irregularis[5]	VTX00105 [TYPE]	AJ505617
	VTX00114	AJ505615

[1] The *Funneliformis* genus is present in the MaarjAM database under the *Glomus* genus designation

[2] VTX00065 is also associated with *F. mosseae*, reflecting that this species hypothesis can be interpreted as more of a complex of species.

[3] VT assignment is not from a 'TYPE' sequence as per the MaarjAM database classification, but from isolated spores classified with this species hypothesis. The 'TYPE' sequence for this VT is *F. caledonium.*

[4] There was no 18S VT attached to this species, nor was a sequence available through the NCBI database to find the closest match.

[5] The VT assignment for *G. intraradices* was chosen over that of *R. irregularis*. There was not an appropriate 18S TYPE specimen in the database for *R. irregularis,* but there was for *G. intraradices.*

The mycorrhizal inoculum was applied in 2014 at the beginning of the field experiment. The granular inoculum was added between the planted rows of wheat after germination and was not re-applied in the 2015 growing season as AMF added through inoculum are known to be detectable two years after inoculation (Pellegrino *et al.* 2012). For the 2015/'16 growing season plots were subjected to minimal tillage (hand cultivation within the top 10 cm depth of soil), with wheat residue from the previous season incorporated into the seed bed. Wheat seeds were sown in five rows between those of the previous year in November 2015. After an initial failure of wheat to germinate in-field, plots were re-sown in February 2016.

Ammonium nitrate fertilizer, (YaraBela, Yara, Grimsby UK) was applied to each plot at a rate of 50 kg ha^{-1} in April 2015. In 2016 fertilizer was applied at a rate of 34 kg ha^{-1} in May and 65 kg ha^{-1} in June (99 kg ha^{-1} total). The fertilizer additions used in this experiment are less than half of those typically recommended for arable fields within NVZs. The maximum amount of nitrogen permitted to be applied to winter wheat in these areas is 220 kg N ha^{-1}.

2.2.2. Wheat Sample Collection and Inoculum Response Measures

Three wheat plants were harvested in June 2017 to assess AMF communities. Whole root systems were collected from the plants, down to a depth of 30 cm for molecular community analysis. Plants were separately harvested in September 2017, coinciding with the end of the winter wheat growing season This was chosen to gather an agriculturally relevant measure of plant biomass. A single wheat plant was randomly selected from one of the two outer rows of wheat within each plot. Each wheat plant was oven-dried for 3 days at 70 °C and split in to two components for measuring mass- shoot (including leaves) and grain. Recorded shoot and grain biomass were combined as the total aboveground (ABG) biomass of the plant and analysed separately to grain biomass. ABG biomass and grain biomass were used to calculate harvest index. This is the ratio of grain to total ABG biomass, and is an agriculturally relevant measure of yield (Hay 1995) reflecting the resource input that a crop puts in to the development of grain. Whole plots were assessed for other agronomic wheat characteristics. A single wheat plant was surveyed at each corner and at the centre of plots, unless a plant was not present within 3 inches of a given sampling point. Wheat plants were assessed for the number of tillers per plant, height of the tallest tiller, and

the number of grain heads per plant. The mean for each plot was calculated for statistical analysis.

An inoculum growth response was calculated for all wheat metrics both across all samples and separated by wheat cultivar and field block. This response metric was calculated as the proportion change between control and inoculum treated plots ((inoculated value – control value) / control value). 95% confidence intervals were calculated around the mean of the inoculum growth response through permutation (R=9999), using the bias-corrected and accelerated ('BCa') method. This approach robustly corrects for bias and skewness in in the distribution of bootstrap estimates which can results from unevenly distributed data. To analyze mycorrhizal growth response, the overlap of 95% confidence intervals with zero was visually assessed and combined with two-tailed t-tests with the alternative hypothesis that inoculated samples will have a mycorrhizal growth response differing from zero in either direction. T-tests were performed over 9999 permutations also using the "BCa" bootstrapping method, and all p values were adjusted to account for the number of pairwise comparisons made by this method, using the Benjamini-Hochberg (BH) correction of family-wise error rates.

2.2.3. Molecular Analysis

DNA was extracted from the roots of wheat plants collected in-field as described in the previous section. Roots were washed with water, frozen, freeze-dried and ground using a Tissuelizer and stainless-steel grinding jars (Qiagen). Total DNA was extracted using MoBio (now Qiagen) PowerPlant Pro DNA extraction kits according to the manufacturers protocol. Resulting DNA concentrations were measured using a NanoDrop (Thermo Fisher) to ensure that there was DNA present without contaminants.

PCRs were performed in the presence of 0.2mM dNTPs, 10pmoles of each primer, 2mM $MgCl_2$, and the manufacturers reaction buffer, using GoTaq G2 Flexi Kits (Promega). PCR was carried out on a TC-512 thermocycler (Techne). Primer sets for two regions of the rRNA operon were used to identify all fungi, and to specifically target AMF species. Amplicon libraries for both targeted regions were created through a semi-nested PCR approach, summarized in *Table 2.3*. The Internal Transcribed Spacer (ITS) region was targeted for assessing the general fungal

community as this is accepted as the universal barcode for fungi but lacks specificity between detecting AMF species resulting in an often under-representation of the community. The SSU rRNA gene alternatively has been shown to capture a greater specificity of AMF species so was targeted for this purpose. (Stockinger, Krüger, and Schüßler 2010; Schoch *et al.* 2012; Thiéry *et al.* 2012).

Following semi-nested PCR amplicons were cleaned using AMPure beads (Agincourt) following the manufacturer's instructions. ITS amplicons and SSU amplicons were pooled in a ratio of 1:3 before NextEra (Illumina) barcoding and sequence library preparation. Libraries were sequenced on the Illumina MiSeq platform.

Table 2.3. Primer sets and PCR protocols used to generate DNA amplicons for the two targeted rRNA regions used in this study.

Amplicon Library	FWD	REV	Conditions	References
ITS Region (General fungi)	ITS1F	ITS4	95C 5m; 35 cycles (94C 30s, 55C 45s, 72C 1m 30s); 72C 10m	(White *et al.* 1990; Gardes and Bruns 1993; Ihrmark *et al.* 2012)
	gITS7	ITS4	95C 2m; 20 cycles (94C 30s, 55C 30s, 72C 1m 30s); 72C 10m	
18S gene (AMF)	AML1	AML2	95C 2m; 30 cycles (94C 30s, 59C 30s, 72C 1m); 72C 10m	(Lee *et al.* 2008; Dumbrell *et al.* 2011)
	WANDA	AML2	94C 5m; 20 cycles (94C 30s, 58C 30s, 72C 30s); 72C 10m	

2.2.4. Bioinformatics and Microbial Community Analysis

2.2.4.1 ASV Generation and Taxonomic Assignment

All bioinformatics analyses were performed in R (Version 3.6.1, R Core Team). Sequence reads generated from combined ITS and 18S amplicon libraries were demultiplexed using *Cutadapt* (Martin 2011). *Cutadapt* was used to trim the primer sequences from the forward and reverse ITS reads, and forward 18S reads, discarding any reads which did not have a segment matching the chosen primer sequence. Paired-end ITS reads were not further trimmed to capture the biological variation in ITS region length observed between fungal species. Single-end SSU amplicons were trimmed to a length of 240 bp, corresponding with a decline in the proportion of reads extending past this length. Reads from both regions were quality filtered (<2 errors per read), dereplicated and assigned to Amplicon Sequence Variants (ASVs) using the *dada2* algorithm (Callahan *et al.* 2016). ASVs are sequence clusters with near 100% similarity.

Taxonomy was assigned to ASVs through the implementation of a Bayesian classifier method (Wang *et al.* 2007) as implemented in the *dada2* package. This classifier compares the *kmer* profile (the DNA of each sequence split into short sequences of length *k*) of sample sequences to those of a given reference dataset of known taxonomy. The sequence with the most similar profile is used to assign taxonomy to the query sequence. This classification is bootstrapped 1000 times with a minimum confidence of 50% required for all assignments. Taxonomy was assigned to ITS sequences using the dynamic UNITE database (Nilsson *et al.* 2018). Taxonomy was assigned to 18S sequences using two reference databases. These were the SILVA (Quast *et al.* 2013) and MaarjAM (Öpik *et al.* 2010; Opik *et al.* 2014) databases. SILVA is a general database of SSU sequences across eukaryotes and prokaryotes whereas the MaarjAM database is a highly curated database specific to AMF. The taxonomic assignments in the MaarjAM database use phylogenetic methods to create 'Virtual Taxa' (VTs) in lieu of traditional species assignments. VTs instead can be thought of as species clusters/complexes, capturing what is thought to be wide intra-specific variation (Opik *et al.* 2014; Thiéry *et al.* 2016)

2.2.4.2 Taxonomic Curation

Taxonomic curation of the ITS and 18S sequences were performed using the R package *phyloseq* (McMurdie and Holmes 2015). ITS sequences were first filtered to remove any ASVs which could not be given a taxonomic assignment to the phylum level (50 out of 786 ASVs). ASVs were subsequently clustered in to 97 % OTUs using the R packages *DECIPHER* (Wright 2016) and *speedyseq* (McLaren 2020), were further agglomerated by their species level assignments, resulting in 437 OTUs and 336 agglomerated taxa. Sequences that were not assigned at a given taxonomic resolution were aggregated into a single 'unclassified' taxon within the highest taxonomic level that they could be assigned to. Following aggregation OTUs with less than 5 reads in at least two samples were removed from the dataset leaving 224 final OTUs in the dataset. ITS sequencing depth ranged from 14,391 to 240,884 reads.

SILVA taxonomic assignments of SSU reads were used to subset only ASVs assigned to the class Glomeromycetes (1155 / 1457 ASVs). ASVs were also clustered in to 97 % OTUs, resulting in 45 OTUs. Any OTUs not assigned to the VT level were reclassified manually against all sequences in the MaarjAM database. OTUs were agglomerated by VT-level taxonomic assignment leaving 23 VTs. Following prevalence filtering 16 final VTs were present in the dataset. SSU sequencing depth ranged from 6148 to 113504 reads.

Rarefaction curves were generated for all samples for both ITS and SSU datasets to visually assess species accumulation with increasing sequencing depth. All inspected curves appeared to be at or near an asymptote (*Appendix 2.6, Figure 2.5*) and ANOVA did not reveal significant differences in sequencing depth between any treatment groups, so the data was not rarefied to a standard sequencing depth. To normalize species abundances across samples with differing sequencing depth, read counts were converted to relative abundances.

2.2.4.3 Community Analysis

Alpha diversity was calculated for samples using *phyloseq* as observed OTU and VT richness, and Shannon index. Shannon Index is a measure which considers both the richness of species in a sample, and the relative abundance of species. This indicates the evenness of species abundances within a community, i.e., whether a community is dominated by one or few species with many rare individuals, or whether species are equally represented. Alpha diversity metrics were compared between groups using ANOVA testing after checking for normality through Shapiro-Wilke tests. The general and AMF community composition was visualized through detrended correspondence analysis (DCA) plots, generated with the *decorana* function in *vegan* with rare species downweighed (Oksanen *et al.* 2019). Community compositions were statistically compared through permutational multivariate analysis of variance (PERMANOVA), conducted using Bray-Curtis dissimilarities between samples. Where PERMANOVA was found to be overall significant, pairwise PERMANOVA between groups was conducted with BH corrections as a post-hoc test. Pairwise PERMANOVA analysis does not always discriminate between groups well when sample size is low, and so the DCA 1 and 2 axis values of samples were also compared through ANOVA as a complementary test to PERMANOVA. This is acceptable as distance along either axis represents turnover (change in community composition) between samples. Post-hoc testing of significant ANOVA outputs was conducted as Tukey's post-hoc with HSD corrections for multiple comparisons. Detrended correspondence analysis is based on an approach to condensing multivariate data (i.e., species abundance tables) into a two-dimensional space that can be used to visualize gradient patterns in turnover (i.e., difference in community composition between samples) called reciprocal averaging (Hill and Gauch 1980). Reciprocal averaging is an iterative algorithm that initially assigns arbitrary numbers to species (called *trial species scores*) and uses these to create *trial sample scores* by calculating a weighted average of all trial species scores. The weights in this case are based on the relative abundance of each species within each sample. These trial sample scores are then used to create new trial species scores through the weighted average of sample scores (hence the phrase *reciprocal*) and are normalized across samples through variance stabilization around zero. This process of reciprocal averaging is repeated several times until there is no change in species and sample scores upon successive iterations. This results in sample and species scores that are maximally correlated (i.e., have maximum

correspondence, which is why this is usually referred to as correspondence analysis, CA) so that the sample score reflects the underlying species composition to the maximum extent. Through this analysis the first axis (CA1) often represents an environmental gradient structuring a community across samples, though does not accurately represent turnover due to compression at either end of the axis. This also results in a characteristic 'arch' pattern in the data along axis 2 (CA2) that is an artifact making the value of the axis uninterpretable. DCA detrends the data firstly by splitting the ordination into sections along CA1 and variance normalizing the values within around zero. It then rescales the axes so that the distance between samples reflects turnover driven by changes in species abundances and presence.

Indicator species analysis was conducted to find OTUs which had a significant association with one or a combination of treatment variables. This is preferred for multivariate data with many comparison factors, as multiple pairwise comparisons can result in false positives. Indicator species analysis was carried out using the *multipatt* function in the *indicspecies* R packages (De Cáceres and Legendre 2009). P-values derived from the analysis were subjected to Bonferroni corrections as recommended by the *indicspecies* protocol.

2.2.4.4 Field Chemistry Characterization

Soil for chemical analysis was collected at the same time as wheat plants were collected in-field. Soil was collected up to a 30 cm depth using a 3.81 cm diameter cylindrical soil core. Soils were sieved to a <2 mm fraction and had roots, soil fauna and stones removed before any chemical analysis was conducted. All analyses of soil chemistry were performed at two depths- 0-15 cm and 15-30 cm to create a composite sample. Statistical analysis was performed on the composite mean value of properties for each plot rather than individual depths.

Fresh soils were subjected to a 1M KCl extraction followed by Whatman 44 filtering to obtain a filtrate that could be analysed photometrically for inorganic nitrate species-N (nitrate, NO_3^- + nitrite, NO_2^-) and ammonium-N (NH_4^+). Photometric analysis was performed using the microplate method described by (Hood-Nowotny *et al.* 2010). NO_3^--N and NH_4^+-N values were corrected for fresh soil moisture content and volume of KCl extractant used to get the mg NO_3^--N and NH_4^+-N per gram of soil as a measure of content (mg kg $soil^{-1}$). Soil moisture content was measured through drying a subset of soil at 105 °C for 24 hours and subtracting the dry soil mass from the original fresh soil mass. Oven-dried soils were then further subjected to temperatures of 550 °C for four hours to estimate loss on ignition (LOI) organic matter (Heiri *et al.* 2001). LOI organic matter was converted to LOI SOC under the principle that carbon makes up 58 % of organic matter stoichiometrically. This value will hereafter be referred to as LOI SOC. Soil pH was measured using 1M $CaCl_2$ solution.

All soil measures were tested for normality through Shapiro-Wilke tests and transformed appropriately where necessary. NH_4^+-N, NO_3^--N and pH were log transformed prior to analysis. Soil chemistry values were compared statistically through ANOVA tests conducted for *Block*, *Inoculum, Cultivar,* and the interaction factor of *Inoculum x Cultivar*. Where global comparisons were found to be significant ($p < 0.05$), pairwise comparisons were conducted using Tukey's post-hoc test with HSD corrections for multiple comparisons.

2.3. Results

2.3.1. Field Soil Chemistry

Moisture content and nitrate content were found to be different between field blocks at the time of harvest (*Table 2.4*). Block B and Block C had a significantly greater moisture content than Block A (ANOVA: F = 5.43, df = 2, p = 0.01), though the absolute recorded difference between blocks was minor (1.87 % and 1.77 % difference respectively). The scale of difference in NO_3^--N content between blocks was greater than this (ANOVA: F = 4.07, df = 2, p = 0.03), with Block A and Block B having increased nitrate contents in comparison to Block C by a factor of 1.74 and 1.95. NO_3^--N values were highly variable in Block A however, resulting in a non-significant difference from Block C. The Live and Control inoculum treated blocks also showed differences in soil chemistry, with live inoculated plots containing on average near half of the NH_4^+-N content observed in control plots (ANOVA: F = 6.93, df = 1, p = 0.02), and a small reduction in water moisture content (ANOVA: F = 5.43, df = 2, p = 0.01). No other differences in soil chemistry were observed across fields or inoculum treatments.

Table 2.4. Field chemical properties presented as mean values ± standard error. Values are presented for all plots used within the field experiment (Total), grouped by field block, and grouped by inoculum treatment. Superscript letters denote statistically different values ($p < 0.05$) for either Kruskall-Wallis tests (Inoculum comparisons) or Wilcox pairwise post-hoc tests where Kruskall-Wallis tests were found to be significant, following Bonferroni corrections for multiple testing (Block comparisons).

	Moisture content (%)	Nitrate content (mg kg^{-1} soil)	Ammonia content (mg kg^{-1} soil)	LOI (%)	pH
Total	9.75 ± 0.30	21.00 ± 2.08	21.20 ± 2.95	3.15 ± 0.03	6.97 ± 0.02
Block A	8.56 ± 0.44 ^{a}	23.04 ± 3.95^{ab}	23.04 ± 3.50	3.16 ± 0.03	7.00 ± 0.02
Block B	10.43 ± 0.26 ^{b}	25.92 ± 6.36^{a}	25.92 ± 3.94	3.19 ± 0.06	6.96 ± 0.04
Block C	10.33 ± 0.63 ^{b}	13.26 ± 4.19^{b}	13.26 ± 1.65	3.10 ± 0.07	6.97 ± 0.02
Control	10.37 ± 0.36 ^{a}	22.92 ± 3.31	29.05 ± 5.13^{a}	3.14 ± 0.04	6.97 ± 0.02
Live	9.18 ± 0.43 ^{b}	9.18 ± 2.61	13.88±1.70^{b}	3.16 ± 0.05	6.98 ± 0.03

2.3.2. Wheat Response to AMF Inoculation

The addition of an AMF inoculum was associated with an increase in ABG biomass ($p = 0.02$) and grain biomass ($p = 0.03$) in live inoculated plots (*Figure 2.2*). This was not however associated with any changes to the harvest index of these plants, being a relatively consistent increase across grain and stem. Other plant characteristics also showed no significant inoculum response.

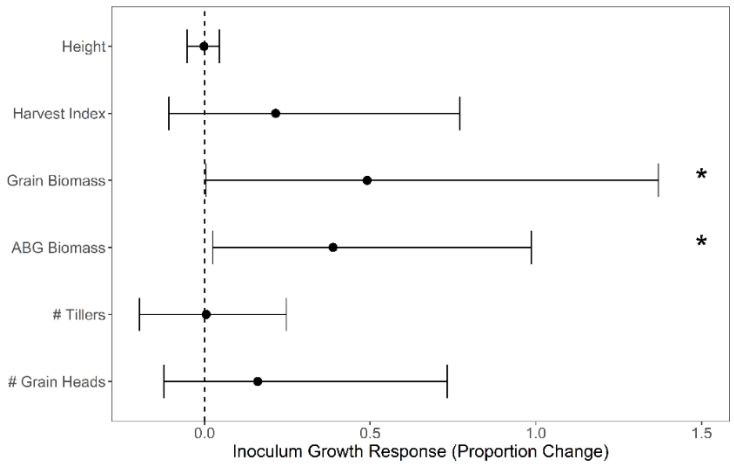

Figure 2.2. The inoculum growth response of wheat across all cultivars and blocks. Data are presented as mean values of growth response ± 95 % confidence intervals calculated by permutation (R=9999) using the 'BCa' method. Asterisks denote wheat characteristics that showed a significant ($p < 0.05$) response to inoculum through one-sample permutation testing after BH corrections for multiple testing.

Wheat cultivars exhibited a range of responses to the inoculum addition (*Figure 2.3 A*). Robigus was negatively impacted by inoculation, with decreases observed in harvest index ($p= 8.02x10^{-10}$), ABG biomass ($p= 0.006$) and grain yields ($p= 0.0002$). Avalon exhibited a reduced number of tillers in inoculated samples ($p= 0.001$), but this did not affect ABG biomass or grain yield, which could be an inference that the biomass per tiller was increased in inoculated plots. Holdfast was positively affected by inoculation, though the effect size of this was small. ABG biomass and grain yield increased by a mean proportion of 0.15 ($p= 8.76x10^{-7}$) and 0.19 ($p= 9.66x10^{-7}$) respectively in inoculated plots. Live inoculated plants also grew taller than control plants ($p= 9.11x10^{-7}$). Cadenza showed an increase in harvest index only ($p= 1.48x10^{-6}$), by a mean proportion of 0.96, though this was also very variable, with confidence intervals ranging from a lower limit of 0.09 to an upper limit of 1.76. All other

cultivars showed a generally positive mean effect sizes of wheat characteristics to inoculation, but were variable in this response, resulting in a statistically insignificant responsiveness. Mercato had the largest positive effect size associated with inoculum addition, but this was not significant due to the wide observed intra-cultivar variability.

When partitioned by field block, there was no variation in inoculum response observed for any wheat characteristics (*Figure 2.3 B*). The mean response across characteristics was generally positive, but with a large degree of uncertainty around these values reflecting intra-block variation between cultivars and demonstrating that responses in no one block specific were responsible for increases in ABG or grain biomass.

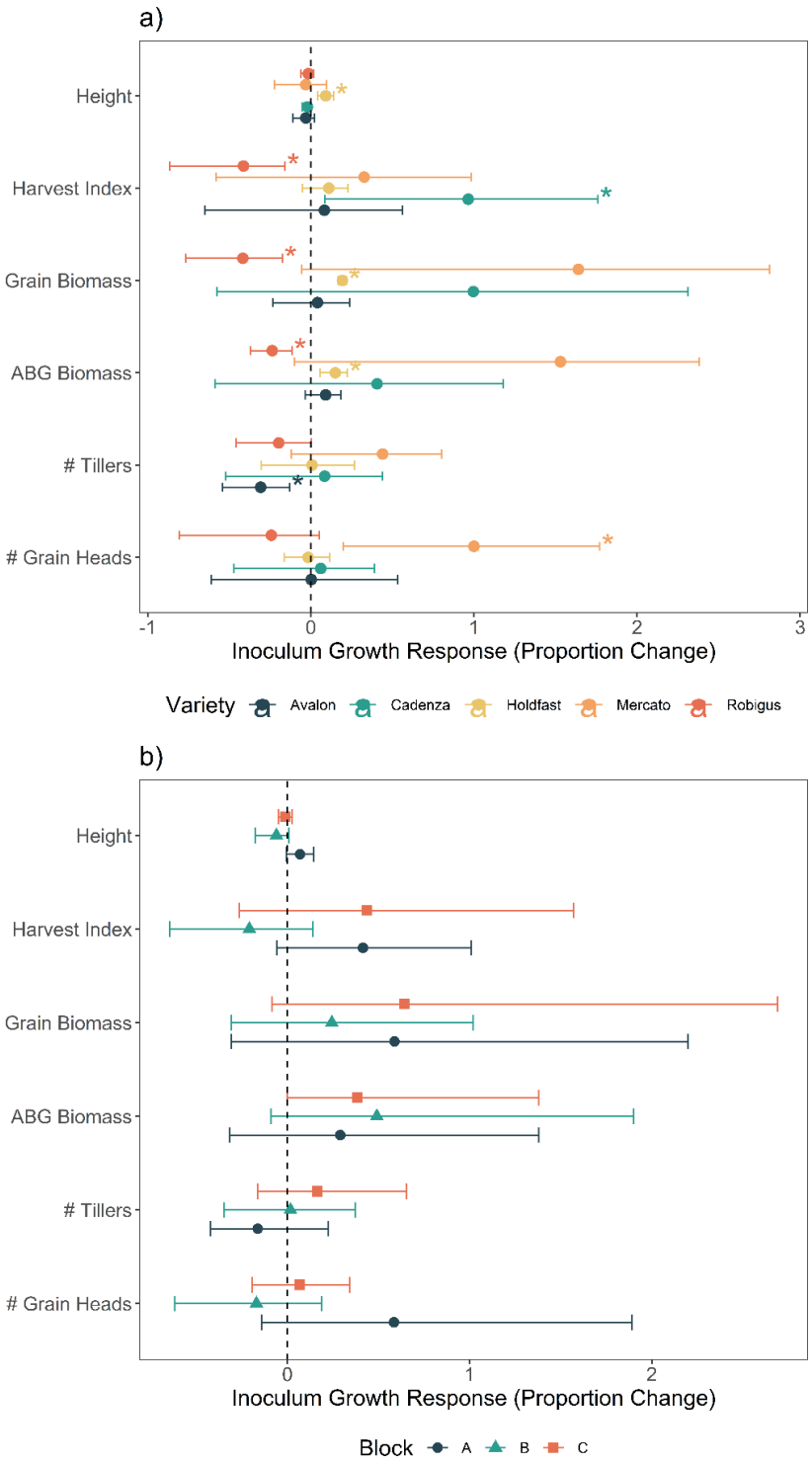

Figure 2.3. The inoculum growth response of wheat across a) cultivars and b) blocks. Data are presented as mean values of growth response ± 95 % confidence intervals calculated by permutation (R=9999) using the 'BCa' method. Asterisks denote wheat characteristics that showed a significant ($p < 0.05$) response to inoculum through one-sample permutation testing after BH corrections for multiple testing.

2.3.3. Fungal Community Composition and Diversity

2.3.3.1 Overview of General and AMF Communities at the Site

Across all samples 196 unique fungal OTUs and 18 AMF VTs were detected. Through ITS sequencing, members from 10 fungal phyla were observed. Plots were dominated by species belonging to Ascomycota (with a relative abundance of 0.76 ± 0.02 SEM), followed by Basidiomycota (0.23 ± 0.02 SEM). The next most abundant sub-phylum was Glomeromycotina (which is presented as a distinct phylum from Mucuromycota in the SILVA database- Glomeromycota). The relative abundance of this was very low in comparison to Ascomycota and Basidiomycota (0.0011 ± 0.0006 SEM). The remaining four phyla accounted for a combined mean relative abundance of approximately 0.0017. This included Olpidiomycota, Mortierellomycota, Chytridiomycota, and Rozellomycota. The AMF subset of the community as determined through 18S sequencing was dominated by members of the *Glomeraceae* family, accounting for a mean relative abundance of 0.67 ± 0.04 SEM, followed by *Paraglomeraceae* (0.23 ± 0.04 SEM). Three other families were present in the community at greater than 0.01 mean relative abundance. *Ambisporacea, Diversisporaceae and Gigasporaceae* accounted for relative abundances of 0.05 ± 0.01 SEM, 0.02 ± 0.02 SEM, and 0.03 ± 0.01 SEM, respectively. *Claroideoglomeraceae* was present in the community at a mean relative abundance below 0.01. No VTs belonging to the family *Archaeosporaceae* were detected at this site.

2.3.3.2 Alpha Diversity

For the general fungal community there were no observed differences in any calculated alpha diversity metrics between the experimental groups. When species richness is similar between communities. Shannon index was close to 3.5 across all samples, ranging between 2.52 and 3.5. Values of Shannon index are typically between 1.5 and 3.5. The field site therefore shows a high evenness for fungi. The AMF community was less even than the general fungal community. An average of 6.33 species of AMF were found in each plot (ranging from 3 to 13 per plot), with a mean Shannon index of 1.18. The inoculum addition did not increase the richness or diversity of the community (ANOVA: Richness, $F = 0.06$, $df = 1$, $p = 0.81$; Shannon index, $F = 0.32$, $df = 1$, $p = 0.58$), nor was there significant variation across the field blocks (ANOVA: Richness, $F = 0.731$, $df = 2$, $p = 0.49$; Shannon index, $F = 0.24$, $df = 2$, $p = 0.79$) or between cultivars (ANOVA: Richness, $F = 2.05$, $df = 4$, $p = 0.13$; Shannon index, $F = 2.09$, $df = 4$, $p = 0.12$). There was further no interaction effect of cultivar and inoculum (ANOVA: Richness, $F = 1.68$, $df = 4$, $p = 0.19$; Shannon index, $F = 0.50$, $df = 4$, $p = 0.73$).

2.3.3.3 Fungal Community Composition

The addition of AMF inoculum was not associated with any change to the composition of either the general or AMF communities colonizing the roots of wheat plants. The general fungal community was found to be structured by host wheat cultivar, while the AMF community was instead structured by field block (*Table 2.5, Figure 2.4*). Post-hoc pairwise comparisons of Bray-Curtis matrices through PERMANOVA were not able to discern differences between the general fungal communities colonizing the roots of specific cultivars. The cultivar effect on general fungal communities was also confirmed by the analysis of DCA axis scores (DCA1 ANOVA: $F = 5.406$, $df = 4$, $n = 30$, $p = 0.003$; DCA2 ANOVA: $F = 1.310$, $df = 4$, $n = 30$, $p = 0.293$) which revealed that the community variation across cultivars was spread across DCA axis 1. Tukey's post-hoc testing with HSD corrections for multiple comparisons showed that the cultivars Holdfast and Mercato were both significantly different from the cultivar Robigus, and that this contributed to the apparent global effect observed between cultivars.

Pairwise PERMANOVA comparisons of AMF distances revealed that the block effect observed was driven by differences between Block A and C, which were the greatest distance from one another. Variation along DCA axis 2 was associated with compositional differences between field blocks (DCA1 ANOVA: $F = 0.233$, $df = 2$, $n = 30$, $p = 0.794$; DCA2 ANOVA: $F = 3.444$, $df = 2$, $n = 30$, $p = 0.047$), though this was only marginally significant. Post-hoc Tukey tests corroborated the findings of pairwise PERMANOVA testing.

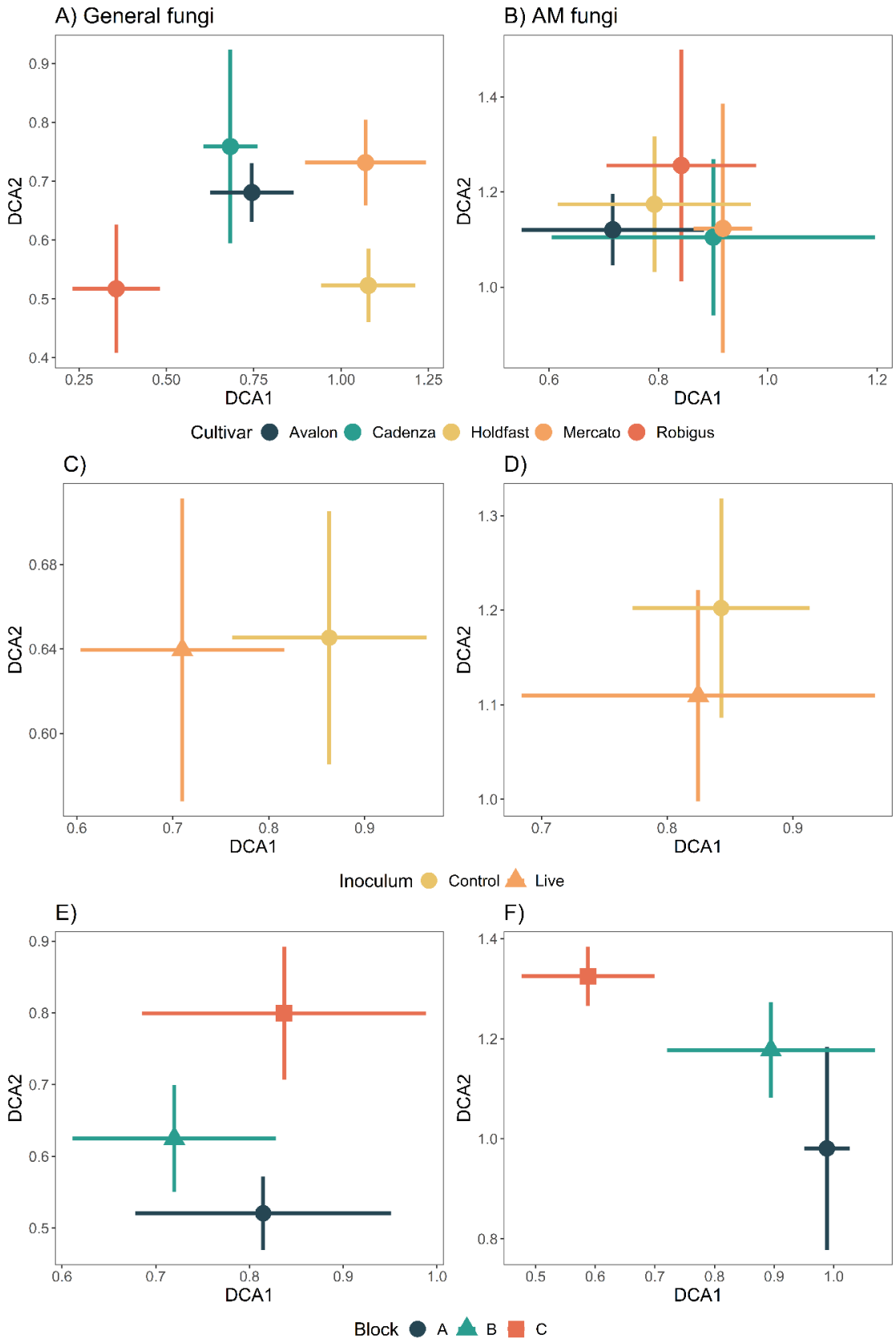
A) General fungi
B) AM fungi
DCA2
DCA1
Cultivar Avalon Cadenza Holdfast Mercato Robigus
C)
D)
Inoculum Control Live
E)
F)
Block A B C

Figure 2.4. Detrended correspondence analysis (DCA) ordination plots of general fungi (left) and AMF (right) community data separated by A) B) Wheat cultivar, C) D) Inoculum treatment and E) F) Field block. Data are presented as the mean centroid values of each wheat cultivar and inoculum treatment ± standard error. For the general fungal community ordination: DCA axis 1 length = 1.47, eigenvalue = 0.13, variance explained = 41.62 %; DCA axis 2 length = 1.27, eigenvalue = 0.06, variance explained = 21.7 %.

Table 2.5. Results of PERMANOVA and Beta-dispersal analysis comparing experimental groups. Significant outcomes are denoted by **bold** values and asterisks.

Taxonomy	Source of Variation	PERMANOVA		Beta-Dispersal
		Variance Explained (%)	p-value	p-value
ITS (General Fungi)	Block	9.78	0.08	0.89
	Cultivar	**21.28**	**0.02 ***	**0.58**
	Inoculum	2.98	0.52	0.70
	Cultivar x Inoculum	8.85	0.98	0.76
SSU (AMF)	**Block**	**14.69**	**0.009 ***	**0.06**
	Cultivar	8.03	0.96	0.89
	Inoculum	1.42	0.88	0.49
	Cultivar x Inoculum	11.60	0.80	0.99

Differences in the relative abundance of AMF taxa were tested at the VT and family level, whereas for the general fungal community this was assessed at the genus and phylum level. This was carried out to capture two resolutions of taxonomic differentiation within each community and to assess whether the functional guild of AMF (represented by Glomeromycota at the phylum level using UNITE taxonomy) differed between treatments. Reflecting the lack of differentiation between the communities of inoculated and uninoculated plots, no fungal genus was found to be significantly different between the inoculation treatments and only one unique genus was found in inoculated samples- *Scutellinia.* The abundance of Glomeromycota (and the nine other tested phyla) also showed no significant association with either inoculated or uninoculated plots. Indicator species analysis also did not reveal any taxa at the genus and phylum level for the general fungal community, or the VT and family level significantly associated with any field block or cultivar.

17 AMF VTs and 145 general fungal genera were shared across both control and live inoculated plots. One AMF VT was found only in control plots (VTX00214 *Glomus sp.*, 0.006 % ± 0.003 SEM) while two were found only in live inoculated plots (VTX00153 *Glomus sp.*, 0.004 % ± 0.003 SEM; VTX00193 *C. claroideum / C. lamellosum*, 0.002 % ± 0.002 SEM). The three unique VTs were all rare VTs present in low relative abundances. VTX000193 is associated with the AMF species group *C. claroideum* and *C. lamellosum*, the former of which was claimed to have been added by the AMF inoculum. Of the three other named species present in the inoculum with associated MaarjAM VTs, at least one representative VT of each species was present in both control and inoculum plots. These were VTX00067 (*G. mosseae*); VTX00065 (*G. geosporum / G. caledonium*) and VTX00114 (*R. irregularis*). VTX00065 was the most abundant VT at the site, present at a relative abundance of 0.587 +- 0.032. VTX00067 and VTX00114 were less common, at relative abundances of 0.349 +- 0.038 SEM and 0.069 +- 0.022 SEM. Other common AMF VTs (> 0.01 relative abundance) at the site included VTX00281 (0.389 +- 0.052 SEM, *Paraglomus laccatum*), VTX00064 (0.255 +- 0.04 SEM, *Glomus sp*), VTX00283 (0.178. 0.028 SEM, *Ambispora fennica*), VTX00062 (0.05 +- 0.027 SEM, *Diversispora sp*); VTX00052 (0.072 +- 0.025 SEM, *Scutellospora sp*) and VT00049 (0.021 +- 0.009 SEM, *Scutellospora sp*).

2.4. Discussion

The inoculation of plots with a multi-species commercial inoculum was associated with increases in wheat biomass at harvest in terms of total ABG biomass and commercially important grain yields. ABG biomass and grain yields increased by an average of 49 % and 88 % respectively across all wheat cultivars. Recent meta-analyses have shown that AMF inoculants can improve yields in many crops, and particularly in wheat, by averages of 15 % and 17 % respectively (Pellegrino *et al.,* 2015, Zhang *et al.*, 2019). By this comparison, our observed field site is high performing in the inoculum response of wheat, though it is notable that there was considerable variation in this response across the study. Soil ammonium and moisture content was also significantly reduced in live inoculated plots, which could be indicative of an increased uptake by AMF and suggestive of increased activity as AMF have been shown to preferentially absorb ammonium to nitrate (Ngwene *et al.* 2013).

Despite the apparent benefit of the inoculum addition, the rhizosphere AMF microbiome was unaffected with no observed shifts in species richness, diversity, or community composition. The relative abundance of AMF within the general fungal community was also similar between control and inoculated wheat. Introduced AMF can modify the rhizosphere AMF community (Koch *et al.* 2011; Elliott *et al.* 2020) but there are still few studies assessing microbiome responses to AMF inoculum in-field to compare this response against. A similar outcome to our study has also been observed in field-inoculated lettuce (Epelde *et al*. 2020). Antunes *et al.* (2009) showed that when maize was co-inoculated by a resident soil community and a single species inoculum at the same time, the single species inoculum did not result in different communities to the resident community alone. Many arable soils contain resident AMF communities that are already well established (Oehl *et al.* 2010), and truly AMF-free plants outside of highly controlled greenhouse experiments are also highly unlikely, though the inoculum potential of these communities- as measured through AMF spores, hyphae and colonized roots- can be hindered by conventional farming practices (Lekberg and Koide 2005). All but one of the AMF VTs attached to a named species within the commercial inoculum were already present at the field site in control plots. VTX00193 (*C. caledonium* / *C. lamellosum*) was present only in live inoculated plots, but only in a very small relative abundance. Due to the already

present AMF consortia at the site, VTX00193 may have failed to considerably colonize the roots due to priority effects and the organisms inability to outcompete members of the pre-existing community, as has been previously observed in inoculation studies (Mummey *et al.* 2009).

While adding redundant VTs to a site may appear counterintuitive, increasing the inoculum potential of soils through so-called 'native' inoculants may have a benefit over standardized commercial inocula treatments (Rowe *et al.* 2007; Frew 2020; Maiti 2011) and be more ecologically conscious in the consideration of reducing alien introductions to the surrounding ecosystem (Hart *et al.* 2018). In the context of this study, the inoculum was not explicitly native, as it was not extracted from the site and cultured though it was mostly complementary to the species pool already present. The inoculum may have added different strains within the same VTs, as AMF are known to have great intra-specific diversity (Mathieu *et al.* 2018). Agricultural soils may also select for the VTs of AMF most able to cope with regular disturbance pressure (Johnson *et al.* 1992; Johnson 1993; Verbruggen and Kiers 2010). This may align with those that are most easily culturable and therefore utilizable in commercial inoculums, such as VTX00065 (*Glomus mosseae*), which is near ubiquitous in worldwide agricultural soils including at our field site, and was one of the potential VTs added in the inoculum (Rosendahl *et al.* 2009). The general fungal community was also unaffected by inoculation, though this is not surprising as this response would be expected to be mediated by AMF interactions with members of the wider fungal community (Filion *et al.* 1999; Johansson *et al.* 2004; Whipps 2001; Lioussanne *et al.* 2009).

An important consideration in interpreting the disparity between wheat inoculum response and that of the AMF rhizosphere microbiome observed in this study is that only fungi colonizing the root compartment IRM of the mycorrhizal symbiosis were assessed. AMF microbiomes at the same site can be drastically different in the root compartment and immediate rhizosphere of plants from that of the surrounding bulk soil (Zhang *et al.* 2018), ERM hyphae and spores (Varela-Cervero *et al.* 2015). The inoculum was applied as a granular suspension of colonized root fragments and spores indirectly between wheat rows in the first year of the trail and mixed across plots through minimal tillage of the upper 10 cm of soil prior to sowing wheat in the second year of the experiment. The increasing number of AMF propagules within the

soil may have contributed to the stimulation of nutrient acquisition without ever directly interacting with the root compartment of the wheat. The mechanism of this may be through the scattered inoculum propagules acting as nodes to fortify and extend the mycorrhizal hyphal reach in soils through interactions with hyphae emanating from the direct connection with the plant host. AMF hyphae are coenocytic- meaning that they are one long cell not divided in to compartments- and spores are multi-nucleate (Helgason & Fitter, 2005, and references therein). Hyphal exploration of the soil may therefore stimulate the activation of spores and hyphae from the inoculum as they come in to contact and fuse, facilitating the transfer of genetic resources and nutrients through these structures. This is conceptually similar to the common mycelial network wherein distinct AMF colonizing different plants can fuse to create a large interactive network that can facilitate signalling and nutrient transport across greater distances (Bücking *et al.* 2016). This is a speculative hypothesis that may be able to be resolved to some degree by considering the soil hyphal density and microbiomes associated with the soil bulk compartment in future inoculation studies.

Experiments concerning inoculation responses are typically defined by the measure of root length colonization as a primary response variable, reflecting the abundance of AMF forming associations with the host plant (Lekberg and Koide 2005; Pellegrino *et al.* 2015). Observing the root inhabiting community therefore provides an insight into the species making up this IRM, and their abundance within the general community as a loose proxy of colonization efficiency. Recent studies have suggested better incorporating compositional changes and functional inference as a more appropriate response variable to inoculum under field conditions than colonization rates (Hart *et al.* 2017). It has further been acknowledged that root length colonization measures inaccurately reflect the contribution of non-Glomus genus AMF (Hart and Reader 2002a) to the symbiosis, as *Glomus* species are more likely to direct biomass production to associated internal structures than other genera which may instead focus on hyphal production or spore production, (Hart and Reader 2002b; Powell *et al.* 2009; Varela-Cervero *et al.* 2015). This inaccuracy may further extend to the assessment of root microbiomes exclusively, which also tend to be dominated by *Glomus* fungi such as in this experiment where 76 % of AMF sequences belonging to this genus. A more comprehensive study of soil microbiomes across both

the rhizosphere and bulk soil compartments and hyphal / spore structures could address a gap in our understanding of the mycorrhizal symbiosis, particularly in the variation between studies where root length colonization shows a neutral or inverse relationship to yields.

Our findings are complementary to the study of Elliott *et al.* (2020) which was conducted using soil also derived from the same farm site as in our study- Leeds University FRU. Their study found a neutral response of both ABG and belowground biomass growth to a single-strain *R. irregularis* inoculum in the wheat cultivars Skyfall, Avalon, and Cadenza despite considerable increases in root length colonization being observed in all three cultivars. (Thirkell *et al.* 2020) also observed similar effects of mycorrhizal association in the three cultivars in terms of AMF-acquired N and P in plant shoots. Through T-RFLP analysis of the AMF community it was revealed that community composition of AMF communities was altered by the mycorrhizal inoculum, and that there was no difference in community composition between the three cultivars (Elliott *et al.* 2020). This is contradicted in our results, where AMF inoculum did not change the community composition across a slightly larger collection of cultivars, though we also observed similarity in AMF communities between the cultivars Avalon and Cadenza.

Wheat cultivars showed divergent reactions to the mycorrhizal inoculum in terms of agronomic traits. Most cultivars responded neutral-to-positively to varying degrees though Robigus had an overwhelmingly negative response to inoculation. Robigus exhibited a reduction in ABG biomass, grain biomass and harvest index in live inoculated plots. Avalon exhibited a small negative response to inoculation in terms of wheat tiller count, though this did not overall impact yields, indicating that each tiller may have had increased biomass gains resulting in a neutral response to inoculation overall. As previously mentioned, differences in cultivar inoculum response did not coincide with cultivar specific microbiome responses. Divergent responses of wheat cultivars to AMF inocula without microbiome specific responses have also been previously observed (Garcia De Leon *et al.* 2020). Divergent cultivar responses to inoculum were expected based on previous studies demonstrating variable root length colonization rates and biomass accumulation across wheat cultivars (Azcon and Ocampo 1981; Hetrick *et al.* 1993; Hetrick *et al.* 1996; Elliott *et al.* 2020; Garcia De Leon *et al.* 2020; Thirkell *et al.* 2020). Hetrick *et al.* (1992)

hypothesized that land race wheat cultivars would be more responsive to AMF, potentially through factors such as selective breeding for cultivars that may thrive under high nutrient conditions and therefore rely less on mycorrhizal associations. While it was not explicitly addressed in our experimental design, an interesting finding from our analyses was that the oldest cultivar Holdfast had modest, but consistently positive responses to inoculum with less variability in comparison to the other cultivars. As many studies have documented across numerous crops, modern cultivars may have lost many adaptive traits such as mycorrhizal associations that would be beneficial to re-integrate and improve crop performance under future stress scenarios (Dwivedi *et al.* 2016; Trethowan and Mujeeb-Kazi 2008).

The wheat cultivars considered in this study harboured distinct rhizosphere fungal communities, but not distinct AMF communities. The species pool of AMF at the site was much lower than that of the general fungal community (16, and 336 OTUs respectively) which may be a contributing factor to the lack of effect seen between cultivars in terms of AMF communities. Wheat cultivars may display variable fungal communities due to variation in their root architecture (Valverde-Barrantes *et al.* 2016; Sweeney *et al.* 2021) or exudates (Broeckling *et al.* 2008). Exudate variation has been observed between wheat cultivars though this has been viewed from the comparison of land race and modern cultivars Land race and modern wheat cultivars have been shown to exhibit varying exudate profiles (Iannucci *et al.* 2017), but there are no available studies on exudate profiles or root architecture comparing between a large suite of modern wheat cultivars. Future studies examining fungal communities across wheat cultivars will benefit from incorporating both sets of measures to unpick the causation of community differentiation. This could be used to inform breeding and cultivar choice in fields to maximise beneficial fungal groups.

2.5. Conclusion

Reducing the dependence of agricultural systems on fertilizer additions is becoming of increasing importance to ensure that yields can remain sustainable and environmental impacts minimal in response to increasing input regulations of farming practices, climate change, and food security. Our results show that using a mixed AMF inoculum in-field can have benefits to wheat yields in low-input farming systems. Further research is required to ensure that the trade-offs associated with this reduction in inorganic fertilizer inputs result in equitable yields to conventional systems, and how AMF inoculum may be used in combination with other environmentally conscious practices such as fallow periods, leguminous crop rotations and intercropping to offset any potential yield gaps. The overlap between AMF species added by the inoculum and those already present across the field site demonstrates that gains to wheat yields can be made using a 'native' / redundant inoculum, potentially through increasing the AMF inoculum potential and reinforcing the existing community rather than through adding novel species to the system (Verbruggen *et al.* 2013). There has been concern voiced over the unintended consequences of introducing 'new' AMF to a community which may outcompete those already present, along with the uncontrollable spread of AMF in field conditions (Hart *et al.* 2017). In situations where an inoculum is necessary to add, using a native or at least redundant complement will reduce this risk. We also observed cultivar-specific controls on the general fungal community, along with divergent responses to AMF inoculum in terms of biomass change, including negative, neutral, and positive responses. This supports the need to further consider the mycorrhizal capacity of wheat cultivars in future wheat breeding programs to ensure that the recommended cultivars can harness the benefits of the AMF mycorrhizal association.

2.6. Appendix

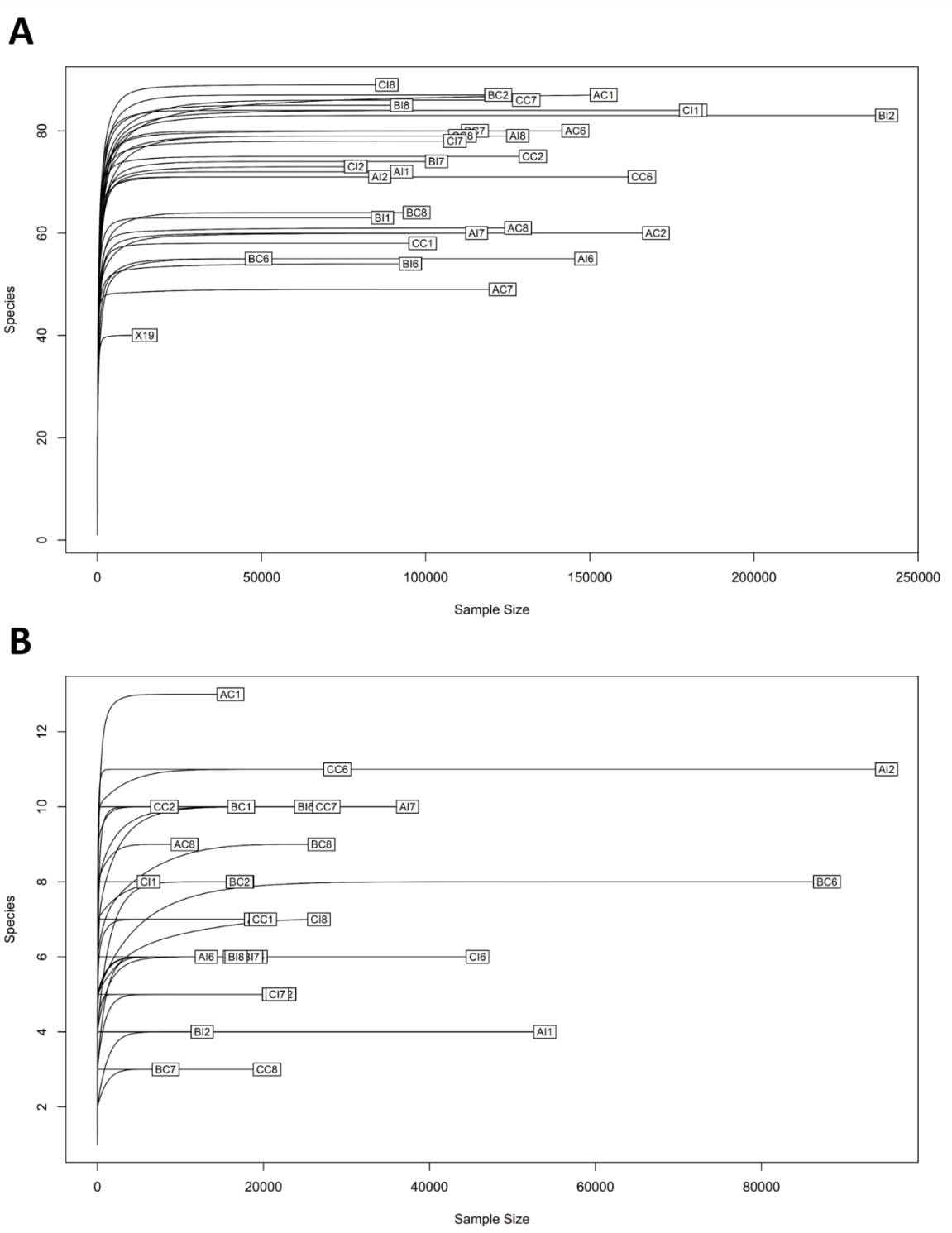

Figure 2.5. Rarefaction curves generated per sample for A) ITS sequences and B) SSU sequences.

Prologue to Chapter 3 and 4

In the following two experimental chapters there is a shift in the study system and the underpinning experimental treatments used to manipulate AMF communities and soil health. The following is an apologia for this abrupt redirection, detailing the scientific rationale and the external factors which also contributed to this choice.

The main hypothesis presented in Chapter 2 was that AMF inoculum addition could be used to increase the abundance, diversity, and functional potential of AMF communities colonizing host crop plants. To address this hypothesis, Chapter 2 explored the use of AMF inoculum addition to increase AMF diversity and host crop performance under 'normal' circumstances in-field. Chapter 2 was intended as an introduction to the experimental system, the methodology of community manipulation, and to generate soils with similar underlying edaphic properties but differing levels of AMF diversity and community composition. From this, further experimental manipulations of water-stress conditions could be made to explore the central research question posed in the title of the thesis.

To re-summarize the findings of Chapter 2- The hypothesis could not be entirely confirmed through the experiment. Inoculum addition did not result in an increase in AMF diversity or abundance and was likewise not associated with any shift to community composition. Despite this, there was an increased functional potential observed, with the inoculated wheat exhibiting an increase in ABG biomass in comparison to control plants. This feature could be due to increasing the inoculum potential of the surrounding soil to the benefit of host crop plants without any observable shift to the presence of AMF within the root compartment itself, therefore resulting in the null response based on sequencing analysis. The importance of the AMF residing in the bulk soil or the ERM hyphal network was not considered in the original hypothesis, and so there are no samples available to confirm this theory. Other studies since have however shown a large discrepancy in root-residing, and hyphae generating AMF communities (Varela-Cervero *et al.* 2016b; 2015) that show this should be a consideration in future studies.

Chapter 2 therefore did not establish a system wherein AMF community composition and diversity were observed to be successfully manipulated or provide a proof-of-concept that this could be done. The focus of the thesis research was subsequently

shifted towards another system through the in-field manipulations conducted as part of the *SoilBioHedge* project also carried out at Leeds University Farm. Holden *et al.* (2019) demonstrated that at this site arable and pasture soils contained distinct AMF communities, with pasture soils containing a greater richness of AMF VTs than arable soils. Reciprocal strips of grass-clover and conventionally managed crop-rotations were subsequently planted in the arable and pasture fields respectively to observe the effects of a ley fallow period in the arable strips, and the effects of tillage on the pasture system (which was the target direction and '*best case scenario'* of the change in soil health expected for the ley fallow period). Preliminary analysis also showed that the AMF communities had indeed begun to shift to different extents within the reciprocal experimental strips in both expected directions from their original field conditions (See Bird and Helgason, unpublished data; *Appendix 3.6*).

Further to the hypothesis-driven rationale underpinning the change in experimental direction presented, there were also logistical and personal reasons for the choice. The field experiment conducted in Chapter 2 was a large undertaking which was physically and emotionally taxing for a single junior PhD student to carry out with minimal experience. Due to burnout, mental health decline and a field-work related injury over this period, the data collection and analysis associated with Chapter 2 was behind schedule. It was considered at the time that joining an established experiment from which the thesis aims could be explored would allow sufficient reprieve without taking an extended leave of absence or requesting an extension to the PhD.

In collaboration with researchers at the University of Sheffield, a research plan was devised that incorporated the thesis aims into an offshoot of the *SoilBioHedge* project. This offshoot featured an experimental design which bore methodological similarity to the study that was being formulated to follow the inoculum field trial in Chapter 2, albeit using a different system. Soil taken from each of the land use strips in-field was to be planted with wheat and subjected to drought and flood stress. The devised research plan incorporated the assessment of AMF, along with nitrogen cycling prokaryotes within a longitudinal analysis across the time course of the wheat growing season. The assessment of nitrogen cycling prokaryotes was included as AMF have been shown to not just influence water relations under stress conditions, but are also particularly important in reducing N loss from arable soils (Bender *et al.* 2015; Cavagnaro *et al.* 2015). One of the potential mechanisms of AMF contribution to

reducing nutrient leaching is through their interactions with nitrogen cycling prokaryotes, though little was known at the time about this interaction, and it is still a developing area of research (Cavagnaro *et al.* 2007; Bowles *et al.* 2018; Storer *et al.* 2018). It was hypothesised that firstly AMF diversity and compositional differences across the land uses would be significantly associated with soil health. Through this and the benefits to host plants associated with the symbiosis, AMF would also confer crop and soil response advantages to drought and flood stressors. It was further hypothesized that the interaction between AMF and nitrogen-cycling bacteria would mediate nitrogen dynamics within the system, affecting the potential for N losses through NH_4^+-N leaching, NO_3^--N leaching and gaseous N_2O emissions. Through competition for nutrients and alterations to water relations affecting nitrogen cycling activity, AMF would moderate the expression of key functional N genes that would be indicative of various process rates and influence both N loss and plant nutrition (Cavagnaro *et al.* 2015). This assessment would have provided key results demonstrating mechanistic interactions between AMF and nitrogen cycling prokaryotes that would begin to resolve our knowledge gaps. AMF and nitrogen cycling bacteria were however not able to be longitudinally studied through the experiment due to the structural disruption this would cause to the soil profile through sampling, and the negative effect this would have on future measures required by the experiment collaborators. This constraint however was not known until a considerable time investment had already been put into the experiment. Without abandoning the experiment and leaving collaborators without the shared measures that were assigned to my aspect of the project (i.e., the longitudinal analysis of soil nutrient contents), an alternate experiment was unfeasible to set up.

With reflection on the completed thesis against the original aims, this may have not been the most beneficial choice. Greater efforts could have been made to maintain the methodological through line of using AMF inoculum addition as the chosen management practice to manipulate AMF diversity and assess the role that this plays in water stress mitigation, nutrient retention, and crop yields. As discussed in Chapter 2, one of the potential reasons for the lack of community or diversity response to the in-field inoculum was the presence of an already established AMF community at the field site paired with the general lack of truly novel VTs added to the community. At the expense of "field-relevance", A series of more controlled and directed

experimental manipulations of AMF and water conditions could have followed the findings of Chapter 2. Through this the mechanistic relationship between AMF species, diversity and community complexity in conferring crop and water stress benefits could be conducted than was able to be done through Chapter 3 and 4.

3. Exploring the impact of arable land use and management strategies on soil health, water and nutrient retention, and crop yields following an artificial drought and flood

3.1. Introduction

Agricultural management practices associated with the '*Green Revolution*' of the 1950s (i.e., deep tillage, high mineral fertilizer, herbicide and pesticide inputs) have ensured that up until now food production could maintain pace with an ever-growing human population (Tilman *et al.* 2002). These short-sighted practices have focused on maximizing grain yield, with little regard for the long-term impacts on soils or the surrounding environment that are necessary for sustainable production and long-term food security (Matson *et al.* 1997; Tilman *et al.* 2002; Foley *et al.* 2005). This has pushed agricultural soils into a state of disequilibrium causing the degradation of key soil properties that are associated with soil functions which support human activities including plant productivity, carbon and nutrient cycling, and water relations (regarded holistically as soil health, Doran 2002; MEA 2005). It is estimated that soil degradation affects approximately 33 % of all land worldwide, with deforestation, overgrazing, and poor agricultural management identified as the key contributors (FAO & ITPS 2015).

In the UK, 71 % of all land is used for agriculture (17.3 ha), of which 6 million ha are classed as 'croppable' land. This includes land under cereal production (50 % of croppable land), temporary grass or grass-clover cover (i.e., ley rotations within cereal producing land, 20 % of croppable land), uncropped land (e.g., grassy margins, low productivity unplanted cropland, hedgerows *etc.*, 9 % of croppable land) and a variety of other vegetable, bioenergy and horticultural crops (DEFRA 2020b). Wheat (*Triticum aestivum*) is the most widely grown cereal crop in the UK, historically covering 1.6- 2.1 million ha of croppable land between 1985 and 2019 (DEFRA 2020b), and is primarily concentrated within the Eastern region of England (Harkness *et al.* 2020).

60 % of arable land in the England is cultivated through the practice of mouldboard plough and harrowing (Townsend *et al.* 2016). This has resulted in considerable SOC decline over time which can be as high as 400 kg C ha^{-1} yr^{-1} under winter wheat cropping systems (Heenan *et al.* 1995; Persson *et al.* 2008), which also affects the fertility of soils (Tiessen *et al.* 1994). SOC losses across UK arable land have been primarily attributed to the conversion of high SOC-containing permanent grassland to continuously cultivated arable land, and a reduction in temporary grasslands within arable rotations that are traditionally used to restore such properties between crops, known colloquially as leys (King *et al.* 2005; Kirk and Bellamy 2010). The disruption of soil structure and SOC losses further affect the hydrological functioning of soils. Conventional intensive arable systems with tillage disturbance are associated with reduced water holding capacity of soils by as much as 26 % (Govindasamy *et al.* 2020), reductions in drainage capacity, and increases in surface water run-off in comparison to no-till systems (Dick *et al.* 1989)

Despite the widescale degradation of soil health associated with intensive management, worldwide cereal yields have never been higher, though the incremental gains in yield year-on-year have been falling in recent years and we are now facing a plateau in the yields of global staple crops (Knight *et al.* 2012; Ray *et al.* 2012; Fischer *et al.* 2014). This observed buffering of the effects of soil health decline is in part due to the continuous high application rates of inorganic fertilizer seen since the *'Green Revolution'* which offset any of the potential negative effects that may be seen on yields due to tillage-induced SOC loss and the cascading effects of this on soil health and innate fertility over the same period (Persson *et al.* 2008). This is not however an economically or ecologically viable strategy, as described in *Section 1.2.2.*

Climate change is further expected to compromise the capacity for reliable increases in food production as changing environmental conditions and weather patterns will lead to both the increased incidence and severity of flooding and drought during key crop development periods (Ekström *et al.* 2005; Fowler *et al.* 2005; Bates *et al.* 2008; Murphy *et al.* 2010). The projected unpredictability of precipitation events will affect food security as yields are expected to fluctuate drastically due to these environmental changes (Porter and Semenov 2005; Schmidhuber and Tubiello 2007; Fuss *et al.* 2015; Ray *et al.* 2015). This is evident across Europe where wheat yields have been estimated to decrease 33 – 50 % due to the extreme expected variations in

temperature and precipitation (Thaler *et al.* 2012; Mäkinen *et al.* 2018). This is a considerable vulnerability considering that wheat is Europe's most important cereal crop, accounting for 128.99 million tonnes of production in 2018, or 43.71 % of all European cereal yields (Eurostat 2018). As a pertinent example of this, UK wheat yields in 2020 were severely depressed due to the erratic weather conditions experienced during the growing season. The UK experienced over +200 % anomalies in precipitation compared to the 1981-2010 average in February, causing widespread flooding. This was paired with a drier than average March, April, and May, particularly across the east of England where most of the wheat is grown (Harkness *et al.* 2020, Met Office, 2020 metoffice.gov.uk/research/climate/maps-and-data/summaries/index). Consequently, the final yield estimates across the UK were 9.7 million tonnes, or 7.0 tonnes ha^{-1}. This is lower than the five year average of 8.4 tonnes ha^{-1} (DEFRA 2020b).

Part of this vulnerability to weather extremes likely stems from the reduced functional capacity of AMF in agricultural soils, which can play a vital role in maintaining soils structure, hydrological function, and increasing the water stress tolerance of host plants (Ruiz-Lozano *et al.* 1995; Augé 2001; Rillig 2004; Rillig and Mummey 2006; Bowles *et al.* 2018). Tillage disrupts soil exploring AMF hyphae which contribute to macroaggregate formation (Tisdall and Oades 1982; Rillig and Mummey 2006), and also reduces the capacity for them to re-establish associations with crop plants, leading to reduced root colonization and nutritional benefit in the long-term (Brito *et al.* 2012; Verzeaux *et al.* 2016). Of the AMF that are able to re-establish year-on-year in conventionally managed arable soils, they are likely optimized for self-preservation and disturbance tolerance, minimizing the efficiency of their symbiosis with plants (Johnson *et al.* 1992; Johnson 1993; Verbruggen and Kiers 2010). There is also high variation across AMF species in their capacity to form the hyphal structures that secrete proteins and physically entangle soil particles to generate macroaggregates and stabilize soil (Tisdall and Oades 1982; Jakobsen *et al.* 1992; Augé 2001; Rillig and Mummey 2006; Maherali and Klironomos 2007; Thonar *et al.* 2011). AMF species that have been documented to have higher investment in hyphal development tend to be the most negatively affected by agricultural pressures including tillage and fertilization (Jansa *et al.* 2003; Zhang *et al.* 2016; van der Heyde *et al.* 2017). This means that the species that are potentially the most important to

recovering soil structural and hydrological functioning are the most reduced in agricultural soils (Miller and Jastrow 2015)

In recognition of the need to develop climate change resilient agro-ecosystems and the economic and environmental impacts of soil degradation, there is a growing impetus to manage agriculture more sustainably through decreasing the intensity of disturbance or number of inputs while maintaining consistent yield increases. This can be referred to as sustainable intensification, where practices are adopted which maintain a balance between sufficiently high crop yields and the environment (Godfray *et al.* 2010; Pretty and Bharucha 2014; Bender *et al.* 2016). Sustainable intensification requires increases to SOC, soil health, and better use of the soils' natural capital by increasing the abundance and functioning of the ecosystem engineers that maintain it. This is supported by global initiatives such as the '4 per 1000' initiative to increase carbon sequestration in the world's agricultural soils, and the UN sustainable development objectives for Zero Hunger, Life on Land, and Climate Action (UN 2015). At the national scale, the UK Government has further committed to sustainably managing all of England's soils by 2030 as part of their 25 year Environment Plan (Committee 2018).

Sustainable development goals can be met in several ways, including the adoption of minimum or no-tillage practices, and the inclusion of SOC and fertility restoring crop cover rotations such as grass-clover leys. These practices reduce the frequency or intensity of disturbance to the soils and provide an opportunity for populations of key ecosystem engineers including earthworms and AMF to recover, thus restoring their contribution to the maintenance of soil function (Rillig 2004; Lavelle *et al*. 2006; Mummey *et al.* 2009; Blouin *et al.* 2013; Powell and Rillig 2018). In the case of grass-clover leys, they further introduce temporal variation in soil root architecture, having much denser roots than cereal crops such as wheat which is important to soil aggregation (Bolinder *et al.* 2002; 2010), and potentially providing novel niche space for AMF communities to develop within and re-colonize wheat from (Sweeney *et al.* 2021).

Reducing the intensity of tillage or adopting no-tillage practices has been well documented to alter soil health indicators that are important to water maintenance including increases to the proportion of water stable aggregates, hydraulic conductivity and water holding capacity, and decreases to bulk density (see the meta-

analysis of Li *et al.* 2019). It has further been shown that this can contribute to the increased colonization of crops by AMF (Bowles *et al.* 2017) and hyphal development in to the soil (Kabir *et al.* 1997). This can contribute to decreases in water loss, nutrient leaching, and increases in crop yields under variable water regimes (Bender *et al.* 2015; Cavagnaro *et al.* 2015; Bowles *et al.* 2018).

The role of grass-clover leys in soil health, water stress resilience, and AMF capacity is less well-studied. In a review of soil health indicators across a wide breadth of available literature, Chapman *et al.* (2018) identified that grass-clover leys are underexplored relative to other practices, and that generally many studies concerned with soil health in these systems did not incorporate yield assessments. This is a puzzling omission as the primary function of arable land is to produce crops, the yield of which is known to be closely tied to SOC (Oldfield *et al.*, 2019). Through the few studies assessing grass-clover leys, they have been shown to be capable of increasing SOC by 5-67 % (Christensen *et al.* 2009; Johnston *et al.* 2017; Prade *et al.* 2017; Albizua *et al.* 2015; Börjesson *et al.* 2018; van Eekeren *et al.* 2008), and where follow-up crop yields were reported, they could also be increased by 8-32 % relative to continuously cultivated crops (Johnston *et al.* 1994; Taylor *et al.* 2006; Christensen *et al.* 2009; Albizua *et al.* 2015; Prade *et al.* 2017).The effects of leys can be diminished by the continuation of conventional practices in the following crop cycle such as conventional tillage and inorganic fertilizer additions (Christensen *et al.* 2009; Albizua *et al.* 2015). Studying the intersection between grass-clover ley rotations and other management practices such as tillage important to better understand how we can optimize their inclusion in rotational crop production.

We also understand very little about how AMF populations develop in grass-clover leys. (Albizua *et al.* 2015) demonstrated that the inclusion of grass-clover leys in crop rotations can increase the abundance of soil AMF (e.g., the spores and hyphae within the soil), and only one study has assessed AMF communities in leys, finding them to be significantly different in soils from land currently under cereal production and soils from long-term grasslands (Manoharan *et al.* 2017). There are no studies showing whether AMF community differences are maintained in the subsequent cereal crops.

The main aims of this study are therefore to explore **(a)** the role that land use and management can play in soil health **(b)** the impact that soil health can have on crop yields and water maintenance under ambient, drought and flood stressed conditions. Through these two aims this study addresses knowledge gaps regarding the effectiveness of grass-clover leys to regenerate AMF communities and soil health lost through conventional agriculture, and for improving the resilience of wheat yield and soil functioning when exposed to extreme weather. This is further compared against tillage regimes of different intensity in a reference grassland to assess to what extent any potential grass-clover ley benefits could be maintained in tilled arable rotations. Soil health parameters are presented here in Chapter 3, and an assessment of the AMF community is presented in Chapter 4.

This study forms part of the larger NERC Soil Security Programme *SoilBioHedge* project. In the *SoilBioHedge* project, fields under arable and pasture land use (see Holden *et al.* 2019) were used as reference states for in-field reciprocal planting experiments designed to test the hypotheses **(a)** that grass-clover leys sown into arable fields would enable key ecosystem engineers (e.g., earthworms and AMF) to recolonize the fields from hedgerows and grassy margins, resulting in improved soil health **(b)** the subsequent conversion of grassland (as a reference / benchmark goal for the 'best case' of arable-to-ley soil health improvement) to crop food production would result in a decline in AMF, earthworms, and soil health based on the intensity of tillage used. This study sets out to verify these two hypotheses as they relate to AMF and soil health. The study tests the further hypothesis that **(c)** land uses and management practices favouring the development of diverse AMF communities and soil health maintenance (i.e., arable-to-ley conversion vs arable, pasture vs arable, pasture vs pasture-to-CT and pasture-to-MT tillage conversion, pasture-to-MT vs pasture-to-CT tillage intensity) will confer a greater resistance and resilience advantage to crop yields and water maintenance following drought and flood stress.

Using intact soil mesocosms extracted from the *SoilBioHedge* field experiment, winter wheat (*Triticum aestivum* cv. Skyfall) was grown under ambient conditions and a simulated 4-week drought and flood during stem elongation in May. This was followed by the drainage of flooded soils and an extreme precipitation event (20 mm rainfall in one day) ending the controlled ambient and drought treatments. Water maintenance was assessed by throughflow collected from all mesocosms following

the end of the experimental water stress treatments. Nutrient loss of NO_3^--N and NH_4^+-N was estimated over the same period. Crop performance was assessed through the endpoint yield of wheat at harvest in September. Soil health indicators spanning physical, hydrological, and chemical parameters were also measured at the end of the experiment to use as explanatory variables in the resistance and resilience responses of the mesocosms. Preliminary analysis of in-field AMF communities showed that the grass-clover ley details a significant shift in AMF community composition away from that of the arable land, but only marginally towards those seen in pasture. (Bird and Helgason, unpublished data; *Appendix 3.6*). This preliminary analysis provides an indication of the direction of AMF community development under the various management practices but is not directly relevant to the mesocosm study at hand. Therefore, the contribution of AMF community diversity and composition to the soil health and functioning considered in this chapter is explored within a follow-up study detailed in Chapter 4.

3.2. Methods

3.2.1. Site Description and Experimental Set-Up

Experimental mesocosms used in this study were derived from six fields within the University of Leeds farm. This is a commercial mixed arable and pasture farm near Tadcaster, Yorkshire, UK (53.52° 52' 19.2'' N, 1° 19' 44.4'' W). The soil is a well-drained loamy, calcareous brown earth from the Aberford series of calcaric endoleptic cambisols (Cranfield University 2020). Three of the fields at the site were conventionally managed arable fields, and three were under permanent grassland pasture (for 6 to > 50 years). Land use cover of each field and location relative to one another is shown in *Figure 3.1.* For this study, the field 'Big Sub Station' was divided in to two replicates ('Big Sub Station West' (BW) and 'Big Sub Station East' (BE)). The fields 'Copse' (C) and BE / BW were last under a grass ley in 1988 and 1994 respectively and have been conventionally managed with arable crop rotations since. 'Hillside' (HS) was converted from grass pasture to arable land in 2009. The management practices of each field have been outlined in Holden *et al.*, (2019), which previously characterised soil chemical, physical and hydrological properties, along with the diversity of soil biota associated with the pasture and arable land use in the fields.

As part of the NERC Soil Security Programme *SoilBioHedge* project, A reciprocal planting study was conducted wherein strips of land from fields under recent conventional arable management were converted to grass-clover fallow, and strips of land from fields under recent pasture management were ploughed and converted to arable land. The reciprocal planting provides an opportunity to assess both the capacity of a short-term ley period to restore soil health through the arable-to-ley conversion, and whether the demonstrated benefit of a long-term fallow period in the case of the pasture fields is maintained upon re-ploughing in the subsequent pasture-to-arable reconversion. All strips extended outward from the hedge boundary of the field and had dimensions of 3 m width by 70 m length. The grass-clover ley strips contained a mixture of ryegrass (*Lolium perenne*), white clover (*Trifolium pratense*) and red clover (*Trifolium repens*) sown in May 2015. Each arable field contained two arable-to-ley strips. Each pasture field also contained two pasture-to-arable strips, which were converted through either minimal tillage (MT) or conventional tillage (CT). Conventional and minimum tillage were administered in line with the conventions established for each in Powlson *et al.* (2012). Conventional tillage includes a form of inversion tillage / mouldboard plough to at least 20 cm depth, followed by a secondary cultivation, whereas reduced / minimal tillage includes non-plough-based cultivation practices. In the UK this is typically done through shallow disc cultivation or tines within the 10-15 cm depth. Tillage strips were subjected to two rounds of tillage in October 2015 and January 2016 for CT and MT respectively, and in October 2016 for both strips. CT strips were ploughed to a depth of 20-25 cm followed by power harrowing. MT strips were cultivated through compact disc harrowing within the first 12 cm depth. A control area of arable or pasture was managed the same as the rest of the field between the two experimental strips (*Figure 3.1*).

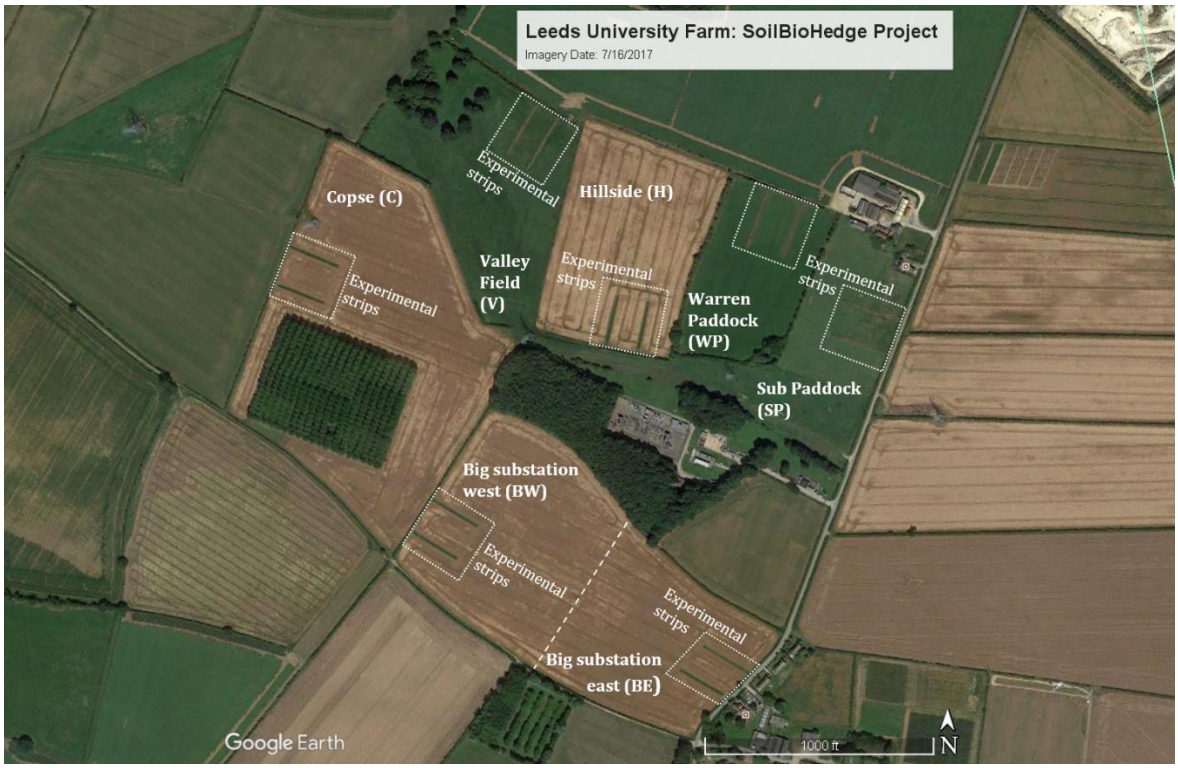

Figure 3.1. Location of experimental and control strips within conventionally managed arable and pasture fields at Leeds University Farm, Yorkshire, UK. Control strips were found between the two experimental strips in each site, though are not visually apparent as they were managed in the same manner as the rest of the field.

3.2.2. Experimental Mesocosm Collection from Field

Intact mesocosms of soil (with dimensions of 37 cm length x 29 cm width x ~ 30 cm height) were collected from the field site in November 2016- near 19 months from the beginning of the arable-to-ley conversion, 13 months from pasture-to-CT conversion and 10 months from pasture-to-MT conversion. Mesocosm boxes each had nine 10 mm drainage holes drilled into the base and were lined with nylon mesh to prevent loss of soil through the holes. Three mesocosms were collected from each of the experimental and control strips at 68 m distance into the field from the hedgerow boundary. One mesocosm from each strip within each field was to be used for one of three water stress treatments, resulting in four arable, eight arable-to-ley, and three pasture, pasture-to-CT, and pasture-to-MT replicates for each water stress treatment, described in the next section. The dataset of this study is therefore imbalanced due to the number of fields and strips used for each land use treatment. Following collection from the field, mesocosms were stored outdoors at the Arthur Willis Environment Centre, Sheffield, UK (53° 22' 52.50'' N, 1° 29' 55.70'' W) for the remainder of the experiment.

Arable fields were sown with winter barley (*Hordeum vulgare*) in Autumn 2016, and pasture-to-CT / pasture-to-MT strips were sown with winter wheat over the same period. At the stage that mesocosms were removed from the fields the barley and wheat seedlings were at an early growth stage and could be removed easily whilst causing minimal soil disturbance. This was not possible for ley and pasture soils where the removal of well-established and dense-rooted grass and clover plants would have resulted in a greater amount of soil disturbance. Instead of manual removal, vegetation was removed from pasture and ley mesocosms via herbicide treatment in December 2016 (Diquat as dibromide in 55 ml water per mesocosm, SyngentaRetro). Arable, pasture-to-CT and pasture-to-MT mesocosms were not treated with diquat, as it is absorbed by plant material and therefore would have remained in the soil until taken up by the wheat subsequently grown.

Thirty winter wheat plants (cv. Skyfall) were sown in each mesocosm in January 2017 and grown until October 2017. All mesocosms received two application of N fertilizer over this time. The first application was in April 2017, and the second in May 2017. Fertilizer was added in the form of YaraBella Prilled N at a rate of 50 kg N ha^{-1}.All mesocosms received unimpeded ambient precipitation for the local area until the 4th

of May 2017 when water stress treatments began. All stress treatments lasted for 28 days. Over this period a transparent rain shelter was installed, preventing all mesocosms from receiving local precipitation. Three water stress treatments were implemented over this period – ambient, drought and flood. Ambient mesocosms were watered three times per week for a total input of 58.7 mm equivalent rainfall over the period, in line with the recorded average rainfall for May (*Table 3.1*). Drought mesocosms received zero precipitation input during this period. Flood mesocosms had their drainage holes sealed with bungs and water added until soil was submerged with approximately 3 cm of standing water. Water was regularly topped up to maintain this level until the end of the water stress treatment. Water stress treatments were ended on the 30st May 2017, when the rain shelter was removed. The ambient and drought mesocosms were subjected to an extreme wetting / rewetting event on 31st May through the addition of 2 L water to each (20.2 mm rainfall equivalent). The flood treated mesocosms did not receive any additional water at this time and were instead un-bunged and allowed to drain. All mesocosms received ambient rainfall for the remaining duration of the study in October 2017.

Table 3.1. Monthly precipitation and long-term averages for Sheffield taken from Sheffield Weather, www.sheffieldweather.co.uk. **Bold** values represent monthly precipitation below the 30-year average, and *italic* values represent monthly precipitation above the 30-year average.

	Jan	Feb	Mar	Apr	May	May 31st	Jun	Jul	Aug	Sep
Average precipitation (mm)	80.3	65.1	65.0	58.7	60.0	-	68.8	60.3	67.7	65.1
2017 precipitation (mm)	**39.3**	65.6	66	**15.5**	**47.7**	-	*102.6*	*72.3*	*79.2*	68.5
Experimental precipitation (mm)										
Ambient	-	-	-	-	58.7	20.2	-	-	-	-
Drought	-	-	-	-	0	20.2	-	-	-	-

3.2.3. Water Throughflow and Nutrient Leaching

Mesocosms were attached to a collection box below with no air gaps to prevent evaporation loss. Water throughflow was collected over the periods of 0-7 days, 8-15 days and 16-29 days following the controlled precipitation period. Eijkelkamp MacroRhizon soil moisture samplers (0.25 cm diameter, 9 cm length) were inserted at the beginning of the experiment and used to extract soil solutions at similar times to water throughflow collection. The samplers were made from a PTFE membrane which pre-filters soil water to <0.1um upon extraction in to a 10 ml leur-lock syringe. The syringe plunger was drawn out to the 10 ml mark to apply a 100 kPa suction and held open with a small board on the day prior to collection. Collected solutions were analysed for solubilised nitrate NO_3^--N and ammonia NH_4^+-Nconcentrations by plate reader (CLARIOstar plate reader, BMG Labtech), following the colorimetric assay described by Hood-Nowotny *et al.* (2010). Solubilised nutrient concentrations were able to be recorded with suitable replication only for arable, pasture and ley treatments. Soil water collection from pasture-to-CT and pasture-to-MT mesocosms did not yield enough solution on several occasions in the experiment, resulting in a lack of replication and preventing the ability to draw conclusions from the data. An estimate of water throughflow and nutrients leached from soils per day (in L day^{-1} and mg day^{-1} respectively) over the three collection periods was estimated from the measured water throughflow (L) and soluble concentration of nutrients (mg L^{-1}). Water throughflow and estimated nutrient leaching following the water stress treatments was considered only for ambient and drought-stressed mesocosms. The water conditions under flood stress were pushed far past their natural moisture capacity through oversaturation and sealed to maximally achieve and maintain flood stress for the wheat. Thus, any subsequent measures of the drainage capacity of the mesocosms is inappropriate as any excess water added past the saturation point of soils would also be collected, potentially masking any land use and soil health relations.

3.2.4. Soil Health Indicator Assessment

All soil health indicators except for saturated gravimetric moisture content were assessed after wheat crop had been harvested in October 2017. Soil moisture content was estimated throughout the experiment from mesocosm weights recorded fortnightly over the growing season from the 1st of February onward. Moisture content was back-calculated from 400g soil cores oven dried at 105 °C, which were collected at the same time as the final mesocosm weighing. Over the first four measurement dates precipitation was consistent with the long-term average, and moisture content measures were consistent. These four measures were therefore taken as a proxy of the water holding content of the soil, reflecting the saturated moisture content when inputs and outputs to the system are balanced.

In October 2017 A single soil core (cylinders of 20 cm depth and 3,5 cm diameter) was collected from each mesocosm to assess bulk density, extractable NO_3^--N content, extractable NH_4^+-N content, and loss on ignition (LOI) SOC. 5 cm^3 of fresh soil was sub-sampled at 5 cm and 15 cm depth and dried at 105 °C for 24 hours to estimate bulk density based on dry mass per volume (Blake 1965). These sub-samples were also used to estimate the moisture content at each depth to correct measured concentrations. The fresh soil from the aggregate topsoil (0-10 cm) and subsoil (10-20 cm) were then sieved to 2 mm diameter, and had rocks and roots removed for chemical analysis. A subset of the sieved soils was oven dried at 105 °C, and then further dried in a muffle furnace at 550 °C for four hours to estimate the LOI organic matter (Heiri *et al.* 2001). LOI organic matter was converted to LOI SOC using the principle that carbon makes up 58 % of organic matter stoichiometrically (Broadbent 1953). 1M KCl extraction with Whatman 44 filtering was performed on fresh soils to obtain a filtrate that could be analysed photometrically for oxidized inorganic nitrogen species-N (nitrate NO_3^--N, nitrite NO_2^--N, the latter of which is expected to be negligible), and ammonium-N (NH_4^+). Photometric analysis was performed using the microplate method described by Hood-Nowotny *et al.* 2010. NO_3^--N and NH_4^+-N concentrations (mg L^{-1}) were converted to content per gram soil (g kg $soil^{-1}$).

A tension infiltrometer was used to measure field saturated hydraulic conductivity K_{sf} at two depths: surface (0 cm depth) and 10 cm depth. The infiltrometer design was based on that of Ankeny *et al.* (1988) and Holden *et al.* (2001), modified as described in Holden *et al.* (2019). A thin layer of fine, moist sand was applied to the measured

areas to aid disk contact. Infiltration measurements for each pressure head continued until well after steady state. K_{sf} values were obtained from steady-state data as outlined by Reynolds and Elrick (1991). Measurements were conducted at tensions of -3 cm and -6 cm. From capillary theory these tensions exclude water flow through pore spaces of <1 mm and <0.5 mm, respectively. Measures were also conducted at -0.5 cm tension, enabling flow through nearly all pore spaces. K_{sf} was estimated from measures taken at -0.5 and -3 cm tension. K_{sf} was further partitioned in to functional macroporosity, mesoporosity and microporosity, reflecting the flow rate of water through pore classes in the range of < 1 mm, 0.5 – 1 mm, and <0.5 mm size respectively, such as has been done in past publications (Wallage and Holden 2011).

3.2.5. Soil Health Index (SHI) Determination

Soil health indicators found to be significantly different between land uses and management types were Z-score transformed and incorporated into principal component analysis (PCA) to determine the minimum dataset (MDS) required for soil health indexing and to calculate SHI. PCA-based determination of the SHI has been shown to perform well when compared to other methods, particularly when evaluating crop yields (Masto *et al.* 2008).

Indicators included in the PCA were NO_3^--N content (averaged across both measured depths), LOI SOC (0-10 and 10-20 cm depth separately), K_{sf} (0 cm depth), functional microporosity (0 cm depth), and bulk density (averaged across both measured depths), and saturated gravimetric moisture content (whole mesocosm). Generally, principal components (PCs) derived from this analysis with eigenvalues ≥ 1 and which explain at least 5 % of the variation are examined, from which indicators are chosen to be included in the MDS based on their factor loadings (contribution) to the examined PCs (Wander and Bollero 1999; Masto *et al.* 2008). In this study an indicators factor loading was considered 'high' if it had a percentage contribution towards a given PC greater than would be expected if the contributions by all indicators to the PC were equal (> 14.29 % contribution). When more than one indicator was retained under a single PC through this method, correlations between significant indicators were calculated to determine which indicators were redundant and could be removed (as per Andrews *et al.* 2002). Correlations were carried out using the *rcorr* function from the *Hmisc* package (Harrel 2020) and visualised using *ggcorrplot* (Kassambara 2019). This was combined with knowledge of the relation

between indicators and soil function to reduce the MDS to LOI SOC (0-10 cm depth), K_{sf}, saturated gravimetric moisture content, and bulk density. Where two indicators were still maintained attached to one PC, they were transformed according to their relative contribution to the PC to ensure that they did not outweigh the indicators attached to other PCs. Together the chosen MDS reflects SOC, soil fertility, water maintenance, and structure.

The chosen indicators were subsequently converted to unitless scores ranging from 0 (low) to 1 (high) based on the critical values expected within the system. This scoring can be calibrated based on known minima and maxima of the same or similar systems from other studies, though in this study it was calibrated using the samples found within the experimental site itself. This was chosen as the study system at the site contains what we would expect to be the '*worst-case scenario*' values of the chosen indicators in the conventionally managed arable sites, and the '*best case scenario*' values of the chosen indicators within the long-term grassland pasture sites. Scoring was carried out using a non-linear scoring function (NLSF) based on the sigmoidal function, which has been shown to more accurately reflect system functions than linear-scoring (Masto *et al.* 2008). Non-linear scoring was carried out with the *sigmoid* package, using the '*logistic*' method with '*SoftMax = TRUE*' to ensure that scores were between 0 and 1 for the system minima and maxima. Two types of scoring functions were generated using a '*more is better'* (i.e., saturated hydraulic conductivity, LOI SOC, gravimetric water content), and a *'less is better*' (i.e., soil bulk density) approach, as has been implemented in previous studies (Karlen and Stott 1994; Karlen *et al.* 1994; Hussain *et al.* 1999; Glover *et al.* 2000; Masto *et al.* 2008). Sigmoidal curves generated through these scoring functions for the parameters within the MDS are shown in *Figure 3.2*.

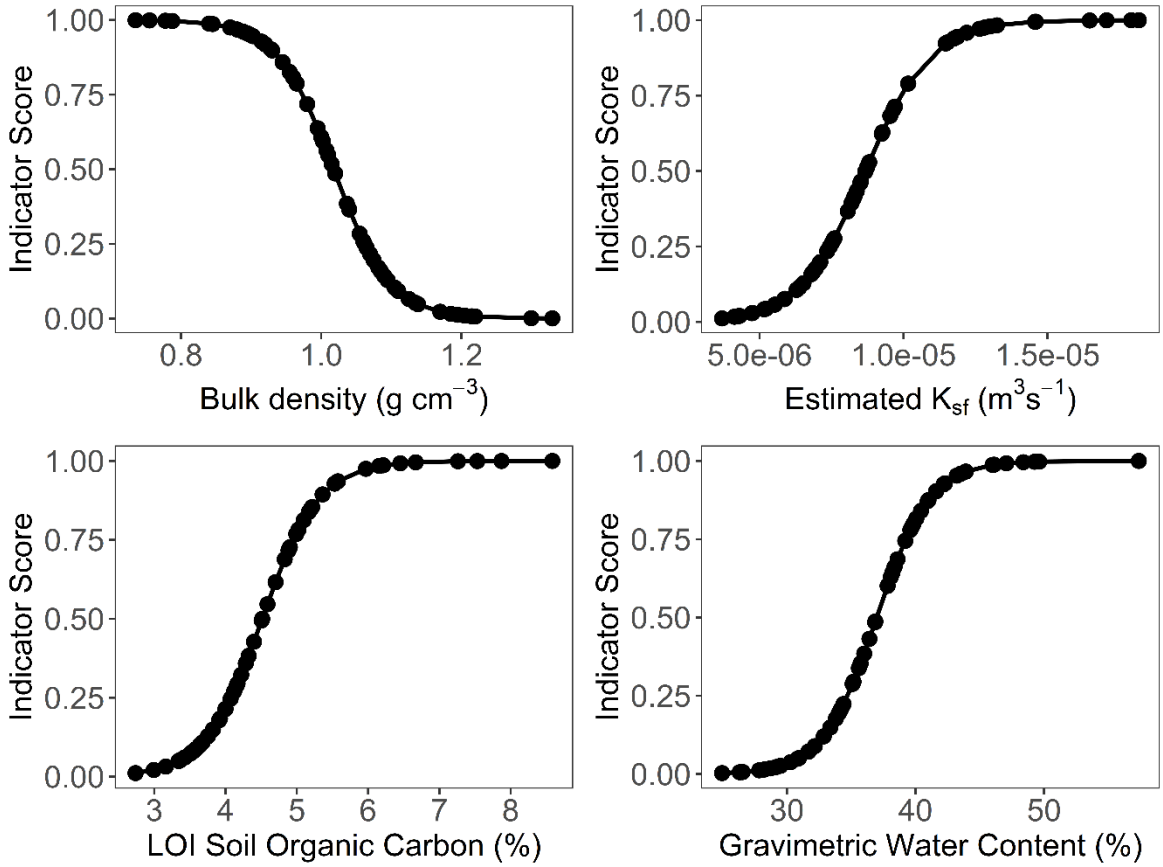

Figure 3.2. Non-linear scoring functions for the soil health indicators maintained within the MDS for SHI calculation.

The results of the PCA were combined with the unitless scores to derive the SHI. Each PC explained a certain amount of variation found within the dataset (%). The unitless indicator scores were weighted based on the total variance explained by the PC for which the indicator they are derived from had the highest factor loading for (%, weighing factor). The final PCA based SHI was calculated as follows, which was then normalized to get a maximum SHI of 1:

$$\textbf{\textit{Soil Health Index (SHI)}} = \sum_{i=1}^{n} W_i . S_i$$

Where n = total number of indicators considered, i = a given indicator, W_i = The weighing factor of the PC attached to indicator i, S_i = the unitless score attached to indicator i.

3.2.6. Statistical Analysis

All statistical analyses were carried out using R v 4.02 (R Core Team). Where R packages used were not developed by the R Core Team they are cited separately.

3.2.6.1 Soil Health Indicators and SHI

Soil health indicators measured at the end of the experiment were statistically analysed individually through ANOVA. Indicators were tested for normality using Shapiro-Wilks tests, and were found to be non-normal were transformed appropriately (Percentage values were asin square root transformed, and other measures were log transformed) prior to ANOVA. Indicators were first compared by depth (i.e., model for comparison: indicator ~ depth). If significant differences were found between depths then they were subsequently analysed separately, and if not were analysed together. ANOVA was then used to assess differences between land uses and managements (i.e., model for comparison: indicator ~ land use). Calculated SHI scores per sample were also compared between land uses using ANOVA based on the same model. Where ANOVA was found to be significant Tukey's post-hoc test was conducted, with BH corrections of p-values.

3.2.6.2 Water Loss and Nutrient Leaching

Solubilized soil chemical concentrations (NO_3^--N, NH_4^+-N) and leachate losses per day were calculated for arable, arable-to-ley and pasture soils over three date periods – 0-7 days post treatment, 7-15 days post treatment, and 15-28 days post treatment. Water loss per day was calculated over the same time periods for all experimental strips. Like with indicators, variables were first tested for normality through Shapiro-Wilk testing and log transformed if found to be non-normal. Models constructed for ANOVA incorporated the date period of variable assessment and water stress treatment into the model: variable ~ date * water stress * land use. Where ANOVA was found to be significant Tukey's post-hoc test was conducted. BH corrections of p-values were calculated where the number of pairwise comparisons were greater than three, and Fisher's least significant difference where pairwise comparisons were conducted only between the combination of arable, arable-to-ley and pasture mesocosms.

3.2.6.3 Structural Equation Modelling

Structural equation modelling was used to unpick the interactions between agricultural land use, soil health, and water / wheat yield maintenance under the differing water stress scenarios. Models were constructed for the flood water stress treatment separately from ambient and drought stress treatments. This was done because of the differences between the scenarios through which water losses were measured over the post-water stress period. In ambient and drought scenarios water loss was measured following an extreme precipitation event marking the end of both controlled precipitation periods, whereas in the flood scenario water was drained following the four-week oversaturation of soils and did not have an extreme precipitation event carried out.

Parameters used in the model were the calculated SHI aggregating the most explanatory soil health indicators, water loss between the end of water-stress treatments and 7 days later, and wheat heights at harvest. The ambient + drought model was fit and evaluated using a linear mixed-effects model with restricted maximum likelihood, from the *nlme* package and *piecewiseSEM* packages (Lefcheck 2016). Water stress treatment was used as the random variable (random = ~1|Treatment). The flood model- containing only one water stress treatment- was fit and evaluated using a generalized linear model and *piecewiseSEM*. For both structural equation models, land use could directly affect SHI and water loss. SHI could directly affect water loss and wheat height in the ambient + drought model, and wheat height only in the flood model where water loss was not included. Finally, wheat height could affect water loss in the drought + ambient model as a representative proxy variable of plant demand for water and potential water interception by wheat over the period. For models containing a categorical variable (land use), marginal means of each factor level were estimated and post-hoc tests were conducted where appropriate using the package *emmeans* (Searle *et al.*, 1980; Lenth 2020).

3.3. Results

3.3.1. Soil Health Indicators

3.3.1.1 Soil Chemistry

There was no statistical support for NO_3^--N content being different between depths measured (ANOVA: df = 1, F = 2.37, p = 0.13), nor for NH_4^+-N content (ANOVA: df = 1, F = 2.24, p = 0.14). Values were subsequently compared without consideration of depth. Land use was a significant determiner of NO_3^--N content (ANOVA: df = 4, F = 17.395, p = < 0.001), though this appears to be driven by historic land use prior to the experimental strips. Both arable and arable-to-ley soils had NO_3^--N contents lower than pasture, pasture-to-CT soils, and pasture-to-MT soils (*Table 3.2*). There was no effect of land use on NH_4^+ content (ANOVA: df = 4, F = 1.64, p = 0.17, *Table 3.2*).

LOI SOC was significantly different between soil depths (ANOVA: df = 1, F = 7.6132, p < 0.01). LOI SOC was driven by land use at both depths (0-10 cm ANOVA: df = 4, F = 29.736, p <0.001; 10-20 cm ANOVA: df = 4, F = 8.7717, p < 0.001). At the 0-10 cm depth arable and pasture soils had the greatest differences in LOI SOC, which was higher in pasture soils. The pasture-to-CT and pasture-to-MT conversions both resulted in a reduction in LOI SOC, with the pasture-to-CT conversion having the most negative effect. The arable-to-ley conversion did not result in any change to LOI SOC percentages. At the 10-20 cm depth, the significant difference between arable and pasture soils was maintained, though lower LOI SOC in pasture soils at this depth made the difference less pronounced. Due to this, the negative effect of pasture-to-CT and pasture-to-MT conversions on LOI SOC was also less pronounced. LOI SOC in the two pasture-tillage conversions was like both arable and pasture soils at this depth (*Table 3.2*).

3.3.1.2 Soil Physical Properties

Bulk density was similar between the two depths studied (ANOVA: df=1, F=1.8728, p=0.17), which were combined for further analysis. Bulk density was significantly different across land uses (ANOVA: df=4, F=5.1887, p < 0.001, *Table 3.2*). Pasture soils had a lower bulk density than arable soils. The pasture-to-MT and arable-to-ley conversions altered the bulk density of the soils to an intermediate state between the two extremes of land use, though the pasture-to-CT conversion did not affect the bulk density of the soils.

3.3.1.3 Soil Hydrology

K_{sf} values measured at the soil surface were significantly faster than those measured at 10 cm depth (ANOVA: df=1, F=211.68, p<0.001), so the two depths were analysed separately. The mean K_{sf} was 8.68 $m^3 s^{-1}$ ± 4.44 SEM at the soil surface, and 2.02 $m^3 s^{-1}$ ± 2.03 SEM at the 10 cm depth. Surface K_{sf} was significantly different between land uses (ANOVA: df = 4, F = 8.06, p < 0.001). Post-hoc tests showed that surface K_{sf} was different between pasture, ley, and arable soils. Arable soils had the lowest value, followed by ley, and then pasture. Pasture-to-CT and pasture-to-MT conversion slightly decreased K_{sf} in comparison to pasture, which was intermediate between the values of pasture and arable-to-ley though this decrease was not significant (*Table 3.2*). At the 10 cm depth, there was no statistically significant differences between land uses (ANOVA: df = 4, F = 0.734, p = 0.57, *Table 3.2*).

Functional macroporosity, mesoporosity and microporosity were all significantly different between depths (ANOVA: functional microporosity, df = 1, F = 59.183, p <0.001; functional mesoporosity, df = 1, F = 40.194, p <0.001; functional macroporosity, df = 1, F = 80.267, p <0.001), which were subsequently analysed separately. Functional macroporosity was significantly higher at the soil surface than at 10 cm depth (surface = 90.90 % ± 0.91 SEM, 10 cm = 67.31 % ± 2.66 SEM). Functional mesoporosity was significantly lower at the soil surface than at 10 cm depth (surface = 4.10 % ± 0.69 SEM, 10 cm = 13.19 % ± 1.57 SEM), as was functional microporosity (surface = 5.01 % ± 0.43 SEM, 10 cm = 19.49 % ± 2.06 SEM). At the soil surface, there was no significant difference observed for functional macroporosity (ANOVA: df = 4, F = 1.2985, p = 0.28) or functional mesoporosity (ANOVA: df = 4, F = 0.7127, p = 0.81) between land uses. Functional microporosity was found to be significantly related to land use (ANOVA: df = 4, F = 9.3841, p <0.001). Pasture-to-CT

and pasture-to-MT conversion resulted in the same non-significant decrease in functional microporosity as it did with $K_{sf,}$ reflecting the close association between the two measures (*Table 3.2*). The composition of functional porosity was more uniform at the 10 cm depth, with no significant differences being seen in any of the three pore classes between land use types (ANOVA: functional macroporosity df = 4, F = 0.6264, p = 0.65, functional mesoporosity df = 4, F = 2.0080, p = 0.11, functional microporosity df = 4, F = 0.2165, p = 0.33, *Table 3.2*).

Gravimetric water content was assessed over four dates where ambient water inputs were high (*Table 3.1*) and water content stayed relatively consistent between time points, from which it could be suggested that soils were at saturation capacity. This saturated gravimetric water content was significantly different between land uses (ANOVA: df =4, F = 50.825, p <0.001). Pairwise testing revealed that arable and arable-to-ley soils had minor, yet significantly different gravimetric moisture content from each other, and from pasture soils. There was no change in water content from pasture soils to the pasture-to-CT and pasture-to-MT conversions (*Table 3.2*).

Table 3.2. Summary of soil health indicators separated by control and experimental strip and depth. Indicator values averaged across both depths (Total) are presented where indicators were assessed irrespective of depth. Superscript letters represent groupings of similarity following pairwise comparisons after ANOVA. Results where significant differences between strips were found are in **bold**. Replication of each land use at each depth: Arable (n = 12); Ley (n = 24); Pasture (n = 9); CT (n = 9); MT (n = 9). Units are g cm^{-3} (bulk density), % (Functional micro/meso/macroporosity, LOI, and gravimetric water content), mg kg^{-1} soil (NO_3^--N and NH_4^+-N content), and mm s^{-1} (field saturated hydraulic conductivity, K_{sf}). The raw data for hydrological indicators presented was provided by Dr. Despina Berdeni through the *SoilBioHedge* project.

	Arable	Arable-to-Ley	Pasture	Pasture-to-CT	Pasture-to-MT
Physical indicator					
Bulk density (0-10 cm)	1.10 ± 0.04	1.01 ± 0.03	0.88 ± 0.05	0.95 ± 0.05	1.02 ± 0.07
Bulk density (10-20 cm)	1.10 ± 0.05	1.06 ± 0.04	0.99 ± 0.04	0.91 ± 0.04	1.04 ± 0.06
Bulk density (Total)	**1.10 ± 0.03^{b}**	**1.04 ± 0.02^{ab}**	**0.93 ± 0.03^{a}**	**0.93 ± 0.03^{a}**	**1.03 ± 0.05^{ab}**
Chemical indicators					
NO_3^- content (0-10 cm)	31.50 ± 4.55	30.97 ± 2.14	70.05 ± 8.31	54.6 ± 6.31	54.83 ± 3.7
NO_3^- content (10-20 cm)	34.47 ± 5.08	27.90 ± 2.62	61.78 ± 11.22	58.15 ± 5.27	35.66 ± 5.57
NO_3^- content (Total)	**32.98 ± 3.35^{bc}**	**29.44 ± 1.69^{b}**	**66.16 ± 6.73^{a}**	**56.27 ± 4.06^{a}**	**45.81 ± 3.97^{ac}**
NH_4^+ content (0-10 cm)	12.89 ± 1.84	15.27 ± 2.54	24.78 ± 6.52	21.84 ± 2.55	25.82 ± 6.34
NH_4^+ content (10-20 cm)	12.66 ± 3.08	13.14 ± 2.14	26.08 ± 6.65	20.76 ± 4.51	12.86 ± 3.04
NH_4^+ content (Total)	12.77 ± 1.79	14.23 ± 1.66	25.39 ± 4.51	21.3 ± 2.51	19.72 ± 3.89
LOI (0-10 cm)	**3.74 ± 0.11^{a}**	**3.82 ± 0.14^{a}**	**6.67 ± 0.41^{c}**	**4.54 ± 0.21^{ab}**	**5.22 ± 0.22^{b}**
LOI (10-20 cm)	**3.66 ± 0.12^{bc}**	**3.54 ± 0.10^{c}**	**4.99 ± 0.35^{a}**	**4.46 ± 0.12^{ab}**	**4.15 ± 0.24^{a}**

Hydrological Indicators					
K_{sf} (0-10 cm)	**$5.63x10^{-6} \pm 3.46x10^{-7}$ c**	**$8.09x10^{-6} \pm 6.17x10^{-7}$ a**	**$1.22x10^{-5} \pm 1.17x10^{-6}$ b**	**$9.41x10^{-6} \pm 8.82x10^{-7}$ ab**	**$1.01x10^{-5} \pm 1.38x10^{-6}$ ab**
K_{sf} (10-20 cm)	$1.63x10^{-6} \pm 3.82x10^{-7}$	$2.13x10^{-6} \pm 2.6x10^{-7}$	$2.42x10^{-6} \pm 7.68x10^{-7}$	$2.22x10^{-6} \pm 7.42x10^{-7}$	$1.64x10^{-6} \pm 4.46x10^{-7}$
Functional microporosity (0-10 cm)	**8.79 ± 1.47 b**	**4.92 ± 0.50 a**	**2.54 ± 0.38 a**	**4.25 ± 0.64 a**	**3.40 ± 0.44 a**
Functional microporosity (10-20 cm)	19.25 ± 4.73	21.60 ± 3.78	19.70 ± 5.69	16.43 ± 4.47	17.06 ± 4.77
Functional mesoporosity (0-10 cm)	5.33 ± 2.45	2.44 ± 0.0.68	5.97 ± 2.10	4.47 ± 2.06	2.44 ± 0.68
Functional mesoporosity (10-20 cm)	15.52 ± 3.31	11.31 ± 2.26	9.57 ± 3.12	9.17 ± 2.33	22.73 ± 6.54
Functional macroporosity (0-10 cm)	85.87 ± 3.45	91.82 ± 0.85	91.49 ± 2.41	91.28 ± 2.25	94.16 ± 1.00
Functional macroporosity (10-20 cm)	65.23 ± 5.02	67.08 ± 4.29	70.73 ± 7.79	74.40 ± 6.29	60.21 ± 9.04
Total gravimetric water content	**31.40 ± 0.59a**	**33.9 ± 0.52b**	**43.50 ± 1.40c**	**42.6 ± 0.60c**	**40.6 ± 0.65c**

3.3.1.4 Soil Health Index (SHI)

Principal component analysis (PCA) was performed to determine the MDS for SHI indexing using soil health indicators that were found to be significantly different between land uses. PC1, PC2 and PC3 together represented a cumulative variance of 80.70 % (*Table 3.3*) and reflect all the variables used in PCA as seen through the determination of relative contributions of each variable per PC. The three PCs were used for SHI indexing as each had an eigenvalue above 1. PC1 (reflecting 50.20 % of variation) was associated primarily with NO_3^--N content (total), LOI SOC (at both depths), and whole mesocosm saturated moisture content. PC2 (reflecting 15.95 % of variation) was associated primarily with K_{sf} (at the 0 cm depth), and functional microporosity (at the 0 cm depth), and LOI SOC (at the 10 cm depth). PC3 (reflecting 14.55 % of variation) was associated with bulk density (total) and functional microporosity (at the 0cm depth). Almost all highly weighted variables under PC1, PC2 and PC3 were correlated with one another (*Figure 3.3*). The MDS was therefore reduced based on knowledge of the system and underlying functions that the variables control. Only LOI SOC from the 0-10 cm depth (where differences between land use were greatest), and gravimetric moisture content were maintained from PC1 for SHI calculation, reflecting both soil fertility and WHC. SOC is indicative of soil fertility as measured through N and P (Tiessen *et al.* 1994), and so NO_3^--N content was not included due to this redundancy. For PC2, only K_{sf} was maintained- reflecting hydrology and soil structure. For PC3 only bulk density was maintained- reflecting soil structure.

The calculated soil health index was significantly different between land uses (ANOVA: df = 4, F = 42.32, p = <0.001). The arable and pasture mesocosms had the most different soil health indices from one another (*Figure 3.4*). Post-hoc testing also showed that the arable-to-ley conversion significantly increased the soil health index relative to the arable soils to an intermediate state between arable and pasture. Both tillage conversions resulted in decreases in soil health, though this was not found to be significant.

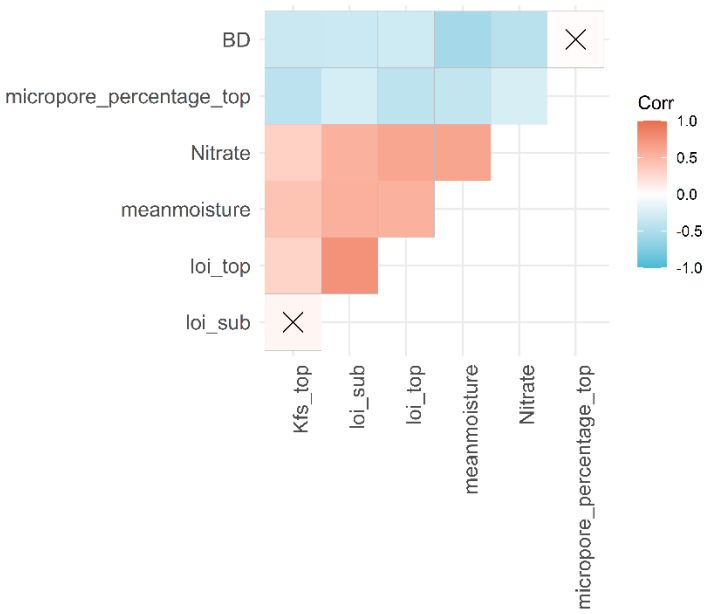

Figure 3.3. Correlation matrix of untransformed soil health indicators selected for PCA analysis. Statistically insignificant correlations ($p > 0.05$) are indicated with an X.

Table 3.3. Results of PCA on soil health indicators under contrasting land uses. **Bold** eigenvalues correspond to the PC's examined for the SHI. **Bold** eigenvectors are considered highly weighed, and **bold-underlined** eigenvectors correspond to the indicators included in the index after removing indicators for redundancy.

	PC1	PC2	PC3	PC4	PC5
Eigenvalue	**3.51**	**1.11**	**1.02**	0.47	0.40
Variation (%)	50.20	15.95	14.55	6.75	5.79
Cumulative variation (%)	50.20	66.15	80.70	87.45	93.24
Eigenvectors					
NO_3^--N content (total)	**0.42**	-0.16	-0.07	0.60	0.55
LOI (0-10 cm)	**0.44**	-0.16	0.33	0.21	-0.41
LOI (10 – 20 cm)	**0.40**	**-0.43**	0.31	-0.17	-0.30
Bulk Density (total)	-0.32	0.12	**0.68**	0.38	0.23
K_{sf} (0 cm)	0.29	**0.66**	-0.26	0.38	-0.38
Functional microporosity (0 cm)	-0.28	**-0.56**	**-0.48**	0.41	-0.21
Gravimetric water content	**0.45**	0.01	-0.19	-0.34	0.45

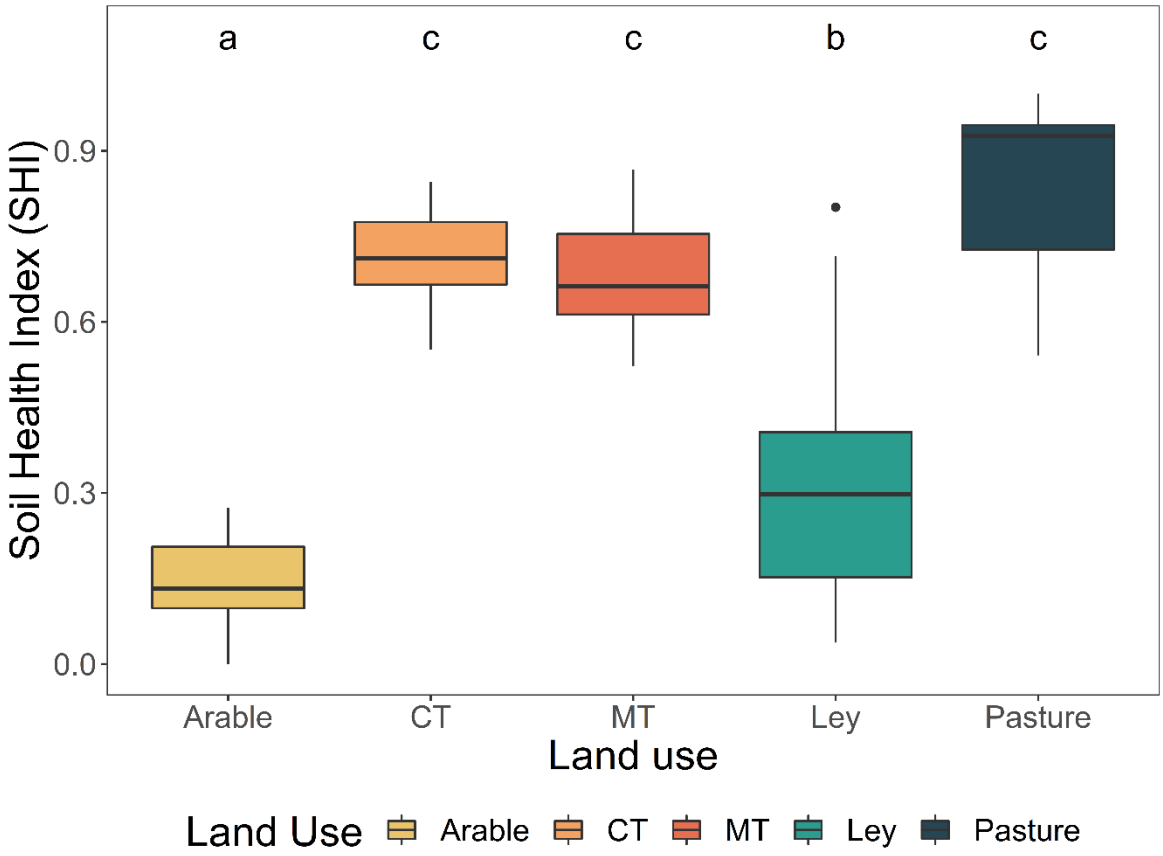

Figure 3.4. Soil health indices calculated for each land use. Box plots show median, interquartile range, maximum and minimum. Outliers are represented outside of this range. Replication for each land use: Arable (n = 12); CT (n = 9); Ley (n = 24); MT (n = 9); Pasture (n = 9).

3.3.2. Wheat Yield and Height

3.3.2.1 Wheat Resilience to Water Stress

The final harvested wheat biomass was only available for three of the five land use treatments in this study. Wheat height at harvest was well correlated to wheat biomass among these three land uses, and so was used as a proxy for ABG biomass accumulation (*Figure 3.*5). Wheat height was impacted by water stress (ANOVA: df = 2, F = 19.9871, p < 0.001). Mean wheat height was slightly higher in flooded than ambient mesocosms, but this was not significant. Wheat height recorded in droughted mesocosms was 23.12 % lower than that recorded in ambient mesocosms. This was a significant decrease showing the negative effect that water limitation had on wheat growth. Wheat height per land use and water stress is shown in *Figure 3.*6. Wheat height was also significantly different between land uses (ANOVA: df = 4, F = 3.54, p = 0.01), though no interactive effect was observed between and use and water stress treatments (ANOVA: df = 8, F = 1.43, p = 0.21). Wheat collected from the arable mesocosms had generally depressed growth under all water stress treatments compared to the other land uses, displaying the shortest height (mean value = 470.95 cm ± 16.12 SEM). Wheat collected from pasture mesocosms had the highest wheat height (mean value = 523.18 cm ± 15.78 SEM). Pasture-to-CT conversion resulted in a reduction in wheat height, closer towards those found in long-term arable soils. This value was significantly different from both arable and pasture means though (mean value = 518. 62 ± 16.12). Pasture-to-MT conversion was associated with a greater mean decrease in wheat yields relative to pasture than the pasture-to-CT conversion, though overall this was a more variable response resulting in a lack of statistically significant difference from pasture (mean value = 506.03 ± 20.26). The arable-to-ley conversion was also associated with a moderate increase in subsequent wheat yields (mean value = 485.00 ± 15.78), with values lying between arable and pasture yields.

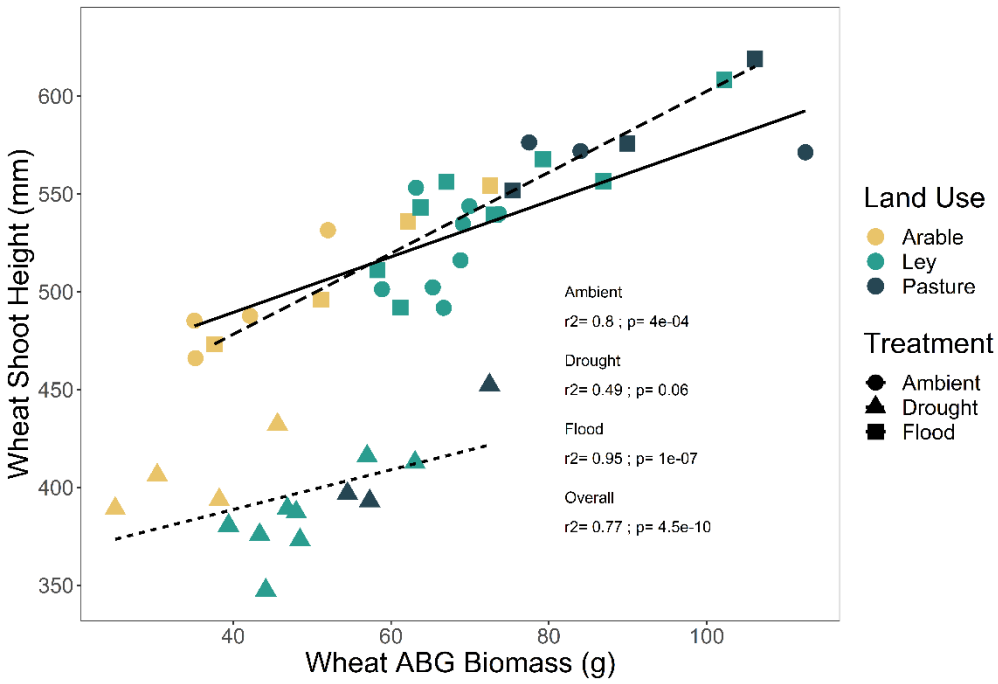

Figure 3.5. Correlation of wheat shoot height with wheat biomass measured at the end of the experiment. Raw data was provided by Prof. Jonathan Leake from measures taken by Dr. Despina Berdeni through the *SoilBioHedge* project.

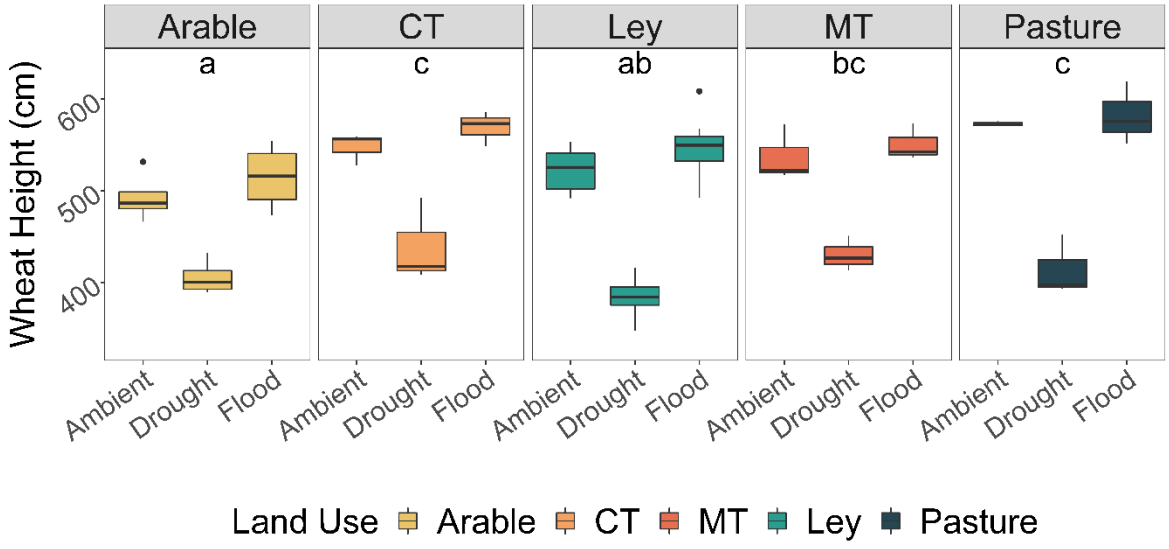

Figure 3.6. Mean wheat stem height at harvest. Box plots show the median, interquartile range, maximum and minimum. Outliers are represented individually outside of the whiskers. Letters denote groups of statistical similarity ($p < 0.05$) after Tukey HSD corrections. Replication for each land use: Arable (n = 12); CT (n = 9); Ley (n = 24); MT (n = 9); Pasture (n = 9). The raw data was provided by Prof. Jonathan Leake from measures taken by Dr. Despina Berdeni through the *SoilBioHedge* project.

3.3.3. Water Maintenance and Nutrient Leaching

3.3.3.1 Water Throughflow Following Ambient and Drought Water Stress

Water throughflow from mesocosms was assessed over three periods following the end of the controlled precipitation period: May 31st – June 7th, June 7th – June 15th, and June 15th – June, and June 15th – June 29th. At the end of the controlled precipitation period, the ambient and drought mesocosms received a shared extreme rainfall event of 20 mm in a single day. Water throughflow was analysed separately for the May 31st – June 7th collection period to capture the resistance response of mesocosms to the extreme precipitation event, and with all three collection periods together to examine water maintenance following the ambient and drought treatments more generally.

Over all three dates water throughflow from mesocosms was found to be significantly driven by the water collection period (date) and land use independently, but not by interaction between the two variables (*Table 3.4*). Water throughflow volume was similar across the first two collection periods, with drought mesocosms seeing slightly higher volumes of water throughflow but not significantly so. Over the third collection period (June 15th – June 29th) the volume of water lost from both ambient and droughted mesocosms was more even, and greater than over the previous two collection periods (*Figure 3.7 A*). This is likely due to a heavy rainfall event over this week collection period, as June was shown to be much more wet than the 30-year average (*Table 3.1).* Across all dates and both water stress treatments, the arable mesocosms lost considerably more inputted water than all other land uses (*Figure 3.7 B)*. Pasture-to-MT tillage had a significantly negative effect on water maintenance over this period, though the increase in water loss was relatively minor.

When only considering the May 31st – June 7th sampling period, It was still found that water throughflow was moderated by land use regardless of whether mesocosms were previously subjected to ambient conditions or drought stress. Arable mesocosms lost substantially higher volumes of water over this one-week period (0.97 L ± 0.16 SEM) in comparison to all other land uses (Arable-to-ley, 0.42 ± 0.11; Pasture, 0.31 ± 0.12; Pasture-to-CT, 0.26 ± 0.11; Pasture-to-MT, 0.34 ± 0.15) which were otherwise similar.

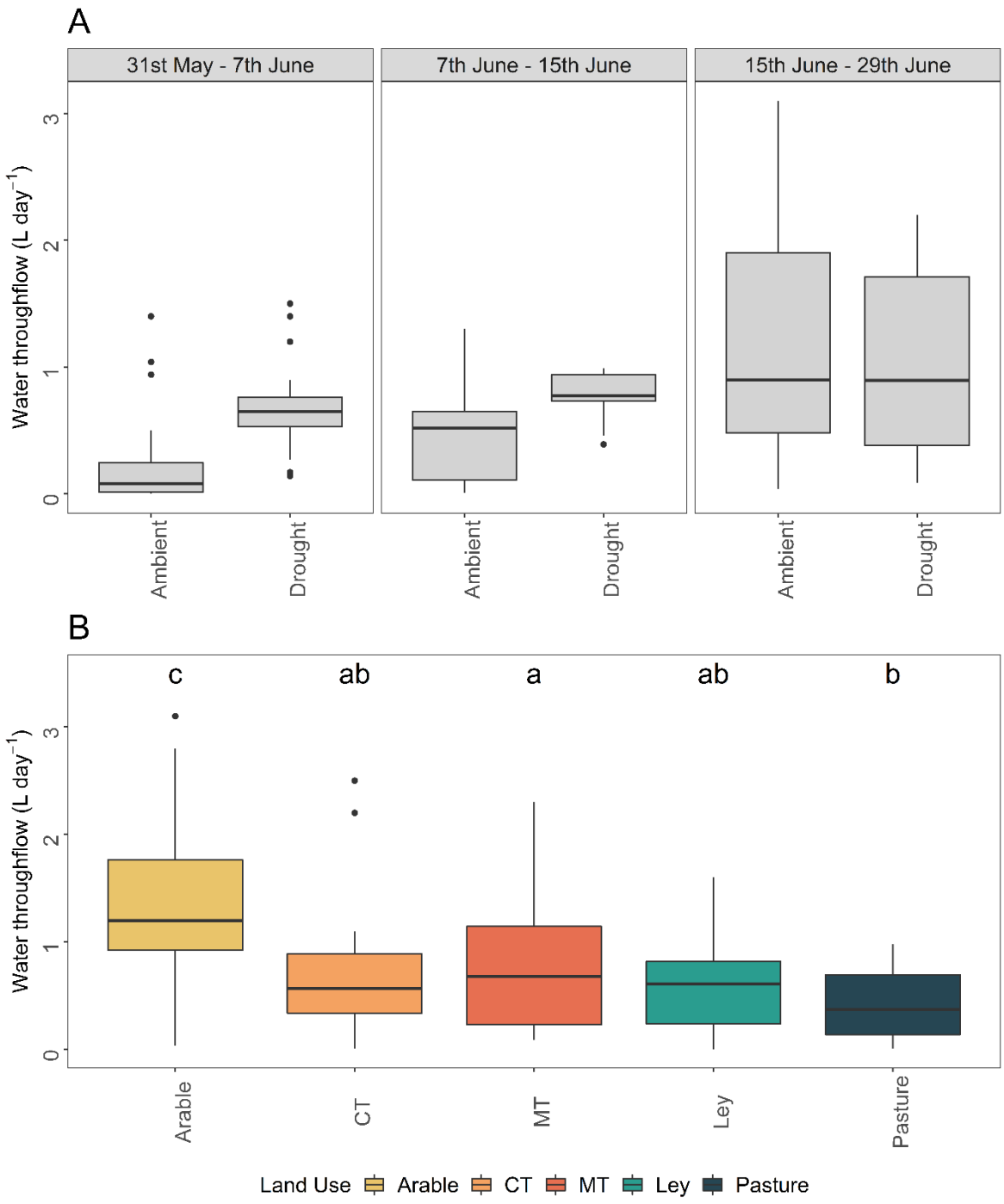

Figure 3.7. Water throughflow from mesocosms A) separated by water stress treatment and throughflow collection period B) separated by land use incorporating all collection periods. Box plots show median, interquartile range, maximum and minimum. Outliers are represented outside of this range. Replication for each land use: Arable (n = 12); CT (n = 9); Ley (n = 24); MT (n = 9); Pasture (n = 9). Replication for each water stress treatment per date: Ambient (n = 21); Drought (n = 21); Flood (n = 21). Raw data was provided by Dr. Despina Berdeni as part of the *SoilBioHedge* project.

Table 3.4. Results of three-way ANOVA model incorporating the effect water stress treatment, collection period (date), and land use (model: treatment * date * land use) on water throughflow (L day^{-1}) over all days; two-way ANOVA model considering only land use and water stress treatment over (model: treatment * land use) for the May 31st – June 7th data subset.

Source of variation	Water throughflow (all dates)		Water throughflow (May 31st – June 7th)	
	F	**P**	**F**	**P**
Treatment (df=1)	1.15	0.29	2.83	0.1
Date (df=2)	**7.84**	**<0.001**	-	-
Land Use (df=4)	**2.60**	**0.04**	**9.83**	**<0.001**
Treatment*Date (df=2)	0.99	0.38	-	-
Treatment*Land Use (df=4)	1.07	0.38	1.94	0.11
Date*Management (df=8)	1.77	0.09	-	-
Treatment*Date*Land Use (df=8)	1.35	0.23	-	-

3.3.3.2 Estimated Nutrient Leaching Resistance

Nutrient concentration data with sufficient replication was only available for the arable, arable-to-ley, and pasture mesocosms. MacroRhizon samplers failed to collect sufficient water volume from mesocosms pasture-to-CT and pasture-to-MT mesocosms on numerous occasions.

Considered over the May 31st-June 7th period, soil NH_4^+-N concentration was similar across both water stress treatments and land uses (ANOVA: Treatment, F = 3.32, df = 1, p = 0.08; Land use, F = 0.06, df = 2, p = 0.94; Treatment x Land Use, F = 0.08, df = 2, p = 0.92). Soil NO_3^--N concentration was significantly increased in droughted mesocosms (Ambient, 1.12 mg L^{-1} ± 0.380 SEM; Drought, 37.10 mg L^{-1} ± 9.35 SEM), and was similar across the three land uses (ANOVA: Treatment, F = 23.82, df = 1, p < 0.001; Land Use, F = 0.03, df = 2, p = 0.97, Treatment x Land Use, F = 0.45, df = 2, p = 0.64). The estimated leachate losses of both NH_4^+-N (ANOVA: Treatment, F = 8.05, df = 1, p = 0.009; Land Use, F = 0.08, df = 2, p = 0.92, Land Use x Treatment, F = 0.69, df = 2, p = 0.51) and NO_3^--N (ANOVA: Treatment, F = 6.93, df = 1, p = 0.01; Land Use, F = 0.001, df = 2, p = 0.999; Land Use x Treatment, F = 0.44, df = 2, p = 0.65) was driven entirely by water stress treatment. Averaged across all land uses, mesocosms lost 0.02 mg NH_4^+-N day^{-1} ± 0.01 SEM and 0.03 NO_3^--N day^{-1} ± 0.01 SEM following ambient conditions, compared to 0.29 NH_4^+-N day^{-1} ±0.03 SEM 4.13 mg NO_3^--N day-1 ± 1.08 SEM following drought conditions. Soil water concentrations and estimated leachate values per land use are shown in *Table 3.5*.

Table 3.5. Mesocosm soil water concentrations of NO_3^--N and NH_4^+-N over the first week after water stress (May 31st – June 7th), and the estimated leachate losses calculated from these known concentrations and the volume of water collected below each mesocosm.

Land Use	**NH_4^+-N (mg L^{-1})**	**NH_4^+-N leachate (mg day^{-1})**	**NO_3^--N (mg L^{-1})**	**NO_3^--N leachate (mg day^{-1})**
Arable	1.81 ± 0.79	0.27 ± 0.11	21.49 ± 14.80	3.00 ± 1.93
Arable-to-Ley	1.76 ± 0.71	0.12 ± 0.06	16.95 ± 7.26	1.74 ± 0.72
Pasture	1.72 ± 0.59	0.10 ± 0.06	24.40 ± 11.76	2.04 ± 1.15

3.3.4. Structural Equation Modelling of Stress Response

The influence of land use on water maintenance across stress scenarios is expected to be ultimately dependent on how land use alters soil health properties such as water holding capacity and infiltration rate which are summarized within the soil health index, along with the biotic component of the soil. In this case shoot height of plants can be indicative of resource needs, transpiration rates and potential for water interception. We teased apart the direct and indirect effects of land use on these properties based on this conceptual framework using structural equation modelling (SEM). Models were created separately on two subsets of the data: flood, and ambient + drought.

In both the ambient + drought and flood SEM models land use had a significant association with SHI (*Figure 3.8, Figure 3.9*). An indirect control of land use on shoot height was found through this under both models. Shoot height was also found to be significantly associated with water loss in the week following the extreme rewetting event in the ambient + drought model. This supports our conceptual framework that increasing soil health improves crop yield (as measured through the shoot heigh proxy) and can subsequently benefit water and nutrient interception by the plant roots through this. However, land use was also directly associated water loss in this model, indicating that there may be an unacknowledged variable associated with land use that could also play an important role in water interception, such as soil microbial activity and abundance which was not accounted for in our calculation of SHI. It is surprising that SHI was not found to have a direct effect on water loss in this model, as increasing water holding capacity should also influence the absolute volume of water that can be maintained within the soil column. Through the two models it is apparent that soil health can play a large role in maintaining plant yields under a variety of water stress scenarios.

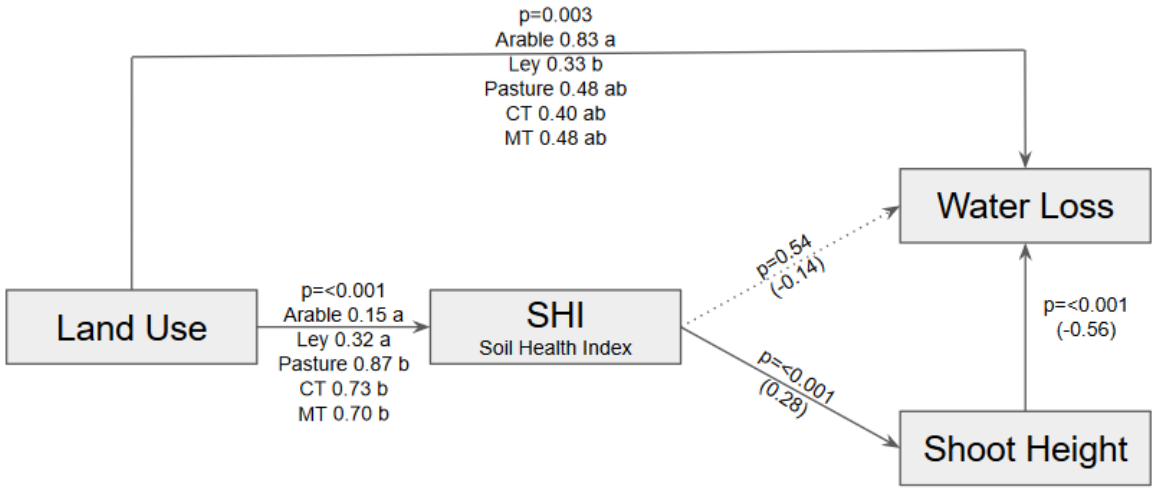

Figure 3.8. Structural equation model of SHI, water loss and wheat growth response across land uses for the ambient + drought treatments. *Solid paths* indicate that the predictor significantly influences the model likelihood. *Dashed paths* indicate that the predictor has no detectable influence on the model likelihood ($p > 0.05$). Model statistics: AIC = 40.58; Fisher's C = 0.58; χ^2 p = 0.75; df = 2. Marginal means and statistical groupings ($p < 0.05$) are shown next to land uses. The whole model summary can be found in *Table 3.6*.

Figure 3.9. Structural equation model of soil health indicators, water loss and wheat growth response across land uses for the flood model. *Solid paths* indicate that the predictor significantly influences the model likelihood. D*ashed paths* indicate that the predictor has no detectable influence on the model likelihood ($p > 0.05$). Model statistics: AIC = 18.69; Fisher's C = 0.69; χ^2 p = 0.708; df = 2. Marginal means and statistical groupings ($p < 0.05$) are shown next to land uses. The whole model summary can be found in *Table 3.7.*

Table 3.6. Model coefficients from structural equation model regression relationships and covariances performed on the ambient + drought data subset. Significant values are indicated by * (<0.05), ** (<0.01), *** (<0.001).

Response	Predictor	Estimate	Std.Error	df	Crit.Value	p-value	Std.Estimate	Significance
Water throughflow	SHI	-0.2174	0.3559	34	-0.6109	0.5454	-0.1421	
Water throughflow	Shoot height	-1.7087	0.3357	34	-5.0898	0	-0.5637	***
Water throughflow	LandUse	-	-	4	15.9063	0.0031	-	**
Water throughflow	LandUse = Ley	0.3266	0.0961	1	3.3975	0.1822	-	
Water throughflow	LandUse = CT	0.4027	0.1561	1	2.5802	0.2354	-	
Water throughflow	LandUse = MT	0.453	0.1502	1	3.0156	0.2038	-	
Water throughflow	LandUse = Pasture	0.4816	0.1878	1	2.5649	0.2367	-	
Water throughflow	LandUse = Arable	0.8344	0.1621	1	5.1463	0.1222	-	
Shoot height	SHI	0.1426	0.0295	39	4.8293	0	0.2824	***
SHI	LandUse	-	-	4	158.0203	0	-	***
SHI	LandUse = Arable	0.146	0.0508	1	2.8771	0.213	-	
SHI	LandUse = Ley	0.3234	0.0359	1	9.009	0.0704	-	
SHI	LandUse = MT	0.7046	0.0586	1	12.0214	0.0528	-	
SHI	LandUse = CT	0.7318	0.0586	1	12.4841	0.0509	-	
SHI	LandUse = Pasture	0.8677	0.0586	1	14.8036	0.0429	-	*

Table 3.7. Model coefficients from structural equation model regression relationships and covariances performed on the flood data subset. Significant values are indicated by * (<0.05), ** (<0.01), *** (<0.001).

Response	Predictor	Estimate	Std.Error	df	Crit.Value	p-value	Std.Estimate	Significance
mean_height	SQI	69.1702	22.0763	19	3.1332	0.0055	0.5837	**
SQI	LandUse	-	-	4	8.5323	$7.00x10^{-4}$	-	***
SQI	LandUse = Arable	0.1308	0.096	Inf	1.3633	0.1728	-	
SQI	LandUse = Ley	0.2594	0.0679	Inf	3.8232	$1.00x10^{-4}$	-	***
SQI	LandUse = MT	0.6106	0.1108	Inf	5.5105	0	-	***
SQI	LandUse = CT	0.6854	0.1108	Inf	6.1859	0	-	***
SQI	LandUse = Pasture	0.7833	0.1108	Inf	7.0693	0	-	***

3.4. Discussion

Mesocosms collected from in-field reciprocal planting experiments were used to explore **(a)** the role that land use and management can play in soil health **(b)** the impact that soil health can have on crop yields and water maintenance under ambient, drought and flood stressed conditions. Based on previously observed differences between arable and pasture soils at the same site (Holden *et al.* 2019) and preliminary analysis of AMF communities within the experimental strips (*Bird and Helgason, Appendix 3.6*), it was hypothesised that **(a)** grass-clover leys sown in to arable fields would result in improved soil health **(b)** using grassland pasture as a representative benchmark of arable-to-ley soil health improvement, gains made under ley may be diminished upon conversion to cereal production. CT tillage will more negatively affect soil health than MT tillage due to the increased soil disruption involved. It was further hypothesised that through the effects of these management practices on soil health **(c)** mesocosms with greater soil health would demonstrate greater crop yield resilience and water maintenance under water stress.

The introduction of a short 19-month grass-clover ley rotation into conventionally managed arable crop fields was associated with substantial short-term gains to soil health, along with the associated benefits of increasing crop yields and water retention under the various water stress scenarios. The relationship between increasing soil health, crop yields and water maintenance were further confirmed through structural equation modelling. This is in support of hypotheses **(a)** and **(c)**, demonstrating that the arable-to-ley conversion can restore both soil health and function.

Crop yields following grass-clover leys have been shown to increase in few studies (Johnston *et al.* 1994; Persson, Bergkvist, and Kätterer 2008; Prade, Kätterer, and Björnsson 2017), with most focusing only on the belowground effects on soil health. In this study yields (as measured through the shoot height proxy) saw only a minor yet significant increase of 3 % following the19-month grass-clover ley. This is lower than can be theoretically achieved (e.g., 8-33 % (Prade *et al.* 2017), though it is likely that the Leeds University Farm field site constrained the maximum yields available and therefore the percentage gain. The reference grassland pasture at this site only demonstrated yields 7.7 % higher than the arable land. In this context the grass-

clover ley resolved approximately 43 % of the yield gap between wheat grown on soils under continuous arable rotation, and those previously under pasture management. Yield gains may have further increased and resolved this gap had they been in place for a longer period. Using 1-6 year grass-clover ley periods Johnston *et al.* (1994) showed that yields of the first crop following ley could continue to proportionally increase with the length of ley until an optimum of three years whereafter yields gains were proportionally minor or absent. Three year grass-clover leys within long-term five-year rotations have also been shown to be beneficial to long-term SOC accumulation (Johnston *et al.* 2017) and so may present an optimum strategy for including grass-clover leys within arable rotations.

This study is the first to demonstrate a functional benefit of grass-clover leys to water maintenance under varying environmental conditions. Only two other studies have assessed the impact of grass-clover ley cover on the water holding capacity of soils and water infiltration rates outside of this study system (Albizua *et al*. 2015; Jarvis *et al*. 2017). Those studies both found null results and used grass-clover ley rotations within otherwise conventionally managed fields that included mouldboard plough tillage. In contrast, this study followed the grass-clover ley period with a direct drilled wheat crop and found a significant benefit. This may contribute to the differing results as cultivation disrupts the soil structure built up during the ley period, affecting hydrological functioning (Mondal *et al.* 2020). While the outcome of this study appears promising for the role of grass-clover ley rotations in stress mitigation under climate change, the lack of further evidence available does not allow us to draw generalizations about the use of grass-clover ley for increased water maintenance. This highlights the need for further studies into the hydrological functioning in grass-clover ley rotations to corroborate these findings.

The functional benefit of arable-to-ley soil health recovery was driven by changes to the soil structure and hydrology. Significant increases in field saturated hydraulic conductivity K_{sf}, and saturated gravimetric water content were associated with the arable-to-ley conversion, along with decreases in functional microporosity. Soil bulk density showed an 8 % decrease in in arable-to-ley mesocosms, which while not significant, does demonstrate a shift in this value towards the lower bulk density observed in the reference grassland pasture mesocosms. van Eekeren *et al.* (2008) showed that bulk density could be highly dependent on the current plant cover as

fields containing ley rotations only demonstrated a decrease in BD relative to continuously cropped fields when they were under the ley rotation- a benefit that was diminished when the fields were in arable rotation. It is unusual that the LOI SOC did not increase in the grass-clover ley, which demonstrates an unprecedented short-term uncoupling of soil carbon dynamics and soil hydrology. The lack of tillage disturbance and mechanical stress during the grass-clover ley period may have allowed AMF and earthworm populations to recover. While AMF presence and activity were not directly measured- It is known from in-field assessment of the AMF communities that arable-to-ley soils harboured distinct AMF communities from the arable soils, and that this was slowly trending towards the target community seen in pasture soils. AMF hyphae can have large effects on the stabilization of aggregates in field experiments (Wilson *et al*. 2009), and this has been shown to also operate as a function of the hyphal network itself, rather than acting through alterations to carbon dynamics (Rillig *et al*. 2010). Increasing earthworm activity would further contribute to macropore space within the soil as they carve out channels through their activity (Francis and Fraser 1998). The grass-clover plant cover itself also likely played an important role in moderating soil structure and hydrology through increased root biomass in comparison to the cereal crop roots found in the arable rotation (Bolinder *et al.* 2002; Fan *et al.* 2016).

SOC changes in soil following a change in management to ley can take a significant amount of time, occurring over several cropping cycles and may take as long as 30 years to reach a stable equilibrium (Johnston *et al.* 2017). Gains or losses to SOC over this period may also be hard to detect and quantify, as they represent relatively small changes within a large pool. Many short-term studies therefore fail to see statistical significance despite minor increases (e.g., 5-8 % change, (Loaiza Puerta *et al.* 2018; Gosling *et al.* 2017).

Unexpectedly, NH_4^+-N content was unaffected by the conversion, while NO_3^--N content marginally decreased. Leys have shown variable effects on total N (which includes organic N as well as the mineral fraction, e.g., increase in van Eekeren *et al.* 2008, neutral response in Christensen *et al.* 2009, increase and decrease depending on N fertilizer additions, Albizua *et al.* 2015). NO_3^--N concentrations specifically have been shown to decrease in response to grass-only leys (Christensen *et al.* 2009). As was seen in Albizua *et al.* (2015) with larger N inputs of 150 kg N ha^{-1}, the 50 kg N ha^{-1}

additions in this experiment may have cancelled out any potential benefit of N-accumulation related to the leguminous clover cover, which paired with the increased fertilizer use in the follow-up crop (Johnston *et al.* 1994) may confer net lower values.

The lack of observed N response may also be indicative of altered nitrogen cycling due to the arable-to-ley conversion. Nitrification (the conversion of NH_4^+ to NO_2^- and NO_3^-) and denitrification (the conversion of NO_3^- to gaseous N_2 and N_2O) are antagonistically controlled by moisture content (Tan *et al.* 2018), so the higher moisture content observed in arable-to-ley mesocosms could have shifted this balance towards denitrification (rates of which are higher under higher moisture conditions), reducing the abundance of NO_3^--N in the soil and diminishing the capacity of its replenishment through nitrification. AMF abundance is also likely to be positively impacted by the conversion (Albizua *et al.* 2015) and may preferentially uptake NH_4^+ (Helgason and Fitter 2005), potentially competing for the same resource as nitrifiers (Cavagnaro *et al.* 2015).

The reciprocal planting experiment conducted highlights the precarious nature of soil health gains made through grass-clover ley rotations in the short term. Hypothesis **(b)** was partially supported as both the pasture-to-CT and pasture-to-MT conversions resulted in a swift decline in SOC over only two tillage passes. This itself is unsurprising as the conversion of grassland to cropped land is associated with SOC declines regardless of the tillage form (Hermle *et al.* 2008). The two tillage forms however did not generally differ in the extent of the soil health decline that they mediated, which may be explained by the similarity of the two tillage methods. Both tillage forms used in this experiment also exhibit similarities in their implementation at the depths observed, as both conducted power harrowing and disc tillage within the top 15 cm of soils. This resulted in disruption and the likely exposure of occluded SOC to decomposers. Both conversions further resulted in a decrease in hydraulic conductivity, which was a key component to the SHI calculated. If either of these tillage forms were to follow the grass-clover ley they may therefore have negated much of the positive effects of the relatively minor increases seen through the arable-to-ley conversion. Therefore no-tillage rotations may be necessary to preserve grass-clover ley soil health gains and functioning into the next wheat crop and beyond.

3.5. Conclusion

Using mesocosms derived from agricultural land uses and management approaches, the results of this study show that the maintenance of higher levels of soil health in agricultural systems has significant benefits to both crop yields and water maintenance under water stress scenarios. This was determined through structural equation models incorporating calculated SHI (based on a suite of chemical, physical and hydrological indicators), crop yield, and water throughflow.

Our results demonstrate that a 19-month grass-clover ley can improve soil health by beginning to restore beneficial soil properties impacted by decades of continuous arable cropping. The improvements seen through arable-to-ley conversion were seen only in soil physical-hydrological properties of functional porosity, saturated gravimetric moisture content and field saturated hydraulic conductivity at the soil surface (infiltration rate). SOC accumulation is expected to occur over greater periods of time as part of a crop rotation rather than after a single 19-month period (Johnston *et al.* 2017). The reciprocal study of pasture-to-arable conversion demonstrates the need to further consider the method by which crops are sown in the cereal-producing years of any arable-ley rotation. While only mildly decreasing soil health after two tillage regimes, both pasture-to-MT and pasture-to-CT mesocosms exhibited sharp declines in SOC and K_{sf}. The benefits of incorporating grass-clover leys into rotations may be diminished by the subsequent re-tillage of the soils. The role of maintaining ley benefits under varying tillage rotations has not been explicitly tested before as far as we know and is therefore an area of research that must be given greater focus to. Finally, as many of the soil health gains within the grass-clover ley rotation may be attributed to AMF populations among other factors, they must also be more greatly considered within this system.

3.6. Appendix

3.6.1. AMF Community Profiling In-Field

To demonstrate the underlying variability in AMF diversity and community composition, unpublished data is presented from the *SoilBioHedge* field study that the mesocosms were collected from (Bird and Helgason, unpublished data). Data was analysed and interpreted by Philip Brailey-Jones for this purpose. Samples were collected at 2 m, 16 m, and 32 m distance into the field within each experimental land use strip in July 2017. DNA extraction, sequencing preparation and bioinformatics processing of DNA reads to aggregate virtual taxa (VTs) was performed as described in (Holden *et al.* 2019) with the exception of rarefying samples to even depths which was not carried out for this comparison. Community composition was broadly compared between land uses through PERMANOVA analysis of Bray-Curtis dissimilarities between samples, and post-hoc pairwise PERMANOVA tests with BH correction. Observed species richness and Shannon diversity was calculated using the phyloseq package (McMurdie and Holmes 2015). Richness and Shannon diversity was compared between land uses through ANOVA, with post-hoc Tukey comparisons conducted where appropriate, with BH corrections.

Land uses were globally distinct from one another (PERMANOVA: df = 4, F = 5.4072, p <0.001). Post-hoc comparisons confirmed this, with most practices being distinct, but with overlap in composition observed between arable-to-CT and arable-to-MT, and the arable-to-CT and the pasture (*Figure 3.10*). Visual inspection of the DCA also shows separation of samples across DCA1 between pasture fields and arable fields. Samples across DCA2 are separated by field management practices, with arable-to-ley communities showing a very slight shift towards pasture communities across this axis, and the two pasture-to-tillage treatments moving in the opposite direction from pasture towards the arable field conditions. This indicates that the tillage has a much more immediate and severe impact than the 'sparing' of disturbance through arable-to-ley conversion does. This appears to be a much slower process in shifting AMF communities towards the target grasslands over the same period though not data is available quantifying the abundance of AMF in the soil. Observed AMF VT richness was significantly higher in pasture, pasture-to-CT, and pasture-to-MT than in the arable and arable-to-ley (ANOVA: df = 4, F = 14.767, p <0.001, *Table 3.8*). There was

no difference in Shannon evenness observed between the land use strips (ANOVA: df = 4, F = 1.219, p = 0.32, *Table 3.8*).

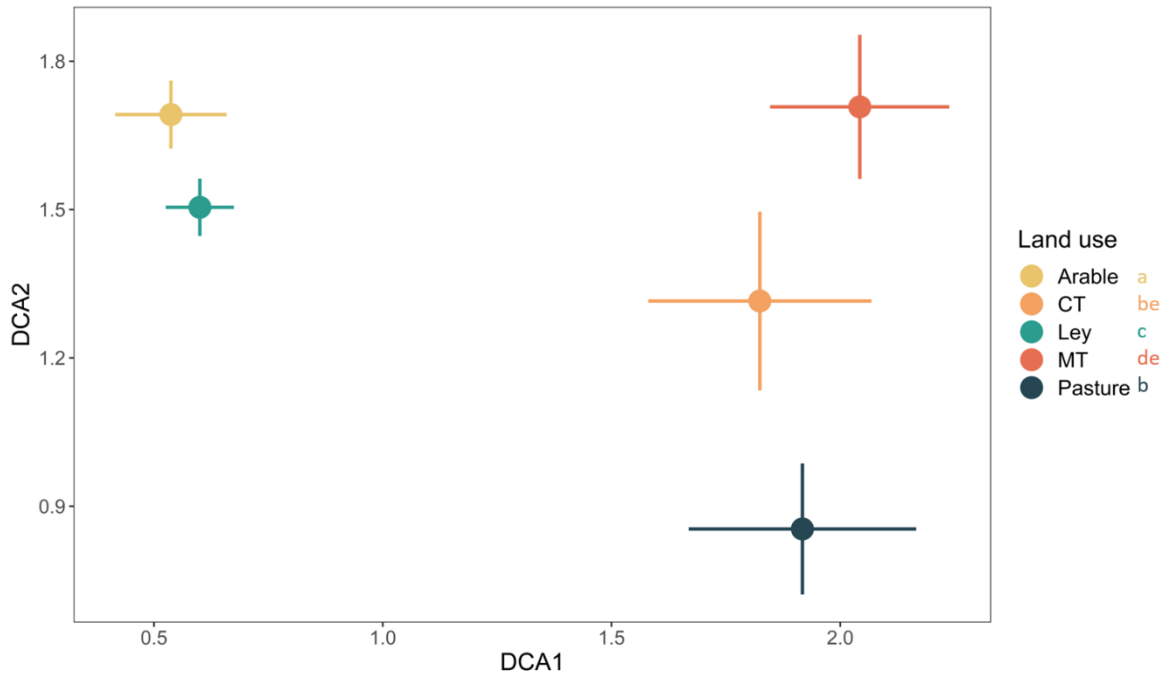

Figure 3.10. Detrended Correspondence Analysis (DCA) of AMF communities from the *SoilBioHedge* project based on field collected rhizosphere samples. letters by the land use variables in the figure legend denote statistically different groups ($p < 0.05$) following pairwise post-hoc PERMANOVA analysis with BH corrections. Data was provided by Prof. Thorunn Helgason and Dr. Susannah Bird.

Table 3.8 Alpha diversity recorded for root samples collected from the SoilBioHedge field experiment that mesocosms in this chapter were collected from. Superscript letters denote statistically different groups ($p < 0.05$) following post-hoc Tukey tests with BH corrections. Data was provided by Prof. Thorunn Helgason and Dr. Susannah Bird.

	Observed VT richness	Shannon's Index
Arable	13.1 +- 0.952 [a]	1.72 +- 0.095
Arable-to-Ley	14.5 +- 0.514 [a]	1.68 +- 0.065
Pasture	21.1 +- 1.12 [b]	1.87 +- 0.142
Pasture-to-CT	21.1 +- 1.78 [b]	1.95 +- 0.133
Pasture-to-MT	19.8 +- 1.01 [b]	1.86 +- 0.142

4. Examining the relationship between AMF communities, soil health, plant productivity and water maintenance following drought and flood

4.1. Introduction

The experiment described here follows directly from Chapter 3 and is built upon the same rationale. In Chapter 3, the impact of land-use and management on soil health properties, crop yields, and water maintenance under water stress scenarios was examined. In the interest of brevity, the specific concepts introduced in that chapter will not be repeated here. Instead, this introduction will focus on the aspects of Chapter 3 that informed this study.

The hypotheses presented in Chapter 3 were partly underpinned by the expected effect of management on AMF community composition. When observed in-field prior to the collection of mesocosms used in Chapter 3, it was found that AMF communities in arable-to-ley soils were beginning to diverge from those found in the arable soils. This was unable to be directly assessed during the experiment. Therefore, this study aims to resolve the gap in our understanding of the system through the assessment of AMF communities from the mesocosm. Subset microcosms were collected after the wheat harvest detailed in Chapter 3, and they were resown with the same winter wheat cultivar. Wheat was grown for 8 weeks to allow the proliferation of AMF and the establishment of IRM into the wheat roots and ERM through the surrounding soil. This was used explore the hypotheses that **(a)** AMF community composition and diversity would be mediated by land use history **(b)** Variation in AMF communities will be significantly linked to variation in soil health and functional differences observed in Chapter 3.

AMF are a key target for their potential to bridge yields gaps between intensive and sustainable agriculture and increase ecosystem functioning (Thirkell *et al.* 2017). The functional diversity found within this group (e.g., ERM hyphae vs IRM root length colonization, (Jakobsen *et al.* 1992; Hart and Reader 2002b; 2005; Maherali and Klironomos 2007; Thonar *et al. 2011)*) and capacity for synergy between species (Jansa *et al.* 2008) increases the importance of monitoring how communities

assemble under different management practices. Increasing our understanding of how land use moderates this assembly and the functional implications will allow us to optimize our approaches to ecological engineering of communities (Bender *et al.* 2016) and ensure that we can cultivate agro-ecosystems that are maximally beneficial and resilient to future climate change scenarios.

4.2. Methods

4.2.1. 5.2.1 Experimental Set-Up

The experiment presented in this chapter follows from that of Chapter 3. Following the harvest of wheat from the experimental mesocosms, they were left bare for 23 days. A single structurally intact microcosm (Cylinders of 20 cm depth and 7 cm diameter) was extracted from each mesocosm on the 4th of October 2020. Five wheat seeds (*cv.* Skyfall, RAGT Seed) were direct drilled approximately 1 cm depth in each microcosm on the 10th of October 2020. Following germination, two wheat saplings were removed from each microcosm to ensure that root growth over the experimental period did not result in overcrowding. The remaining saplings were grown for 48 days under a standardized temperature (20 °C) and light cycle (12 hour on/off) to allow AMF to proliferate and colonize the wheat roots.

Moisture content was estimated from soil cores (cylinders of 20 cm depth and 7.6 cm diameter) collected directly next to the microcosms on the same day. This was used to standardize the watering regimes for microcosms. In Chapter 3 it was shown that soils from the contrasting land uses had different levels of water holding capacity and bulk density, and so a watering regime was chosen that accounted for this innate soil property and to ensure that no microcosms were under or over-watered for the duration of the experiment. Estimated water-filled pore space (WFPS) was chosen as the standardizing variable due to the effect this has on microbial activity (Franzluebbers 1999). Microcosms were watered regularly by weight to 50 % WFPS. At the end of the experiment, a subset of microcosms was sampled for WFPS, which were found to be generally overestimated but only by small margins. Actual WFPS measured at the end of the experiment was 91 % +- 0.03 % SEM proportionally compared to the estimated value. This discrepancy did not violate the original intent of the standardization as microcosms had an average actual WFPS of 47.81 ± 1.30 SEM.

4.2.2. Molecular Analysis

Collected roots and surrounding rhizosphere soil were frozen, freeze-dried and ground using a Tissuelizer and stainless-steel grinding jars (Qiagen). Total DNA was extracted using MoBio (now Qiagen) PowerPlant Pro DNA extraction kits according to the manufacturer's protocol. Resulting DNA concentrations were measured using a NanoDrop (Thermo Fisher) to ensure that there was DNA present without contaminants.

PCRs were performed in the presence of 0.2mM dNTPs, 10pmoles of each primer, 2mM $MgCl_2$, and the manufacturer's reaction buffer using GoTaq G2 Flexi Kits (Promega). PCR was carried out on a TC-512 thermocycler (Techne). A semi-nested PCR approach was taken using the primer pair AML1-AML2 (Lee *et al.*, 2008b) for the first PCR, followed by a secondary PCR using the WANDA-AML2 primer pair (Dumbrell *et al.* 2011). Conditions for the first reaction were as follows: 95C 2m; 30 cycles (94C 30s, 59C 30s, 72C 1m); 72C 10m, and for the second: 94C 5m; 20 cycles (94C 30s, 58C 30s, 72C 30s); 72C 10m. Next, the semi-nested PCR amplicons were cleaned using AMPure beads (Agincourt) following the manufacturer's instructions and prepared for sequencing using NextEra (Illumina) barcoding and sequence library preparation. Libraries were sequenced on the Illumina MiSeq platform.

4.2.3. Bioinformatics and AMF Community Analysis

4.2.3.1 ASV Generation and Taxonomic Assignment

All bioinformatics processing and analyses were performed in R (Version 3.6.1). Sequence reads were demultiplexed using *Cutadapt* (Martin 2011). *Cutadapt* was used to trim the primer sequences from the forward 18S reads discarding any reads which did not have a segment matching the chosen primer sequence. Single-end SSU amplicons were trimmed to a length of 240 bp which corresponded with a decline in the proportion of reads extending past this length. Reads were quality filtered (<2 errors per read), dereplicated and assigned to Amplicon Sequence Variants (ASVs) using the *dada2* algorithm (Callahan *et al.* 2016). ASVs are sequence clusters with near 100% similarity, which represent the estimated true biological variation within each sample.

Taxonomy was assigned to ASVs through the implementation of a Bayesian classifier method (Wang *et al.* 2007) as implemented in the *dada2* package. This classifier

compares the kmer profile (DNA sequences split into lengths of *k*) of sample sequences to those of a given reference dataset of known taxonomy. The sequence with the most similar profile is used to assign taxonomy to the query sequence. This classification is bootstrapped 1000 times with a minimum confidence of 50% required for all assignments. Taxonomy was assigned using two reference databases. These were the SILVA (Quast *et al.* 2013) and MaarjAM databases (Öpik *et al.* 2010; Opik *et al.* 2014). SILVA is a general database of SSU sequences across eukaryotes and prokaryotes. The MaarjAM database is a highly curated database specific to AMF. The taxonomic assignments in this database use phylogenetic methods to create 'Virtual Taxa' (VTs) in lieu of traditional species assignments.

4.2.3.2 Taxonomic Curation

Taxonomic curation was performed using the R package *phyloseq* (McMurdie and Holmes 2015). SILVA taxonomic assignments were used to subset only ASVs assigned to the class Glomeromycetes (125 / 416 ASVs). ASVs were subsequently clustered in to 97 % OTUs using the R packages *DECIPHER* (E. S. Wright 2016) and *speedyseq* (McLaren 2020). They were further agglomerated by their species level assignments resulting in 112 OTUs and 32 agglomerated taxa. Any OTUs not assigned to the VT level were reclassified manually against all sequences in the MaarjAM database. OTUs were aggregated by VT-level taxonomic assignment leaving 32 VTs. Following aggregation, OTUs with less than 5 reads in at least two samples were removed from the dataset. The final dataset contained 26 VTs. One sample was removed from the dataset due to low sequencing depth (Sample: BE Arable Drought, 6 reads). Sequencing depth ranged from 501 to 23638 reads. Rarefaction curves were generated for all samples to visually assess species accumulation with increasing sequencing depth. This was complimented by comparisons of sequencing depth between land use, water stress legacy and the interaction of the two treatments to ensure that treatment groups had equal sequencing depths. All inspected curves appeared to be at or near an asymptote (*Appendix 4.6, Figure 4.4)*, and there was no significant difference of sequencing depths between any treatment groups. Due to this, the data was not rarefied to a standard sequencing depth. To normalize species abundances across samples with differing sequencing depth, read counts were converted to relative abundances.

4.2.3.3 Community and Statistical Analysis

Alpha diversity was calculated for samples using *phyloseq* as observed OTU richness, Shannon index. Alpha diversity metrics were compared between groups using ANOVA. The general and AMF community composition was visualized through detrended correspondence analysis (DCA) plots, generated with the *decorana* function in *vegan* with rare species downweighed (Oksanen *et al.* 2019). Community compositions were statistically compared through permutational multivariate analysis of variance (PERMANOVA) which was conducted using Bray-Curtis dissimilarities between samples. Where PERMANOVA was found to be overall significant, pairwise PERMANOVA between groups was conducted with BH corrections as a post-hoc test.

Indicator species analysis was conducted to find OTUs which had a significant association with one or a combination of treatment variables. Indicator species analysis was carried out using the *multipatt* function in the *indicspecies* R package (De Cáceres and Legendre 2009). P-values derived from the analysis were subjected to Bonferroni corrections. At the family level, relative abundances were also compared between land uses through ANOVA with Tukey's post-hoc tests where appropriate.

ABG wheat biomass was recorded when roots were collected for molecular analysis, which was assessed for normality using the Shapiro-Wilkes test and subsequently analysed through ANOVA. To assess the role that AMF communities may play in determining soil health, generalized additive models (GAMs) were used to fit soil health indicator data generated from the same soils from Chapter 3 and the ABG wheat biomass observed in this chapter as smooth response variables over the AMF community DCA ordination. This was conducted with the *ordisurf* function in vegan (Oksanen *et al.* 2019) which is a wrapper for the *gam* function from the *mgcv* package (Wood 2017) while adding plotting capabilities to the fitted splines. This method was chosen over other established methods as GAMs allow for both linear and non-linear fitting of values to the community composition (unlike *envfit* analysis which assumes linear relationships) and allows the raw values to be fitted against community composition (unlike Mantel tests, wherein values are transformed into distance matrices to be compared against community dissimilarity matrices). Pearson correlations were also calculated from the stated response variables and measures of alpha diversity calculated.

4.3. Results

4.3.1. Fungal Community Diversity and Composition

Mean observed VT richness was similar between land uses (Arable: 12.2 ± 1.16; Arable-to-Ley: 12.0 ± 0.64; Pasture: 13.7 ± 1.19) as was Shannon Index (Arable: 1.34 ± 0.49; Arable-to-Ley: 1.18 ± 0.07; Pasture: 1.23 ± 0.18). There was no significant association between land use, water stress treatment or the interaction between the two on this measure (ANOVA Land Use: df = 2, F = 0.4050, p = 0.67; Treatment: df = 2, F = 0.57, p = 0.57; Land Use x Treatment: df = 4, F = 0.94, p – 0.45, *Figure 4.1 A*). The same relationship was observed for Shannon Index (ANOVA Land Use: df = 2, F = 0.43, p = 0.65; Treatment: df = 2, F = 1.20, p = 0.28; Land Use x Treatment: df = 4, F = 1.95, p = 0.16, *Figure 4.1 A*).

PERMANOVA analysis showed that there was a significant effect of the land use that soils were extracted from on AMF community composition (PERMANOVA: df = 2, F = 2.31079, p = 0.027). Pairwise comparisons revealed that this was driven by a significant difference between the arable and pasture samples (PERMANOVA: df = 1, F = 3.089, corrected-p = 0.03). Samples from the arable-to-ley land use contained AMF communities which were intermediate to both arable and pasture (PERMANOVA ley vs pasture: df = 1, F = 2.313867, corrected-p = 0.09; ley vs arable: df = 1, F = 2.009092, corrected-p = 0.10). This is apparent from the visual inspection of the DCA ordination which shows a separation between arable and pasture samples across DCA2 with ley samples between these two (*Figure 4.1 B*). There was no observed legacy effect of water stress treatments on AMF community composition observed (PERMANOVA: df = 2, F = 0.87772, p = 0.5208), nor was there any interactive effect between land use and water stress legacy (PERMANOVA: df = 4, F = 0.85405, p = 0.6162).

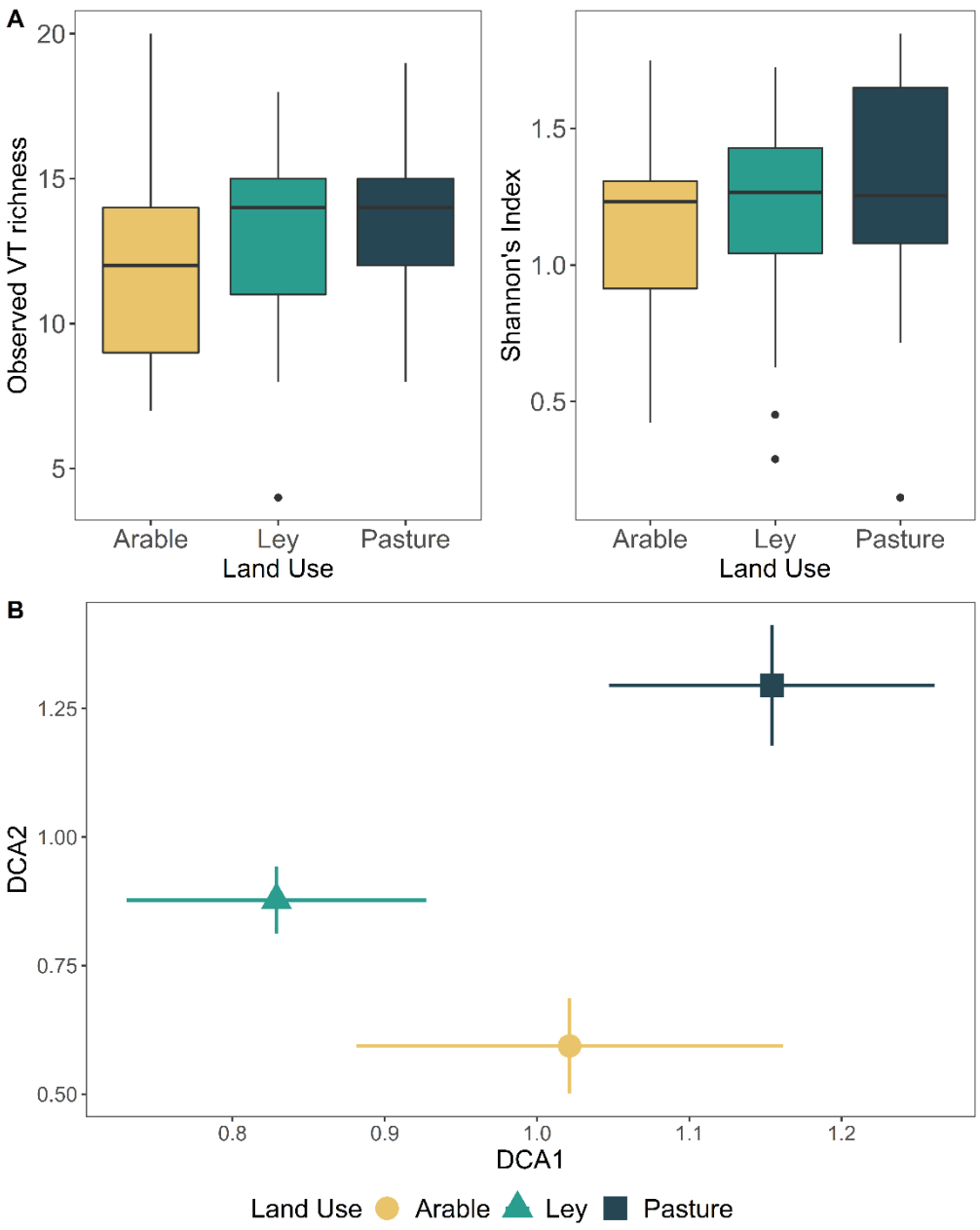

Figure 4.1. A) Diversity metrics separated by land use. Box plots show median, interquartile range, maximum and minimum. Outliers are represented outside of this range. B) Detrended correspondence analysis (DCA) ordination plots of AMF community data separated by land use. Data are presented as the mean centroid values of each wheat cultivar and inoculum treatment ± standard error. DCA axis 1 length = 1.74, eigenvalue = 0.29, variance explained = 38.1 %; DCA axis 2 length = 1.88, eigenvalue = 0.22, variance explained = 29.44 %.

4.3.2. Species and Family Differences across Land Uses

21 VTs (comprising 95.92 % of all sequence reads detected) were shared between all soils, with four being found only in arable and ley soils, and one being found only in ley and pasture soils (*Figure 4.2*). The *Gigasporaceae* (comprising VTX00049 and VTX00052) and *Acaulosporaceae* (comprising VTX00030) families were also found only within arable and ley soils. Indicator species analysis subsequently revealed five VTs and one family which were indicative of the pasture soils (*Table 4.1*). There were no indicator species or families for arable, or arable-to-ley samples. At the family level, VTs belonging to *Glomeraceae* had greater relative abundances in pasture soils than arable soils and were intermediate in arable-to-ley soils (*Figure 4.3,* ANOVA: $F = 3.405$, $df = 2$, $p = 0.043$). *Paraglomeraceae* showed the opposite relationship (ANOVA: $F = 3.28$, $df = 2$, $p = 0.048$). *Claroideoglomeraceae* were most abundant in pasture soils than both arable and arable-to-ley samples (ANOVA: $F = 20.02$, $df = 2$, $p < 0.001$). The other tested families did not show any difference between the three land uses (ANOVA: *Ambisporaceae*, $F = 1.07$, $df = 2$, $p = 0.35$; *Archaeosporacea,* $F = 1.49$, $df = 2$, $p = 0.24$) or between the arable and arable-to-ley samples (ANOVA: *Acaulosporaceae*, $F = 1.24$, $df = 1$, $p = 0.27$; *Gigasporaceae*, $F = 0.05$, $df = 1$, $p = 0.83$). *Paraglomeraceae* and *Glomeraceae* likely were not found to be indicator taxa as the analysis assessed both abundance and specificity to a given treatment. These two families may have been in greater abundance in arable soils but were present in nearly all samples across the three land uses.

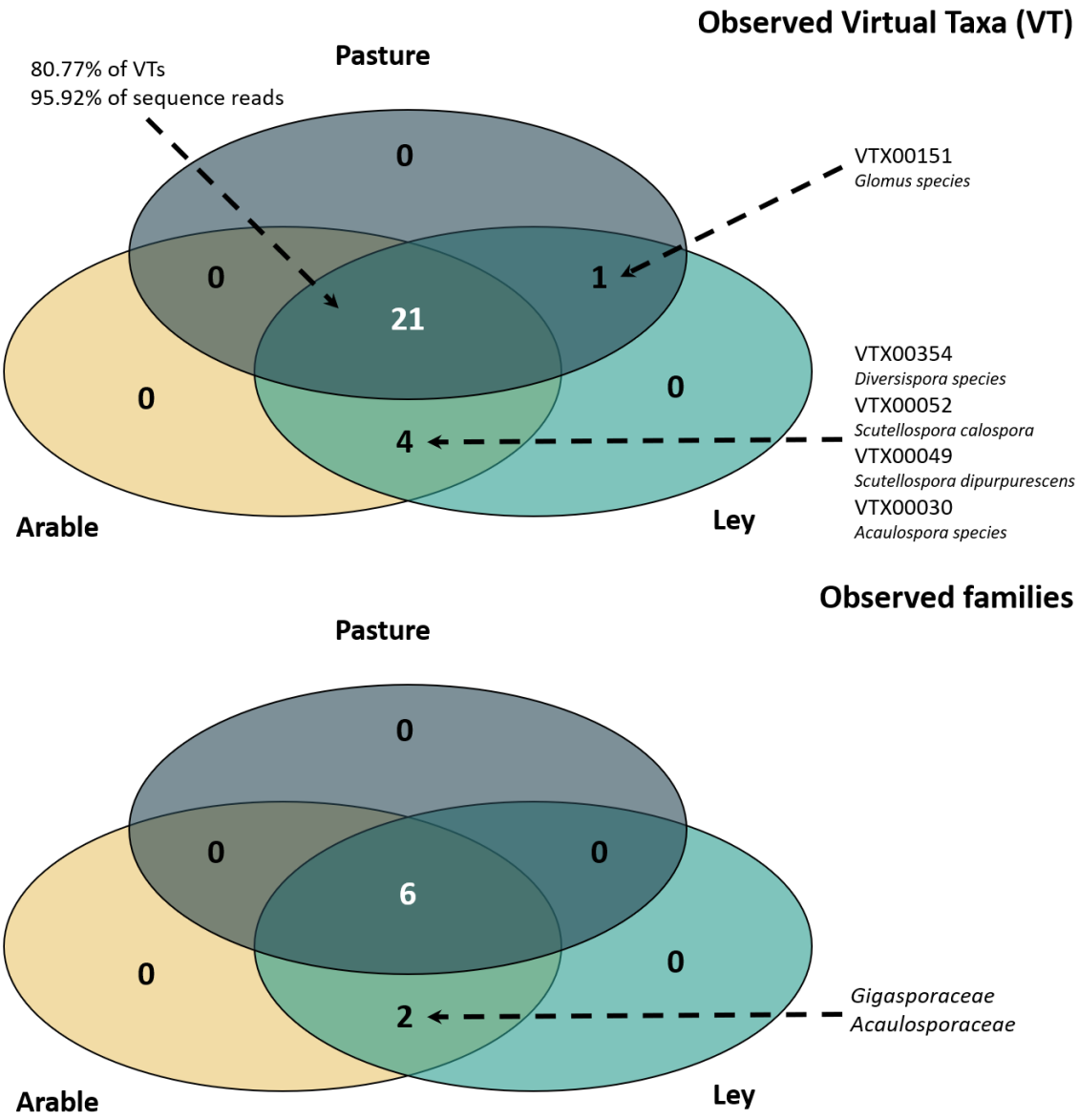

Figure 4.2. Venn diagrams of AMF VTs and families shared between land uses and unique to each.

Table 4.1. Relative abundances of AMF VTs and families found to be significant indicator taxa ($p < 0.05$) for pasture soils. No indicator taxa at either the VT or family level were identified for arable, arable-to-ley or any combination of land uses.

	Relative Abundance			**Indicator**	**Statistic**	**Corrected**
Indicator taxa	**Arable**	**Ley**	**Pasture**	**Status**		**p-value**
VT Level						
VTX00057 *Claroideoglomus sp.*	0.001 ± 0.001	0.002 ± 0.08	0.02 ± 0.006	Pasture	0.87	0.0006
VTX00335 *Paraglomus majewskii*	0.002 ± 0.002	0.001 ± 0.0001	0.006 ± 0.002	Pasture	0.77	0.0088
VTX00056 *Claroideoglomus sp*.	0.004 ± 0.002	0.004 ± 0.002	0.033 ± 0.007	Pasture	0.90	0.0006
VTX00155 *Glomus sp.*	0.0002 ± 0.0001	0.0002 ± 0.0002	0.007 ± 0.004	Pasture	0.56	0.0391
VTX00193 *Claroideoglomus lamellosum*	0.0005 ± 0.0003	0.0006 ± 0.0005	0.007 ± 0.003	Pasture	0.88	0.0006
Family Level						
Claroideoglomeraceae	0.006 ± 0.003	0.006 ± 0.002	0.058 ± 0.01	Pasture	0.91	0.0008

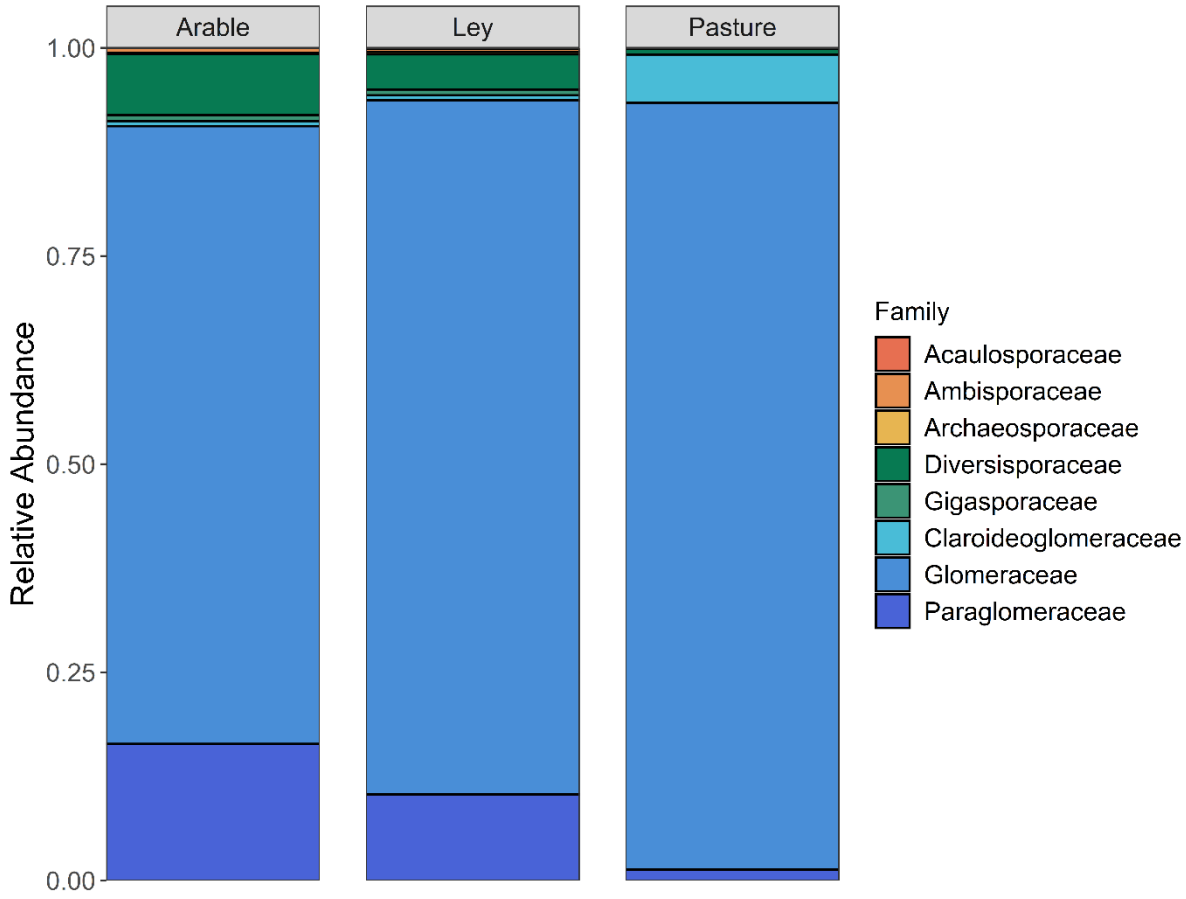

Figure 4.3. Taxa charts of the relative abundance of families within the AMF clade across the three land uses. Families are colour coded per their putative functions based on expected biomass allocation preference (Weber *et al.* 2019). Families assigned to the Ancestral functional group are coloured red (Acaulosporaceae), orange (Ambisporaceae), and yellow (Archaeosporaceae). Families assigned to the edaphophilic functional group are coloured in shades of green (Diversisporaceae, Gigasporaceae), and those assigned to the rhizophilic functional group are coloured in shades of blue (Claroideoglomeraceae, Glomeraceae, Paraglomeraceae).

4.3.3. AMF Community Composition and Function

The SHI calculated in Chapter 3 was shown to be an important determiner of crop yields under all three water stress scenarios and soil water maintenance under the ambient and drought scenarios. Here we use these measures from Chapter 3 to assess the relationship between the soil function and AMF community composition derived from the same mesocosms. AMF community composition and diversity were also assessed against the ABG biomass of the host plants used to propagate the AMF in this experiment.

Overall, there was no significant effect of land use legacy found on wheat ABG biomass of the host plants used in this experiment (ANOVA: Land use, df = 2, F = 1.27, p = 0.29; Water stress legacy, df = 2, F = 0.97, p = 0.39; Land use x water stress legacy, df = 4, F = 0.13, p = 0.97). Unlike what was observed with end-point yields in Chapter 3, at 45 days of growth the wheat plants had similar biomass (mean ABG biomass per land use legacy: arable = 0.78 ± 0.08, arable-to-ley = 0.62 ± 0.08, pasture = 0.84 ± 0.14).

There were no significant correlations between AMF diversity (VT richness and evenness) with soil health, wheat ABG biomass or water throughflow from Chapter 3 (*Table 4.2*). There was however a significant negative correlation found between species richness and ABG biomass of the wheat grown in this experiment (*Table 4.2*). Relationships were also found between community composition and soil health, as SHI was significantly associated with AMF communities. When the individual soil health indicators that were used to calculate the index were compared against the AMF community, it was revealed that this relationship was driven by LOI SOC content, K_{sf} and gravimetric moisture content (*Table 4.3*). Bulk density however had little relevance to the observed AMF communities reflecting the minor comparative contribution the property also made to the calculation of the SHI (Chapter 3, *Table 3.3*). AMF community composition was also not found to be a significant explanatory variable for wheat ABG biomass or water throughflow (*Table 4.3*).

Table 4.2. Pearson correlation coefficients (r^2) and associated p-values for comparisons between AMF diversity metrics, soil health indicators, SHI, ABG wheat biomass and water throughflow. ABG biomass is labelled depending on whether it is from the previous mesocosm experiment (Ch3), or the current experiment (Ch4). Wheat ABG biomass (Ch3) and hydrological data was provided by Dr. Despina Berdeni through the *SoilBioHedge* project.

Property	**n**	**VT Richness**		**Shannon Index**	
		r^2	**p-value**	**r^2**	**p-value**
Soil Health Index (SHI)	44	-0.06	0.69	0.13	0.42
LOI	44	0.06	0.69	0.13	0.41
Ksf	44	-0.03	0.86	-0.06	0.72
Moisture content	44	0.01	0.97	0.22	0.15
Bulk Density	44	0.11	0.49	-0.15	0.33
Wheat ABG biomass (Ch3)	44	-0.30	0.051	-0.13	0.41
Wheat ABG biomass (Ch4)	44	**-0.35**	**0.02**	-0.20	0.20
Water throughflow (*Ambient + Drought only*)	29	-0.11	0.25	0.55	0.19

Table 4.3. The relationship between AMF community composition and soil health indicators, SHI, ABG wheat biomass and water throughflow revealed by general additive modelling (GAM). ABG biomass is labelled depending on whether it is from the previous mesocosm experiment (Ch3), or the current experiment (Ch4). Wheat ABG biomass (Ch3) and hydrological data was provided by Dr. Despina Berdeni through the *SoilBioHedge* project.

Property	**Deviance Explained (%)**	**n**	**p-value**
Soil Health Index (SHI)	**46.9**	**44**	**$9.64x10^{-5}$**
LOI	**31.5**	**44**	**0.005**
Ksf	**36.0**	**44**	**0.003**
Moisture content	**33.7**	**44**	**0.005**
Bulk Density	0.35	44	0.388
Wheat ABG biomass (Ch3)	8.41	44	0.183
Wheat ABG biomass (Ch4)	1.76	44	0.311
Water throughflow (*Ambient + Drought only*)	0.21	29	0.342

4.4. Discussion

This study foremost demonstrates that the inclusion of grass-clover leys in arable rotation can result in a shift in AMF community composition away from that seen in continuously cropped arable land towards that seen in grassland pasture. While communities were more distinct across land uses in-field when soils were under cereal, grass-clover, and pasture grassland cover (Bird and Helgason, unpublished data; *Appendix 3.6*), the results here show that the differences observed can also be maintained in the subsequent wheat rotation. It has been further shown that the AMF community can be significantly associated with soil health properties that are also restored through arable-to-ley conversion, supporting the two hypotheses of this chapter. This was shown through generalized additive modelling which tested the non-linear associations between community composition and traits. Through this, community composition was be significantly linked to the variation in soil health across mesocosms but not to wheat yields or water throughflow. As soil health was shown to be a direct determiner of both properties in Chapter 3, this may be suggestive of an indirect association with these properties through moderation of the soil physical environment by AMF.

There is increasing interest in further using biological indicators of soil health beyond the traditional suite of chemical and physical measures employed, including AMF (Abbott 2014; Mahdi *et al.* 2017). This study confirms that AMF community composition can in fact be indicative of soil health, and therefore warrants further consideration in future soil health studies. Comparing the individual soil health indicators, AMF community composition was associated with differences in infiltration rate K_{sf}, moisture content and LOI SOC. This reflects the strong causal link seen between AMF, soil structure and hydrology (Rillig 2004; Rillig and Mummey 2006). AMF stabilize soil structure and increase the aggregation of soil particles through their dense network of soil hyphae and exudates such as *GRSP* (e.g., Tisdall and Oades 1982; Rillig and Mummey 2006; Wilson *et al.* 2009). This contribution to aggregation enables the long-term development of soil organic matter, affects pore spaces and water flow through the system therefore increasing water holding capacity (Guber *et al.* 2003; Lipiec *et al.* 2007). The architecture (Drew *et al.* 2003; Hart and Reader 2005), density (Hart and Reader 2002b; Maherali and Klironomos 2007)and spread of hyphae (Jakobsen, Abbott, and Robson 1992; Thonar *et al.* 2011)

through the soil profile differs among AMF species It would be expected that species will differentially contribute to soil aggregation, and that synergy between species that can occupy different niches (e.g., short vs. long spread length, thick vs. thin hyphae able to bind particles or travel through pore space within macroaggregates etc.) would be of benefit to soil aggregation and stability. In this study, we show that this may indeed be the case through the broad comparison of whole communities which prompts the need for further in-depth mechanistic study to evaluate the effects of individual species and complements to this functional output.

Additionally, we found no differences in the diversity of AMF in mesocosms from different land uses. This was unexpected as diversity was higher in samples taken from the pasture than from arable or arable-to-ley strips when assessed in-field, and soils from grassland pasture are often shown to have greater diversity than those under arable management (Alguacil *et al.* 2008; Oehl *et al.* 2010; Xiang *et al.* 2014; Manoharan *et al.* 2017). It is important to note that AMF communities were observed in wheat roots in this study rather than the preceding land cover. Under low disturbance conditions where the ERM and previous plants roots are left intact to act as an inoculum sources, it would be expected that subsequent wheat root communities reflect those of the preceding host plant (Campos *et al.* 2018). This expectation is reflected in the community composition, where arable and pasture AMF communities colonizing wheat roots remain completely distinct, but clearly there is some evidence of host-fungus compatibility determining diversity. AMF are known to have some degree of host-specificity (Helgason *et al.* 2002), and this has been linked to the plant functional guild of the host plant (Yang *et al.* 2012; John Davison *et al.* 2020). The long-developed grassland pasture fields likely contained multiple plant functional groups (e.g., Sternberg *et al.* 2000) providing unique niche spaces that foster greater levels of diversity which were lost upon conversion to wheat monoculture.

AMF species richness has been previously observed to be significantly correlated with plant biomass up to an optimum of 8 species (van der Heijden *et al.* 1998; Vogelsang *et al.* 2006; Maherali and Klironomos 2007). In this experiment, species richness ranged from 4 to 20 VTs per sample, and only two samples contained less than 8 species. No significant variation between land use treatments was found, which demonstrates that at similar levels of species richness across land uses, the

composition of AMF communities may play an important role in determining soil function.

Increasing species richness and evenness independent of any land use history was not associated with any positive functional outputs. Increasing species richness was in fact found to negatively correlate with plant ABG biomass, but only for the 45-day old wheat plants grown in this experiment and not the final harvest wheat plants from Chapter 3. This negative association may therefore be due to the relatively short time frame that wheat was grown within. AMF species may take between 1-58 days to colonize the host plant roots (Hart and Reader 2002b). As AMF establish within the roots of plants it would be expected that they would require a greater carbon resource input to form the various IRM and ERM structures required in the symbiosis that may be re-balanced later in plant development through the benefits of the symbiosis. This is reflected in the model of sink-source relations in the symbiosis posited by (Walder and Van Der Heijden 2015). A greater number of co-colonizing AMF species may therefore act as a greater sink of carbon that would otherwise be directed to plant growth during this period.

Several AMF were identified as indicator species for pasture soils. Indicator species analysis identifies species that have both higher abundances within, and higher specificity to treatment groups. As pasture soils were identified as having the highest values of soil health, these species may therefore be associated with this. Indicator species included three *Claroideoglomus* species (VTX00056 *C. sp.*, VTX00057 *C. sp.*, VTX00193 *C. lamellosum*), one *Paraglomus* species (VTX00335 *P. majewski*) and one *Glomus* species (VTX00155 *G. sp.*). There was also one species found only in pasture and arable-to-ley mesocosms (VTX00151, *G. sp.*). While species belonging to *Claroideoglomus* have been functionally categorized as *rhizophilic* based on their phylogenetic closeness to *Glomus* (Powell *et al.* 2009; Weber *et al.* 2019), their presence has also been found to be mainly associated with the soil exploring ERM and soil spores ((Varela-Cervero *et al.* 2015; 2016b; 2016a). They may therefore have some hyphal benefit to soil structure. Notably absent from the pasture samples was the family Gigasporaceae, which was unexpected given their known devotion to hyphal development. Soil fertility (as measured through LOI SOC, NO_3^--N and NH_4^+-N, see Chapter 3 *Table 3.2*) is considerably lower in the arable and arable-to-ley mesocosms than in the pasture mesocosms. Species within the *Gigasporaceae* family

have very dense hyphal networks that only extend around 2 cm from the plant roots before exponentially declining in density such as was seen by Thonar *et al.* (2011) where hyphal length density of *Gi. margarita* was approximately 5 times higher than *G. intraradices* or *C. claroideum* around the plant roots. Through this dense hyphal network *Gigasporaceae* presence has been associated with significant increases in plant nutrient uptake relative to other AMF clades (Jakobsen, Abbott, and Robson 1992; Hart and Reader 2002b; Maherali and Klironomos 2007; Thonar *et al.* 2011) though this has only been demonstrated for P uptake. As nutrients are more accessible in the pasture mesocosm soils, there may therefore be less benefit to the plants maintaining associations with *Gigasporaceae*.

An important caveat in the interpretation of data within this study is that the experiment is not adequately controlled to pick apart AMF specific contributions from those of other actors within the system. The *SoilBioHedge* project was not designed to explicitly test the mechanistic role of AMF to soil health development so much as it was planned to conduct a holistic top-down assessment of the role of shifting land use practices to restore several soil health properties (including AMF) While this study has demonstrated an association between AMF and soil health, there are several groups and factors other than AMF abundance, diversity and community composition which can be contributing to soil health gains seen in Chapter 3 and 4. For example, earthworms also contribute considerable advantages to soil structure and organic carbon accumulation through their activity including bioturbation of organic matter (Fahey *et al.* 2013), soil aggregate enhancement (Sharma *et al.* 2017), and the creation of macropores as they travel through the soil (Francis and Fraser 1998). This can further be of great benefit to water maintenance under drought scenarios and crop yield resilience (Chen *et al.* 2018). Soil fungi other than AMF also produce dense mycelia which can also contribute to soil aggregation (Lehmann *et al.* 2019; 2020). Further to this, the grass-clover ley period removes the disturbance pressure of tillage from the soil which is hypothesized to allow earthworm and AMF communities to re-develop (Chan 2001; Alguacil *et al.* 2008). Without a no-till continuously cropped treatment strip within the arable fields to complement the grass-clover strips, we again cannot conclusively unpick whether this redevelopment is through reduced disturbance (and therefore driven exclusively shifts in soil biota as they recover) or land cover change (and therefore driven by a combination of soil

biota, cover crop plants, host-symbiont specificity). Bowles *et al.* (2017) examined the effect of short cover crops against winter fallow finding that cover crop (and cover crop identity) increased the colonization of roots in subsequent cash crops. This shows that land cover may play a synergistic role with disturbance alleviation.

Hallam *et al.* (2020) was able to unpick the contribution of earthworms more mechanistically to soil health development in the grass-clover leys used by the *SoilBioHedge* project through the defaunation of mesocosms collected from the field site and subsequent observation of soil health changes over time after the implementation of grass-clover leys with and without earthworms added back. Through this they detailed that soil health improve within one year even without earthworms, though their presence led to a much larger improvement. Soil fauna were omitted through a freezing treatment of -20 °C. AMF are strongly frost resilient, and are able to recolonize plants following temperatures as low as – 130 °C (Kilpeläinen *et al.* 2016). It is therefore likely that through the earthworm exclusion treatment, they captured the combined effect of a reduced frost-tolerant subset of AMF and grass-clover plant cover on soil health development in the absence of earthworms though no measures of AMF abundance through root length colonization or soil hyphal length were taken.

Using the *SoilBioHedge* project, a similar approach could have been used to better explore the specific contribution of AMF diversity and community composition to soil health development and water stress resistance within grass-clover ley rotations. Through this approach, soil would be frozen to remove earthworms and other fauna, and inoculated with AMF spores, hyphae and root fragments sourced from the reference grassland to supplant the reduced AMF community with an increased diversity and abundance of AMF. This could use a factorial design of land cover by AMF inoculum (no-till continuous wheat crop vs grass-clover ley by reduced AMF community vs enhanced AMF community). Following the grass-clover ley rotation period, winter wheat would be planted in the mesocosms matching the continuously cropped mesocosms. This would allow us to explore how AMF communities develop undisturbed under wheat and grass-clover cover, and how the starting abundance, diversity and composition of these communities affects soil health development over time. Functional assays could then be conducted from the final wheat rotation to

assess crop productivity, water, and nutrient retention under contrasting water stress regimes.

4.5. Conclusion

The maintenance of robust soil biotic communities, particularly AMF, has been repeatedly proposed as a necessary part of ensuring sustainable agriculture through their capacity to enhance yields, stabilize soil structure and increase the resilience of hosts to a range of abiotic and biotic stressors including flood and drought ((Bender *et al.* 2016; Thirkell *et al.* 2017; Rillig *et al.* 2019; Zhang *et al.* 2020)). However, very little is still known about the genetic and functional diversity of AMF in agricultural environments. This is particularly true in terms of our understanding of non-nutritional response variables which restricts our ability to optimize communities to balance trade-offs in function due to our lack of underlying mechanistic knowledge (Rillig *et al.* 2016). This study provides evidence that AMF communities can be associated with non-nutritional soil responses through their direct effect on soil health properties and potentially indirect effect on yields and water maintenance. There are however numerous confounding factors that limit our ability to draw any definitive comparisons or suggestions from this study. Future research aimed at evaluating the presence, life history, morphological characteristics, and functional traits of a wide suite of AMF species under contrasting scenarios will greatly progress our knowledge of the context-dependence of functional traits and provide a more solid framework to begin integrating AMF analysis into more complex holistic studies such as the one presented.

4.6. Appendix

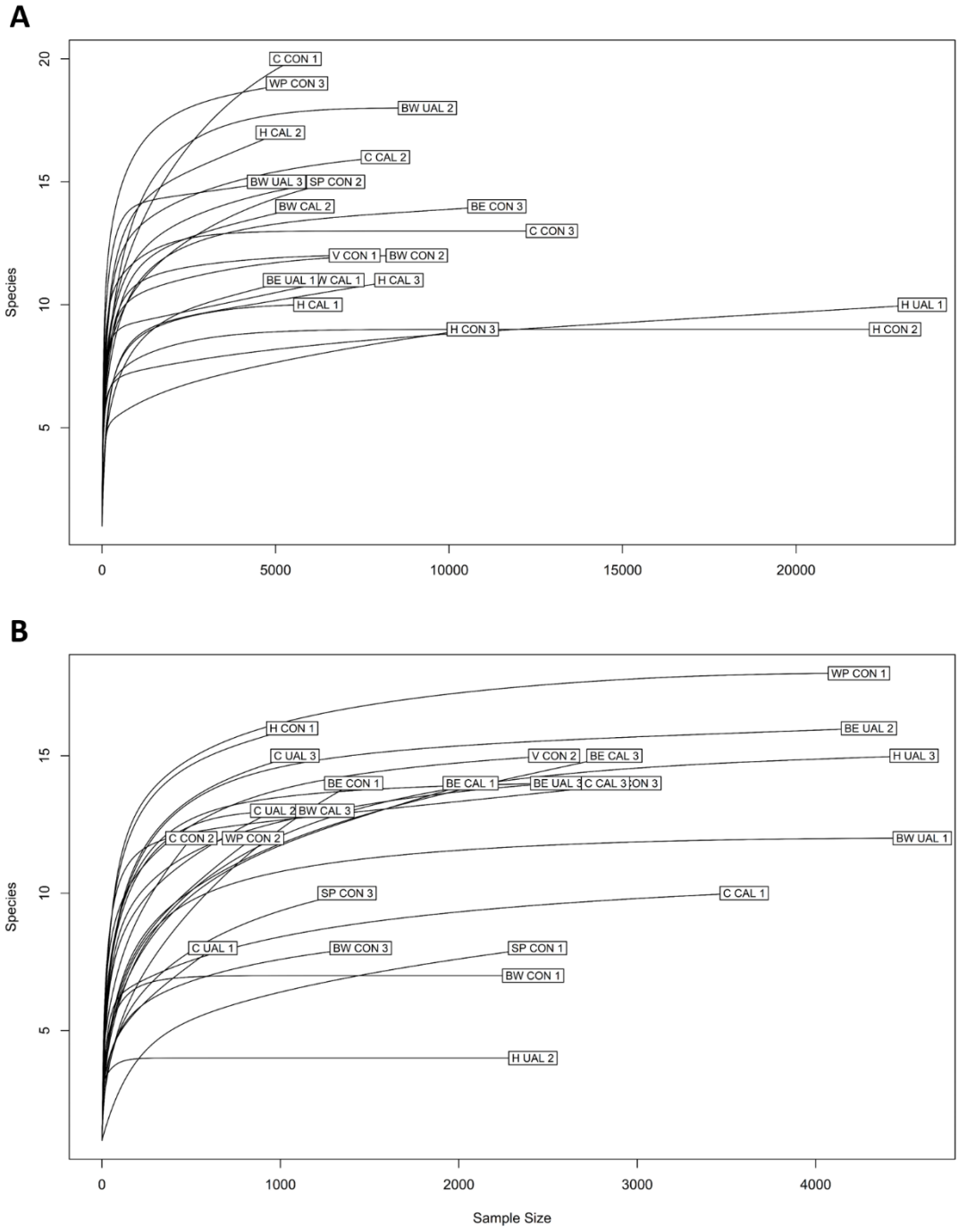

Figure 4.4. Rarefaction curves generated per sample for A) samples with sequencing depths above 5000 and B) samples with sequencing depths below 5000 reads.

5. General Discussion

5.1. Introductory Remarks

In this thesis the central question of "Can soil microbial diversity mitigate water stress and maintain crop yields in agriculture?" was explored. This is a broad topic and so was initially narrowed down to a key component of microbial diversity- AMF. This group contribute to a wide breadth of ecosystem functions (e.g., plant productivity, soil structural maintenance, water relations, (Rillig 2004)) that play an expected role in not just improving crop yields, but moderating water relations that may be vital to water stress mitigation (Augé 2001).

The introduction chapter found that there is a lack of studies investigating the role that AMF community composition can play in conferring both nutritional and non-nutritional benefits to plants and surrounding soils. Past experiments studying the connections between AMF and ecosystem functions have overwhelmingly done so by manipulating the presence or absence of the entire lineage. This allows a broad insight into the potential for the symbiosis to affect various functions. It provides relatively little insight into how individual AMF species or assemblies can contribute to those same functions in real world scenarios where AMF are ubiquitous (Öpik *et al.* 2010; Davison *et al.* 2015) but their abundance and composition within sites are moderated by factors including land use (Schnoor *et al.* 2011; Oehl *et al.* 2017; van der Heyde *et al.* 2017). Further to this, due to the growing need to ensure that agro-ecosystems can be resistant and resilient to climate change related stressors that can impact food security (e.g., Schmidhuber and Tubiello 2007; Gornall *et al.* 2010; Harkness *et al.* 2020; Tilman *et al.* 2011), studies regarding AMF function must begin to consider their capacity to function under not just a range of management scenarios but also water stress scenarios. From this the two central aims of the thesis were conceived to examine AMF communities in close to real-world scenarios that could be used to inform future management decisions based around maximizing AMF communities and soil health.

The specific objectives of this thesis were **(a)** to investigate how agricultural management impacts AMF communities and **(b)** explore the interaction between AMF diversity and community composition with key soil health properties and the functional outputs of plant productivity and water stress mitigation.

Through a combination of experimental approaches at different scales and levels of complexity and across two study systems containing contrasting land uses and agricultural practices, the following main hypothesis was investigated:

1. Agricultural management practices will be a key determinant of AMF communities:
    a. Adopting AMF inoculation of soils will increase the richness and abundance of AMF under minimum tillage agriculture, thus increasing the functional potential of the symbiosis to positively impact host crop performance in-field. (*Chapter 2*).
    b. Higher agricultural land use intensity (e.g., grassland vs arable, low vs high intensity tillage, grass-clover crop rotation vs continuous cropping) will have a greater negative impact on AMF community diversity and composition (i.e., the presence of members with complementary functions). (*Chapter 3, 4*).
2. AMF community properties will be significantly associated with soil health properties (e.g., SOC, bulk density, hydrology SHI) and the functional outputs of crop yield and water maintenance under water stress scenarios (*Chapter 2, 3, 4*).

An overview of the specific chapter aims hypotheses and main findings is detailed in *Table 5.1*.

Table 5.1. Summary of the thesis aims and hypotheses, compared with the main findings of each experimental chapter. Abbreviations: ABG, aboveground. SHI, soil health index.

Chapter	Aims and objectives	Hypotheses and predictions	Main findings
Two	To determine how a commercial multi-species AMF inoculum performs in-field. The main questions were: 1. Does the inoculum increase the functional potential of AMF to the benefit of wheat agronomic properties? 2. Does the inoculum increase the diversity of AMF present or alter composition? 3. Do wheat cultivars a) exhibit different responses to inoculation? b) contain different endophyte AMF communities?	1. Introducing the inoculum would increase the functional potential of the AMF community through introducing novel species or increasing the inoculum potential of the system through bulking the stock of spores / hyphae / colonized roots from which wheat can take place. 2. The inoculum would add novel AMF species, thus increasing community richness. Interactions between the added and native AMF present will result in an altered community state. 3. Wheat cultivars will have different levels of ability to interact with AMF, resulting in differential inoculum responses and AMF community compositions. Cultivars will recruit exhibit different general fungal endophyte communities through expected differences in root architecture, exudate outputs etc.	1. The inoculum addition significantly increased the ABG total biomass and grain yield of inoculated plants. 2. The mechanism-of-action of the inoculum benefit could not be ascertained. No novel AMF species were added to the site, AMF communities remained consistent between control and inoculation, and the relative abundance of AMF in the general fungal community showed significant alteration. 3. Cultivars exhibited negative, neutral, and positive responses to inoculation. 4. Cultivars appear to selective for endophyte fungal communities, but not the AMF subset.

Three	To assess soil health in samples taken from contrasting land uses with known AMF community diversity and composition. 1. Do land use and management practices emphasising reduced disturbance and improved AMF functioning result in gains to soil health? To explore the impact that land use, management and soil health has on water / nutrient leaching and crop yield resilience under water stress scenarios? 2. Does greater soil health benefit *crop performance* under ambient, drought and flood scenarios. 3. Does greater soil health benefit *water maintenance* under ambient and drought scenarios?	1. Arable-to-ley conversion will result in soil health increases by reducing disturbance to soils, replacing wheat roots with more complex and deep rooting clover and grass roots, and allowing key ecosystem engineers such as AMF and earthworms to recover and carry out beneficial ecosystem functions. 2. Conversion of grassland to cereal production through tillage will reduce soil health by disturbing soil structure, replacing complex multi-species root systems with cereal roots, and damaging the soil biota responsible for soil maintenance. This will be more apparent where disturbance is greater in pasture-to-CT conversion than in pasture-to-MT conversion. 3. Increasing soil health (through alterations of soil chemistry, structure, and hydrological functioning) will increase crop yields, and decrease the potential for water / nutrient leaching under ambient and drought scenarios.	1. Soil Health as measured through a unified soil health index (SHI) was significantly different between the two extremes of land use (arable < pasture). 2. The introduction of a short grass-clover ley (approx. 19-months) into arable rotation conferred a significant increase in SHI, crop yields and water maintenance, but not nutrient retention. 3. Tillage of grassland soils had a significantly negative short-term effect on LOI SOC stored by the soil over two tillage cycles, but only slightly negatively impacted other soil health indicators and SHI without significance. 4. Structural equation modelling showed that land use directly influenced SHI., and through this indirectly influenced crop yields and water maintenance.

Four	To resolve the gap in understanding of the role that AMF play in soil health restoration that was not addressed by Chapter 3 of the thesis. Land use was found to impact soil health, water maintenance and crop yields, but the relationship between this and AMF was not able to be considered. The questions presented in this chapter therefore follow on from those of Chapter 3: 1. Do mesocosms from arable, arable-to-ley and pasture contain different AMF communities in follow-up wheat crops? 2. Can the AMF community diversity and composition be empirically linked to observed differences in soil health between land uses observed in Chapter 3?	1. AMF communities will be significantly different between the three land uses. a. Arable and pasture will be the most distinct. Based on in-field assessment arable-to-ley should be intermediate to the two, but still more like arable. 2. AMF community composition differences between arable, arable-to-ley and pasture soils will be significantly associated with the crop yields, water throughflow, and SHI values recorded in Chapter 3. They will also be associated with the ABG wheat biomass recorded in Chapter 4.	1. AMF community composition was found to be distinct between soils from arable and pasture and was intermediate in arable-to-ley. This shows that the community development seen through grass-clover ley rotations can be somewhat maintained in the follow-up wheat crop. 2. AMF community composition- but not diversity- was significantly associated with soil health, but not crop yields and water throughflow. This may be due to an indirect effect on these through soil health and hydrological properties.

5.2. Synthesis of Results from Empirical Chapters

5.2.1. Overview of the Study Systems Used in this Thesis

Within this thesis the control that agricultural management has on AMF community composition and function was assessed using two study systems. Both study systems were used to consider the two main hypotheses that management practice would alter AMF communities, and that through these alterations we would observe functional variation. As the two systems were distinct, in this section we will consider both hypotheses as they relate to the specific system.

1. Inoculation: Existing AMF communities were supplanted with a commercial inoculum in no-till wheat stands containing multiple cultivars.
2. Land use change: Grass-clover ley cover crop was introduced into a conventionally tilled arable field for 19 months. Mesocosms from the arable field, the grass-clover ley and a reference grassland were planted with no-till winter wheat to assess crop yields and water stress resilience following drought and flood.
    a. A reciprocal study was conducted in the reference grassland using contrasting tillage intensities, but AMF were not assessed in these mesocosms.

5.2.2. Did Inoculation Alter AMF Community Composition and Function?

In Chapter 2, the inoculum chosen was a commercially available variety containing five named AMF species, of which four were able to be matched by name to an AMF VT within the MaarjAM database to look for their presence in-field. The field site used for this experiment contained 18 species of fungi overall, which depending on the wheat plant assessed resulted in colonization of wheat roots by 3 to 13 species. Against what was expected from the first hypothesis, no novel species were added by the inoculum that were not already present at the field site, nor did the inoculum addition affect the root-inhabiting AMF community of any wheat cultivars considered. Further to this, inoculation did not increase the relative abundance of AMF within the wider root endophyte community.

There is a wealth of research documenting the positive effect of AMF inoculum on plant root colonization and yields under field conditions where an already established population of AMF would be present (See the meta-analyses of Y. Lekberg and Koide 2005; Pellegrino *et al.* 2015; S. Zhang *et al.* 2019) though these studies do not consider the original resident or resulting AMF populations following inoculation. The positive impact of AMF inoculation to yields was further corroborated by this study as inoculation did confer a functional benefit in terms of ABG wheat biomass and grain yields observed, though colonization was not measured.

The few other studies that have considered the impact of inoculation on resident AMF communities found variable results (Antunes *et al.* 2009; Elliott *et al.* 2020). (Elliott *et al.* 2020) observe an increase in root length colonization in plants grown in inoculated soils and shifts in community composition. This did not increase the ABG biomass of inoculated plants or mycorrhizal P acquisition and had variable effects on mycorrhizal N acquisition. (Antunes *et al.* 2009) alternately observed no change in colonization of roots, community composition or P acquisition when comparing the resident community to the resident community + inoculum addition. The findings in Chapter 2 present a conundrum, wherein we observed an AMF-mediated functional change that appears to operate independently of the observed community composition and relative abundance of AMF. This both raises a limitation of the work presented in the study and creates questions that may be explored to further our understanding of this disconnect. Chapter 2 did not quantify the abundance of AMF in the system and only qualitatively assessed this using compositional relative abundance data. Further quantification of the functional structures of AMF through root length colonization, soil hyphal and spore mass, qPCR (König *et al.* 2010) or NLFA (M. P. Sharma and Buyer 2015) could have better revealed what underpinned this apparent benefit beyond community composition in this instance.

While this outcome goes against our overarching hypothesis that variation in AMF communities will be related to variation in the expressed functional phenotype of the community, it raises more questions as to *why* we did not see this relationship in this study:

(a) *Were novel isolates of the AMF already present at the site introduced, thus increasing the intra-specific diversity present?*

AMF have been shown to have as wide intra-specific genetic diversity (Koch *et al.* 2006a; Börstler *et al.* 2008; 2010), and variation in their investment of hyphal development in to root length colonization or soil exploring ERM (Munkvold *et al.* 2004). Intra-specific variation in these traits has further been associated with variation in functioning through P uptake and host biomass accumulation (Munkvold *et al.* 2004; Koch *et al.* 2006b; Koch *et al.* 2017). It is possible that while no novel species *sensu stricto* were added to the inoculated plots, novel genetic variants of prior established could have been added that were complementary to the existing community. For this to be accounted for in future studies, the community of the inoculum added needs to be molecularly identified so that it can be compared against the in-field changes. It has been shown that using higher phylogenetic resolution (i.e., clustering DNA sequences at similarities above 97 % OTUs) can reveal divergent ecological drivers structuring AMF communities (Roy *et al.* 2019) which may also be useful to picking out intra-specific taxonomic resolution in future studies.

(b) *Does the inoculum effect extend beyond the root environment? How important are hyphal communities to nutrient acquisition? Can inoculum benefits be explained by interactions with other organisms?*

The disparity observed between wheat inoculum response and AMF communities may be due to the limited scope of analysis in the chapter since only the root-colonizing AMF were assessed. AMF communities within the same site can be drastically different in the root compartment and immediate rhizosphere of plants from that of the surrounding bulk soil (Zhang *et al.* 2018). This is likely informed by the capacity of different AMF species to form IRM and ERM structures (Hart and Reader 2002b) resulting in different consortia of AMF found within the IRM and ERM (Varela-Cervero *et al.* 2015). Analyzing the root compartment may therefore

only represent a limited subset of the total AMF community. The inoculum in this experiment was applied as a granular suspension of colonized root fragments and spores indirectly between wheat rows in the first year of the trail and mixed across plots through minimal tillage of the upper 10 cm of soil prior to sowing wheat in the second year of the experiment. Hyphae producing fungi may still be conferring a benefit to nutrient uptake without directly interacting with or colonizing the roots of the wheat plants. The mechanism of this may be through the scattered inoculum propagules acting as nodes to fortify and extend the mycorrhizal hyphal reach in soils through interactions with hyphae emanating from the direct connection with the plant host. AMF hyphae are coenocytic which means that they are one long cell not divided in to compartments- and their spores are multi-nucleate (Helgason and Fitter, 2005, and references therein). The fusion of hyphae from different AMF 'individuals' often occurs leading to the formation of large common mycelial networks which facilitate nutrient transfer (Mikkelsen *et al.* 2008; Bücking *et al.* 2016). Hyphal exploration of the soil may therefore stimulate the activation of spores and hyphae from the inoculum as they come in to contact and fuse, in the process facilitating the transfer of genetic resources and nutrients through these structures.

Further to the role of soil exploring AMF in facilitating plant biomass growth there is also a potential interaction between other soil and plant dwelling organisms and AMF that could be responsible for the observed benefit of inoculation. AMF have been shown to have complementary interactions with other organisms that can benefit nutrient uptake, biomass growth and pathogen suppression in the host plants. Examples include co-inoculation with plant growth promoting rhizobacteria such as *Pseudomonas* (Pérez-De-Luque *et al*. 2017) and the fungal lineage *Darksidea* (dark septate endophytes, He *et al.* 2020). AMF have been further shown to initiate a priming of plant defences in host plants through which they can be more resistant to both fungal and bacterial pathogens (Pozo and Azcón-Aguilar 2007; Jung *et al.* 2012). The previous examples show that the effects of AMF observed at the host-plant level are likely to be mediated by their interaction with other fungi and bacteria present within both the plant and soil environment. This may also explain some of the variation seen in inoculum response between cultivars in Chapter 2 beyond their ability to form AMF

associations. The cultivars were observed to have distinct fungal communities, particularly between Robigus and the pair of cultivars Mercato and Holdfast. The three cultivars exhibited generally negative, neutral-to-positive, and positive responses to inoculation respectively which could have been informed by the distinct fungal consortia that they recruit.

The unknown contribution of soil exploring AMF and other organisms to the success of an inoculum could be resolved to some degree by considering soil hyphal density and microbiomes associated with hyphae separately to the root-colonizing AMF, and through considering non-AMF organisms such as plant endophytic and soil bacteria and fungi.

5.2.3. Did the Conversion of Arable Land to Grass-Clover Ley Alter AMF Community Composition and Function?

Chapter 3 and 4 function as one joint study over two experiments. In Chapter 3, through the assessment of soil health properties following no-till winter wheat planting the grass-clover ley conversion was shown to significantly improve soil health to an intermediate state between the continuously cropped arable land and the reference grassland. This was driven primarily by improvements to soil hydrology, which did not occur in tandem with SOC gains and therefore were likely driven by alterations of structure through physical entanglement by grass and clover roots, and potentially regenerating AMF hyphae (Tisdall and Oades 1982; Tisdall 1994; Rillig and Mummey 2006). Through structural equation modelling land use was found to directly affect soil health, indirectly affect wheat yields through this, and indirectly affect water through flow under ambient and drought scenarios. Water throughflow was not statistically assessed for the flood scenario as explained in chapter 3 but the same relationship was maintained between land use, soil health, and crop yields. In chapter 4 we consider the AMF communities that were present within the mesocosms. In confirmation of the first hypothesis of the thesis the grass-clover ley resulted in AMF communities intermediate to those observed in arable and pasture. As grass-clover leys are relatively understudied there was only one other study to compare these results to, which found that grassland, grass-clover ley and arable fields had distinct communities, though communities were assessed when soil were still under the given plant cover rather than in the follow-up crop like in Chapter 4 (Manoharan *et al.* 2017). Comparing

these results to the in-field assessment of AMF communities within the *SoilBioHedge* where the same distinction as (Manoharan *et al.* 2017) was seen demonstrated the importance of *current* crop cover, but also that shifts in AMF community composition could be somewhat maintained in the follow-up crop to the potential benefit of the host plant. AMF communities were compared to the soil health properties, crop yields and water throughflow values from Chapter 3, from which it was found that AMF community variation could be linked to variation in soil health, but not crop yields and water throughflow. The caveat with this analysis is that the experiment did not explicitly control for other confounding factors that could also contribute to soil health gains such as earthworms (Hallam *et al.* 2020), and so cannot conclusively unpick the extent to which AMF may contribute to soil health increases under grass-clover leys. This limitation was addressed in the chapter and an alternative experimental design was proposed.

5.3. General Conclusion

5.3.1. Field tests need to be underpinned by mechanisms

With reflection on the completed thesis against the original aims and the central question, the experimental shift in Chapter 3 and 4 took the research of the thesis in a direction that greatly complicated the ability to answering the original question and aims by introducing a more complicated system with many confounding factors that reduce the ability to draw firm conclusions about the importance of AMF within the grass-clover ley conversion presented. The results of Chapter 2 raised a set of interesting questions which if followed through could have more greatly increased our mechanistic understanding of AMF functioning under variable conditions. Greater efforts therefore could have been made to maintain the methodological through line of using AMF inoculum through a series of more controlled and directed experimental manipulations of AMF and water conditions which could increase our mechanistic understanding of the AMF symbiosis and particular members / consortia under variable scenarios.

The experiments featured in this study looked at whole community level functional phenotypes. This gave us an insight into the relationship between microbial diversity (i.e., AMF diversity and composition), wheat yields and water maintenance, but could not identify specifically the members of the community that could be important to these processes. This is in part due to the lack of community response seen in Chapter 2, and the confounding factors overshadowing the results of Chapter 4. This unfortunately may be difficult to resolve in any large-scale holistic experiments such as those carried out in this study (Ray *et al.* 2020). Therefore, the work conducted in this study may have 'jumped a step' in the approach to community level functioning by focussing on larger concepts rather than tackling the key mechanistic questions that can then be upscaled. For instance, we still have very little knowledge of the traits exhibited by AM fungal species in regard to their biomass, life history etc. outside of the studies that have detailed this with a relatively limited subset of species (e.g., Hart and Reader 2002b; Klironomos and Hart 2002; Hart and Reader 2005; Maherali and Klironomos 2007), which impedes our ability to truly assess AM fungal communities through their functional traits (Chagnon *et al.* 2013). Assessing single species and simple consortia may therefore have been a more opportune starting

point to the thesis research, from which the trait-based assessments could have been further built upon in larger scale field experiments. It is recommended based on this that future studies employ a reductionist approach to assessing AMF function under variable situations using a combination of targeted mechanistic experiments and larger holistic experiments. These experiments would assess individual AMF functions under different environmental contexts that will provide a trait-based framework of individual function to be complimented by larger scale community experiments that can expand upon the mechanistic knowledge gathered to begin to predict community-level function.

5.3.2. Contributions of the thesis to knowledge gaps

Overall, the three experimental chapters presented in this thesis begin to fill the knowledge gap identified in the introduction by considering AMF communities against functional phenotypes rather than focussing on presence/absence studies. They further increase our knowledge of the link between management practices, soil health and crop yields that can be used to inform management choice in real-world agricultural situations. A particularly important outcome of this is the demonstration that short-grass clover leys can allow for the regeneration of both soil health and AMF communities towards a closer state to those seen in 'healthier' reference grasslands, and through this can improve yields in the subsequent crop and water maintenance under variable precipitation patterns. This finding is of great importance as grass-clover ley rotations are a understudied management practice that can be of great importance to sustainable agriculture (Chapman *et al.* 2018). As previously discussed, this finding does not necessarily further elucidate the role that AMF community diversity and composition can play in contributing to improved yield and water stress, which must be addressed in more controlled experiments. Despite the apparent null result, the findings of Chapter 2 also provide possibly the first in-field assessment of the impact of AMF inoculum (or lack thereof) on AMF communities and lead to further questions that will be able to expand our understanding of the functional variation within the AMF symbiosis.

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