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Soil invertebrate and mycorrhizal activity in diverse grasslands: benefits for ecosystem service provision

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School of Agriculture, Policy and Development

Sarah Shepperd

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Declaration of Original Authorship

I declare that this research is my own original work and all citations from other sources have been properly and fully acknowledged

Sarah Shepperd

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Thesis Abstract

Sustainable development goals and net-zero target policies necessitate the progression of livestock production from ryegrass monocultures to grassland diversity. Regenerative agricultural farming techniques could meet nitrogen requirements for grassland systems and enhance their resilience to climate change. Little is known, however, about how plant diversity in farming systems affects the soil and its biota in the short term. This work addresses the knowledge gap by researching the impact of commercially viable diverse forage mixtures on soils and soil biota, all measured within one system.

A conventionally fertilised ryegrass monoculture was compared to three unfertilised diverse grasslands differing in plant diversity. Multiple key differences in soil biota were observed as a result of enhancing plant diversity: higher earthworm densities, higher arbuscular mycorrhizal fungi (AMF) root colonisation and higher fungal diversity. A model predicting aboveground biomass production showed that AMF colonisation was the only factor explaining the increase in aboveground biomass at low soil water availability. Additionally, AMF community composition was similar under all diverse mixtures suggesting benefits achieved by AMF presence are equal under a 6 and 17-species grassland.

Plant mixtures positively affecting soil microbial community due to their diversity and interspecific competition, especially in environments where future droughts are more likely, will be better at maintaining their productivity as a result of AMF symbiosis increasing the root surface area. Further, the diverse forages investigated in this research reduce the need for external inputs, an outcome with clear and positive implications for farm profitability and environmental impact whilst improving soil health by enhancing abundance and diversity of ecosystem engineers.

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1.1. Introduction

UK land use is dominated by grassland systems, with permanent, temporary and rough grassland covering 51% of the UK (Norton *et al.*, 2019). Grasslands' primary purpose is to feed ruminants and deliver many additional ecosystem services (ESs). Agricultural intensification, aided by the availability of synthetic fertiliser, has reduced grassland diversity due to the dominance of ryegrass (*Lolium* spp.), considered the most profitable species in these systems (Hopkins and Wilkins, 2006). Currently, ryegrass monocultures require intensive interventions to maintain productivity, which often involve an unsustainable investment of energy and fertilisers, causing environmental damage (Tilman, Fargione, *et al.*, 2001; Crews and Peoples, 2004; Foley *et al*., 2005; Schindler, 2006; Cordell, Drangert and White, 2009; Elser, 2012). Environmental issues resulting from reduced grassland species richness include the reduction of many ecosystem functions such as nutrient cycling, flood prevention and climate regulation (Fillery, 2001; Cong *et al.*, 2014; Wagg *et al.*, 2014).

Diverse forage mixtures contain species with various properties, potentially beneficial for both the above and belowground components of grassland systems (Spehn *et al.*, 2000; Brockwell *et al.*, 2005; Sanderson *et al.*, 2005; Skinner, 2008; Steinbeiss *et al.*, 2008; Hammond *et al.*, 2014; Van Groenigen *et al.*, 2014; Wagg *et al.*, 2014; Skinner and Dell, 2016). This literature review covers the current understanding of diverse forage mixture effects on important soil chemistry and soil biota, and identifies knowledge gaps for research areas. The review also considers the natural capital profitability of diverse mixtures, as this parameter is

the ultimate requirement to encourage a wider implementation of diverse forages in productive systems.

1.1.1. Sustainable intensification

Predictions by the United Nations (UN) suggest a global population of 9.7 billion by 2050 (DESA, 2022); this will require an increase in food production by 70% (Food and Agricultural Organization (FAO), 2009). Increased food production will continue to exert environmental and land resource pressure, facing competition from other land use needs (Lüscher *et al.*, 2014). The introduction of synthetic nitrogen fertiliser through the invention of the Haber Bosch process in the early 20th Century led to agronomic benefits such as increased forage production, enabling a reduction of grassland diversity due to ryegrass dominance (Hopkins and Wilkins, 2006). Current ryegrass monocultures require intensive management to maintain peak productivity, such as the recommended 50-100 kg N/ha per cut (Agriculture and Horticulture Development Board, 2022), which is unsustainable (Crews and Peoples, 2004).

Innovation in sustainable agricultural production systems is essential to produce food from existing land without reducing productivity; sustainable intensification (Royal Society of London, 2009; Godfray *et al.*, 2010). There is an ongoing discussion about sustainable intensification being an oxymoron term or paradigm shift (UK Parliament, 2022; Mahon *et al.*, 2017). Either way, with the predicted rise in meat and dairy consumption, forage production requires intensification with a reduction in environmental damage (Crist, Mora and Engelman, 2017).

With the increasing human population and related demand for food, along with less land area available for agriculture, producers must also face climate change factors of drier, warmer summers, plus increased extreme weather events of droughts and flooding (Lowe *et al.*, 2019). Organisations and governments are aware of the threats to food production at the global and national scales. Recent extreme UK weather events include the summer drought of 2018 and the Yorkshire summer floods of 2019. These events resulted in the affected farming community relying on charitable forage donations from less hard-hit areas to ensure livestock had sufficient feed due to lack of pasture production within their land, alongside the government setting up an emergency farming recovery fund (Rural Payments Agency, 2020). Producing livestock on diverse grasslands could be an answer to future proofing forage production as increases in grassland plant diversity is shown to extend the grazing season (Tilman, Reich, *et al.*, 2001; Hammond *et al.*, 2014), increase aboveground biomass even in dry years (Tilman and Downing, 1994; Tilman, Wedin and J. M. H. Knops, 1996; Hector *et al.*, 1999; Tilman, Fargione, *et al.*, 2001; Hooper *et al.*, 2005; Sanderson *et al.*, 2005) and increases ecosystem stability (Tilman, Reich and Knops, 2006; Bardgett and Caruso, 2020).

The diversity-stability hypothesis states that ecosystems with higher diversity are more stable (McNaughton, 1977; Harrison, 1979; Pimm, 1984; Doak *et al.*, 1998; McCann, 2000; Tilman, Reich and Knops, 2006; Griffin *et al.*, 2009; Mougi and Kondoh, 2012). Diversity in this context can be split into five different types: ecosystem diversity, species diversity, genetic diversity, phenotypic diversity, and functional diversity (Jensen, Torn and Harte, 1990). Early support for the hypothesis involves species having different optimum physiological ranges along with ecosystem resources fluctuating wildly in nature both spatially and temporally. If species overlap in their physiological ranges, the community can remain constant as declines in one species is compensated by an increase in another species (McNaughton, 1977).

McNaughton (1977) showed that this is true at the primary producer level, where plant community diversity stabilised ecosystems functional properties, concluding co-occurring species compensate for environmental fluctuations maintaining ecosystem stability due to species diversity (McNaughton, 1977). A system is assumed more stable with increased population density (McCann, 2000).

Stability also has a variety of meanings in ecology (Tilman, Reich and Knops, 2006) and broadly can be classed into two groups: dynamic and resistance/resilience stability (McCann, 2000; Oliver *et al*., 2015). Dynamic stability is an ecosystem remaining at or within the equilibrium range after perturbation, i.e. a stable ecosystem has little to no variability in response to perturbation. Resilience stability is when a system returns to pre perturbation levels of ecosystem functioning, or indeed a new steady state equilibrium. The less time the system takes to return to a stable state, the more stable the system is (Oliver *et al*., 2015). The degree to which an ecosystem function has changed after a perturbation, with a more stable system showing a lower degree of change, defines resistance stability (McCann, 2000).

Harrison (1979) summarised that community diversity increased ecosystem resistance, but no significant increase was seen in ecosystem resilience. However, Mays' (1973) mathematical reasoning against diversity-stability showed diversity destabilises the dynamics of a community, i.e. the resilience of the ecosystem. May (1973) concluded that diversity is not the driver but that ecosystem stability arises from the ability of communities to contain specific species which contribute towards stability and which have the capacity of differential response to perturbations (McCann, 2000), the insurance effect.

Early studies indicated more complex systems are more stable (McNaughton, 1977; Pimm, 1984). Later studies tended to show the opposite, where more complex systems produce less community stability (Cottingham, Brown and Lennon, 2001). More recently, Mougi and Kondoh's (2012) theoretical model demonstrated a positive complexity-stability relationship, confirming that multi-species coexistence can be maintained. One study in Pimms' (1984) review article found plants in a species-poor system recovered quicker from drought than those in a species-rich field (Lepš, Osbornová-Kosinová and Rejmánek, 1982). Concluding total biomass or community density may correlate more closely with ecosystem functioning than the abundance of species. This again points towards May's (1973) justification against diversity-stability in that stability comes from specific species, not necessarily the diversity of species.

Empirical studies on soil and grassland ecosystems include Laakso and Setälä (1999) soil fauna manipulation research into composition effect on ecosystem functioning measured using plant growth and nutrient mineralisation. Soil fauna species composition manipulated across trophic levels was far more critical regarding nutrient mineralisation and plant growth than species composition manipulated within trophic levels (Laakso and Setälä, 1999). However, manipulation of soil faunal species richness was a poor predictor of soil respiration and rates of litter turnover (Heemsbergen *et al.*, 2004).

Implications of diversity-stability research are essential for practical application as a reliable and sustainable supply of livestock forage can be improved using biodiversity (Tilman, Reich and Knops, 2006). Tilman, Reich and Knops (2006) long-term grassland experiment concluded that the temporal stability of aboveground primary production resulted from a

greater number of plant species and was positively correlated with root biomass. Tilman, Reich and Knops' (2006) research showed that plots with the highest diversity are 70% more stable than monocultures, showing the insurance value of diversity in primary production. A lower proportional biomass change in plots with greater biodiversity was seen, meaning greater ecosystem stability (Tilman, Reich and Knops, 2006). Many empirical studies have concluded increasing diversity increases stability via temporal variation (McNaughton, 1977; Tilman, Wedin and J. Knops, 1996), measuring ecosystem stability by overall community biomass (Tilman, Reich and Knops, 2006). In more species-rich communities, there are higher rates of species abundancy fluctuations, which over time allows for more stable community biomass (Cottingham, Brown and Lennon, 2001). Thebault and Loreau (2003) research, however, indicated that plant diversity does not always lead to an increase in plant biomass. Statistical averaging helps explain this, as diverse species plots have increased temporal variability in growth rates and will therefore have reduced fluctuation of primary production (Doak *et al*., 1998). Therefore, if the measurement of stability is the sum of community biomass through time, then of course species differing in their growth regimes will average out the biomass, alluding to the assumption that diversity means stability may not be the case (Doak *et al*., 1998). Both Griffin *et al*. (2009) and Petchey, Hector and Gaston (2004) suggest the best way to measure the functional diversity of a community is continuous, as this measurement better explains primary productivity than species richness. Variations in ecosystem processes can be explained by functional diversity, however, functional diversity does not explicitly describe any one ecosystem mechanism (Griffin *et al*., 2009). The diversity of the above and belowground grassland systems is an important measure for predicting ecosystem service stability.

In context, the UN has set Sustainable Development Goals (SDG), one of which aims to end hunger and promote sustainable and stable food production systems by 2030 (UN, 2019). SDG targets include food systems that maintain ecosystems, are adaptive to climate change, including extreme weather events, and forage production that improves soil quality. At the national scale, the UK agricultural policy aims to have sustainable agricultural systems which combine improved productivity with environmental enhancement (Department for Environment Food & Rural Affairs (DEFRA), 2018). The top agricultural practices with the maximum potential to deliver UK farm-scale sustainable intensification were designated animal husbandry and soil management (Dicks *et al.*, 2019). Therefore, research and improvements in forage systems are well placed globally and nationally to produce food sustainably with benefits to above and belowground ESs. Farming systems increased resilience to climate change can be achieved through the benefits of regenerative agricultural farming techniques (Tilman, Reich and Knops, 2006; Bardgett and Caruso, 2020).

1.1.2. Regenerative agriculture and Ecosystem services (ESs)

Regenerative agriculture is defined as using conservation techniques to improve topsoil by restoring soil health and increasing biodiversity, leading to enhanced delivery of ESs such as carbon sequestration (Schreefel *et al.*, 2020). Soil health is defined as the *ability of the soil to maintain ecosystem stability while delivering multiple functional traits* (Pawlett, Hannam and Knox, 2021). Biodiversity is the variability of living organisms from all sources, including diversity within species, between species and *ecosystems* (Lawrence, 2008). Grassland systems provide the majority of feed for ruminants and, along with the soil beneath them, offer many ESs (Hopkins and Wilkins, 2006; Milne *et al.*, 2015). ESs are defined as the direct and indirect benefits of goods and services humans obtain from the natural environment (Millennium

Ecosystem Assessment (MA), 2005). The MA (2005) has classified ESs into four functional categories: provisioning, regulating, cultural and supporting services.

Among the many services they provide, grassland and soil ecosystems are involved in an important supporting service of nutrient cycling; endorsing the continued flow of the chemical between biotic and abiotic states (MA, 2005). Nutrients are converted via various biological, physical and chemical processes allowing transformation into different compounds through cycling both above and belowground. Nutrients, particularly nitrogen for crop growth, need to be available at the right time and in correct quantities, as their excess can lead to eutrophication - the leaching of nutrients into waterways stimulating dense growth of algae which in turn depletes oxygen in the water body (Schindler, 2006; Elser, 2012). Due to issues of nutrient excess, governments have drawn stringent fertiliser application guidelines to reduce environmental damage (DEFRA & EA, 2017).

Efficient nutrient cycling requires a diversity of organisms at all trophic levels, known as functional biodiversity (MA, 2005). Well-functioning soil with suitable trophic diversity modifies plant nutrient availability (Milne *et al.*, 2015). Multiple trophic groups containing high species richness were shown to have stronger positive effects on ecosystem services, particularly regulating services of soil carbon sequestration, than individual trophic group species richness, showing the importance of species richness across the whole ecosystem (Soliveres *et al.*, 2016). The timing of water supply and plant nutrient availability is crucial for productive pasture growth (Lebauer and Treseder, 2008; Lowe *et al.*, 2019). Loss of biodiversity in grassland systems has resulted in nutrients passing faster through the cycle as leachates (Cong *et al.*, 2014; Wagg *et al.*, 2014), negatively affecting environments

(Schlesinger and Andrews, 2000; Schindler, 2006; Elser, 2012). Wagg *et al.'s* (2014) novel experiment showed that reducing soil organism diversity reduces ecosystem functions such as nutrient cycling. Soliveres *et al.*, (2016) showed that species abundance or biomass of multiple trophic groups positively affects supporting ecosystem services such as mycorrhizal colonisation. Research has also shown that plant species diversity is more influential in soil nutrient accumulation than plant functional diversity (Steinbeiss *et al.*, 2008), an important finding for forage mixture species selection in reaching net zero targets (NFU, 2019).

Aside from the obvious forage production provisioning ES grasslands supply, a further and often overlooked regulating ES provided by soils is climate regulation through carbon sequestration. Through the process of photosynthesis, plants capture atmospheric carbon dioxide. Excess carbon not converted into plant tissue enters the soil through root exudates, and via various processes, carbon is stored in the form of soil organic matter (OM). Soils can retain OM for months to decades and constitute the third-largest global carbon pool (Archer, 2010). Managing agricultural soils for higher carbon sequestration is currently of significant political interest (Department for Environment Food & Rural Affairs (DEFRA), 2018; UN, 1998, 2015a).

1.2. Forage Species

Experimental results show grassland productivity increases with increasing plant species diversity (Tilman, Wedin and J. Knops, 1996; Hector *et al.*, 1999; Tilman, Reich, *et al.*, 2001). Other benefits include reducing nitrogen leaching in more biodiverse grasslands due to the utilisation of soil mineral nitrogen from increased species richness (Tilman, Wedin and J. Knops, 1996).

Initially, diverse grasslands often become dominated by a few species within a few years. Typical dominant species include tall fescue (*Festuca arundinacea*), cocksfoot (*Dactylis glomerata*) and white clover (*Trifolim repens*) (Sanderson *et al.*, 2005; Deak *et al.*, 2007; Skinner, 2008). Chicory (*Cichorium intybus*), an extremely beneficial forb in forage mixtures due to its high protein content, anthelmintic properties and deep root system, decreases substantially in mixture studies over a short time frame (Sanderson *et al.*, 2005; Skinner, 2008; Skinner and Dell, 2016). Sanderson *et al.* (2005) also showed declines in red clover (*Trifolium pratense*) after two years, so much so that benefits from including these species, such as yield maintenance during dry years, are lost.

To maintain species mixture composition, less dominant forb and legume species must be re-established due to being outcompeted by dominant grass species found in forage mixtures (Sanderson *et al.*, 2005). With an emphasis on agricultural sustainability and farmer participation, forage mixtures must be designed where beneficial species are not out-competed and pasture reseeding is not required. Declines in species composition within experiments described above may be due to the legacy effect (Van der Putten *et al.*, 2013), where plant species cause changes to soil conditions, such as nutrient availability. This historical contingency may have resulted in too high a concentration of soil nutrients at the start of experiments allowing dominant forage species to outcompete less dominant pasture species easily.

With drier, warmer summers (Lowe *et al.*, 2019), deep rooting systems will be favoured during drought stress events; by enabling plants to reach water held deeper in the soil, allowing

continued forage production, and delivering sustainability within agricultural systems. Such deep-rooting forage species include sainfoin (*Onobrychis viciifolia*), lucerne (*Medicago sativa*), yarrow (*Achillea millefolium*) and sheeps parsley (*Petroselenium crispum*). Beneficial not just for their deep roots, sainfoin and lucerne are legumes able to fix nitrogen. Sainfoin has also been shown to possess anthelmintic properties (Hayot Carbonero *et al*., 2011). Studies have shown that low-diversity mixtures of just two species have a reduced root depth compared to more diverse mixtures (Skinner and Dell, 2016). Plant phenological differences mean that low-diversity mixtures produce less forage biomass during dry years than more diverse mixtures (Sanderson *et al.*, 2005). Differences in growth habits in forage mixtures extend the grazing season and show synergistic yield responses resulting in better yield maintenance compared to grass monocultures (Tilman, Reich, *et al.*, 2001; Hammond *et al.*, 2014).

Implementing diverse forages for pasture production results from the interaction of forage mixtures to both aboveground top-down feedback into the soil ecosystem and belowground bottom-up feedback into plant biomass growth (Clarholm, 1985). All soil systems are complex. Soils under a diversity of grassland plant species will affect soils composition and functioning differently to soils under monocultures, mainly due the complexity of plant physiology, natural history, root exudation, or the timing of growth (Tilman, Wedin and J. Knops, 1996; Sanderson *et al.*, 2005; Skinner and Dell, 2016). Researching soil components affected by top-down feedback under diverse mixtures compared to monoculture is key in terms of ecosystem service provisions.

For a succinct overview of biodiverse grasslands' impact on soil, key biota and chemical soil components have been chosen for review due to their importance in ESs and climate change mitigation; these are described in the following sections below.

1.3. Earthworms

Earthworms are important ecosystem engineers and a keystone species symbolic of soil health (Lavelle, 2004). Soil health is defined as the ability of the soil to maintain ecosystem stability while delivering multiple functional traits (Pawlett, Hannam and Knox, 2021). Earthworms are important soil fauna to research in terms of diverse forage mixture application in pasture production systems. Their diversity influences plant diversity, and plant species diversity influences earthworm diversity (Eisenhauer and Scheu, 2008; Eisenhauer *et al*, 2009).

Earthworms alter the belowground structure and nutrient availability by ingesting and engesting soil and moving and mixing OM between soil horizons. This enables organically bound nutrients to become available, feeding the soil food web, impacting organisms at all trophic levels. Through the egestion of soil aggregates, nitrogen previously organically bound in soil residues are mineralised, promoting aboveground production (Van Groenigen *et al.*, 2014). As a result, earthworms can increase crop yield and aboveground biomass by 25 and 23%, respectively (Van Groenigen *et al.*, 2014). The increased release of nitrogen in the presence of earthworms was shown to favour the expansion of fast-growing competitive grasses over slower-growing forb and legume grassland species, thus reducing the plant diversity of grasslands (Scheu, 2003). Alphei, Bonkowski and Scheu (1996) research showed the presence of the endogeic earthworm species *Aporrectodea caliginosa* increased the amount of extractable soil mineral nitrogen, increasing the concentration of nitrogen in shoots and roots

of the host plant. However, the presence of earthworms reduced shoot biomass of the grass host plant *Hordelymus europaeus* with shoot biomass unaffected by earthworm presence, with the researchers putting this conflicting finding down to the grass investing less in root development with earthworm presence.

Reducing plant species richness decreases earthworm density and biomass (Spehn *et al.,* 2000). Low plant species diversity decreases OM input, which Spehn *et al.* (2000) suggested is the reason for the decreased earthworm abundance in these systems. Low earthworm abundance in low-diversity pastures alters nutrient availability, which alters microbial activity, microbial abundance and the microbial structure of soil systems, resulting in microbial biomass also decreasing with plant species loss (Spehn *et al.*, 2000). Lower nutrient availability for plant growth from reduced earthworm and microbial activity thus reduces plant growth in a feedback mechanism. Spehn *et al.* (2000) showed that soil mesofaunal activity, measured using bait-lamina tests, was not reduced with general plant species loss as much as microbial and earthworm biomass. Instead, mesofaunal activity only decreased when specific plant species functional groups of legumes were removed from the systems. Forage species mixture choice, for example, including or excluding legumes can affect all soil faunal activity. Van Groenigen *et al.* (2014) showed that the beneficial effects on pasture production achieved with earthworm presence are not seen when legumes are grown in the forage mixture. The ability of ecosystem engineers to help elevate climate change, along with their reaction to pasture management, such as fertiliser application and subsequent response to pasture production, are critical areas to research for functional pasture soils.

The burrowing activities of earthworms alter soil structure and bulk density by creating stable macro-pore spaces for gas exchange and water filtration (Bastardie *et al.*, 2003; Lavelle, 2004). Earthworm burrows are beneficial in flooding scenarios to encourage water drainage away from the soil surface. Other future climate change mitigation benefits earthworms provide through soil modification is the sequestration of carbon into stable macro-aggregates, resulting in the maintenance of higher soil carbon content (Bastardie *et al.*, 2003; Lubbers *et al.*, 2013; Sánchez-de León *et al.*, 2014). However, earthworm presence can also increase soil carbon dioxide emissions through aerobic respiration by 37% (Lubbers *et al.*, 2013).

Due to ecological differences amongst earthworm functional groups (Bottinelli *et al.*, 2020; Table 1.1), each group can respond differently to external perturbations. Endogeic earthworms, for example (Table 1.1), show a positive effect on plant growth in experiments where soil moisture is maintained at field capacity throughout the duration of the experiment, but in water-deficient environments, contribute negatively to plant growth (Blouin, Lavelle and Laffray, 2007). Nitrogen application in pasture production or the inclusion of legume species instead of fertiliser application can positively and negatively affect the earthworm population structure. Edwards and Lofty (1982) showed that organic and inorganic nitrogen fertilisation increases earthworm populations. However, response to fertiliser application within earthworm populations differ, both in ecological grouping and species. Endogeic earthworms (Table 1.1) increased in inorganically treated plots fertiliser with urea, superphosphate and potash, whereas no response is seen in the other two earthworm ecological groups (Murchie *et al.*, 2015). Although a response is seen in earthworm abundance amongst earthworm ecological groups, Murchie *et al* (2015) showed that inorganic fertiliser had no overall effect on earthworm biomass, explained by the high abundances seen in the *Aporrectodea* juveniles, which contributed little to biomass. Acidification of soil in highly inorganically fertilised systems

causes a negative effect on earthworm abundance and total biomass (Ma, Brussaard and de Ridder, 1990).

Further benefits of earthworms in pasture production include increased arbuscular mycorrhizal (AMF) colonisation which is stimulated by phytohormones produced by earthworms (Azcon, Azcon-G De Aguilar and Barea, 1978; Zarea *et al.*, 2009). Zarea *et al*. (2009) showed the presence of earthworms (*Pheretima* sp) and AMF (*Glomus mosseae)* increased the crop yield of the two clover species mixture Berseem clover (*Trifolium alexandrinum)* and Persian clover (*Trifolium resupiantum*). The highest AMF colonisation rate occurred under the clover 1:1 ratio mixture where earthworms and AMF were present. This 1:1 ratio also showed the greatest soil microbial biomass compared to the other ratio combinations and presence/absence experimental combinations of the earthworms or AMF. Zarea et al. (2009) concluded the positive effects diversity has on agricultural systems, yet more research is required to understand the soil biota interactions and how they affect crop growth.

Table 1.1 Basic earthworm ecological functional groups interpreted from Bottinelli *et al.* (2020)

	Epigeic	**Endogeic**	**Anecic**
Food	Litter	Soil	Litter and soil
Habitat	Litter	Soil	Soil
Burrow	None	Horizontal	Vertical
Size	Small	Small to large	Large
	1-7cm long	2-12cm long	8-15cm long

1.4. Arbuscular Mycorrhizal

Arbuscular mycorrhizal (AM) are obligatory mutualistic microflora structures that aid nutrient uptake in plants, predominantly soil phosphorus, in exchange for carbon. Research into the colonisation ability of AM in diverse forage systems is important as some grassland plant species require AM to be successful. These include important forage mixture legumes of birdsfoot trefoil (*Lotus corniculatus*), black medick (*Medicago lupulina*) and red clover (*Trifolium pratense*).

AM presence in grassland systems can enhance plant species diversity by reducing plant competition from ruderal species, including reducing dominant competitive grass species (*Festuca ovina*) in favour of grassland herb species (*Scabiosa columaria, Hieracium pilosella* and *Plantago lanceolata*; Grime *et al.*, 1987). The ability of AM to colonise diverse mixtures is therefore important as previous forage mixture studies show dominance by tall fescue and cocksfoot after a few years (Sanderson *et al.*, 2005; Deak *et al.*, 2007; Skinner, 2008). Other benefits of AM-colonised communities include reducing nitrogen leaching (de Vries *et al*., 2006) and reducing the impact of plant species richness loss on plant productivity (Klironomos *et al.*, 2000).

Grassland systems with increased rooting depth and root biomass, i.e. diverse grasslands (Steinbeiss *et al.*, 2008), create greater opportunities for AM hyphae to come into contact with a recipient host root, increasing the chances of AM to colonise. Soils rich in AM species also increase the infection potential due to the length of hyphae increasing in such systems (van der Heijden *et al.*, 1998). By having an extended rooting surface area in the soil through mutualism with AM, plants increase phosphorus uptake and water uptake (van der

Heijden *et al.*, 1998). This is an important forage system requirement - to maintain pasture production in the drier, warmer summers we are already experiencing (Lowe *et al.*, 2019).

Diverse forage grasslands with a high legume density accumulate soil nitrogen (De Deyn *et al.*, 2009). Increased soil nitrogen enhances plant productivity until phosphorous becomes the limiting plant growth nutrient. The limited soil phosphorus availability increases the AM inoculum potential and stimulates AM colonisation, aiding further plant growth. This cycle heavily relies on the growth rate of the host legume, taxonomy, and plant density, as these can all affect nitrogen fixation rates and, therefore, directly affect the AM community (Brockwell *et al.*, 2005).

The microbial composition of the soil is an important parameter to measure regarding future predicted climate change extreme weather events. For example, soils rich in fungi are more stable and better adapted to retain nutrients under disturbance events of drying-rewetting than soils with a high bacterial contribution (Gordon, Haygarth and Bardgett, 2008). AM can resist a wider range of drought and heat conditions than bacteria (Acosta-Martínez *et al*., 2014) due to AM's slower autochthonous reproductive strategy. However, bacteria are more tolerant to heat than fungi (Riah-Anglet *et al.*, 2015), and can respond to the disturbance event faster due to their rapid zymogenous strategy. The chemical composition of the soil can also alter the contribution of fungi or bacterial microbial biomass to the soil. Fungi, for example, tend to dominate when the C:N ratio is high, whereas bacteria dominate when the C:N ratio is narrower.

1.5. Soil carbon and nitrogen

Nitrogen and carbon are important in biological terms, as they are required for organism growth (MA, 2005) and political agenda, such as the Paris Agreement for greenhouse gas reduction and mitigation (UN, 2015a). However, carbon and nitrogen cycling has become unbalanced due to anthropogenic exploitation, increasing the carbon cycle by ~15% and doubling the nitrogen cycle to pre-industrial revolution figures (Falkowski *et al.*, 2000; Mackenzie, Ver and Lerman, 2002). Greenhouse gas emission mitigation plans have been drawn to rebalance carbon and nitrogen cycles (UN, 1998, 2015a). Signed countries have agreed to reduce emissions and increase carbon sequestration. The Intergovernmental Panel on Climate Change (IPCC) has categorised greenhouse gas emissions sources into four main economic sectors, with agriculture also categorised as a sink (Smith *et al.*, 2014). Currently, UK agriculture contributes ~10% of the total UK greenhouse gas emissions (DEFRA, 2017). Reduction in emissions and increase in carbon sequestration can occur within pasture production as the choice of forage species sown, along with forage species diversity, can aid in climate change mitigation plans through changes in soil nutrient cycling and retention (Steinbeiss *et al.*, 2008; Smith *et al.*, 2014). For example, macroaggregates created in soil by root and earthworm ecosystem engineers under red clover contained higher carbon and nitrogen contents compared with macroaggregates in soil under ribgrass plantain (*Plantago lanceolata*) (Zangerlé, Pando and Lavelle, 2011). The increased soil nitrogen achieved by red clover means synthetic nitrogen fertiliser application can be reduced. The higher carbon sequestered in the soil under red clover reduces carbon in the atmosphere, also aiding climate change mitigation plans.

1.5.1. Legumes and Nitrogen

Soil nitrogen accumulation and nitrogen plant uptake is highly dependent on forage species composition as grassland species differ in growth rates both spatially and temporally (Sanderson *et al.*, 2005; Fornara and Tilman, 2008; Cong *et al.*, 2014). Popular nitrogen-fixing legumes used in simple grass-clover pastures include red clover (*Trifolium pratense*) due to its protein levels in silage and white clover (*Trifolium repens*), which establishes well in long-term leys. Including legumes in pasture mixtures reduces the need for synthetic nitrogen fertiliser application due to biologic nitrogen fixation. Legumes harbour *Rhizobium* bacteria that fix atmospheric nitrogen for the plant in exchange for carbohydrates in a symbiotic relationship. De Deyn *et al.* (2009) showed in a modelled pot experiment, monoculture forbs and grass species experienced a net loss of soil nitrogen, whereas legume monocultures gained nitrogen in soil. De Deyn *et al.* (2009) concluded that fluxes in nitrogen pools were by the presence of specific species, such as legumes, rather than plant species richness diversity. The conclusions are, however, limited in scope by the short two-year duration. Cong *et al.* (2014), however, suggests that legume species are not key components in increasing nitrogen in the soil, in fact, more nitrogen soil stocks at greater species richness are due to greater plant productivity and soil nitrogen retention from enhanced soil nitrogen mineralisation rates.

Improvements created by legumes in grassland systems include increased soil structure, rooting depth and depths of soil pores (Steinbeiss *et al.*, 2008; De Deyn *et al.*, 2009). The optimal legume percentage in a grass-legume forage mixture to supply sufficient nitrogen is between 30-50% (Lüscher *et al.*, 2014). Legume addition into agricultural rotation increases not only soil nitrogen but also soil carbon. This increase in soil carbon feeds into the whole soil ecosystem and supports a greater abundance of microbiota (Drinkwater, Wagoner and Sarratonio, 1998). Known as the microbial loop (Clarholm, 1985), an increase in root exudates

increases soil microbial populations, which in turn increases their consumers and predators, which increases the release of available nitrogen in the soil, which enables plant growth, which increases root exudates, and thus the loop continues. The presence of soil invertebrates such as earthworms further increases nitrogen availability to plants (Scheu, 2003). However, this may not necessarily be beneficial if a diverse forage system is the desired outcome, as the presence of earthworms increasing the availability of nitrogen favoured fast-growing grasses, reducing plant species diversity in the system (Scheu, 2003). The assumption that legume addition in agricultural systems is solely beneficial is inaccurate. The increased soil pore depths can lead to increased nitrous oxide release (MA, 2005; Holtham, Matthews and Scholefield, 2007). Nitrate leaching, even by nitrogen fixed from legumes, can also occur in grass-clover mixed grazed pasture systems, which can be environmentally damaging (Fillery, 2001; Schindler, 2006; Elser, 2012). Additionally, a global cost-benefit review of using legume plant species for nitrogen versus acquiring nitrogen through synthetic pathways within agricultural systems suggested that some countries depend on artificial nitrogen fertiliser application to maintain productivity and meet demand (Crews and Peoples, 2004). High nitrogen in the soil from legume presence may hinder the colonisation of AMF, reducing the benefits AMF create, such as enhanced plant resistance to drought and pathogens (Jia *et al.*, 2021).

1.5.2. Soil Carbon

Plants sequester atmospheric carbon into the soil. Grazing systems with a high richness of plant species can accumulate more soil carbon than a lower diverse forage mixture (Skinner and Dell, 2016; X. Chen *et al.*, 2019). Biodiverse grasslands increase plant productivity (Tilman and Downing, 1994; Tilman, Wedin and J. Knops, 1996; Hector *et al.*, 1999; Tilman, Reich, *et al.*, 2001; Hooper *et al.*, 2005), increasing litter inputs and food resource for soil invertebrates, enhancing soil organic carbon content and stimulating microbial respiration.

However, even with the increased stimulation for microbial respiration from increased carbon inputs, soil carbon content is greater under diverse grasslands (X. Chen *et al.*, 2019). Soil organic carbon accumulation increases with deeper root systems and high root biomass, reducing mineralisation and carbon loss (Skinner and Dell, 2016; Rutledge *et al.*, 2017). Greater root biomass and deeper (>15cm) roots occur in plant species-rich plots, increasing AMF infection potential, whereas grass species generally develop shallow (<5cm) root systems (Steinbeiss *et al.*, 2008). Steinbeiss *et al.* (2008) concluded that soil carbon accumulation is due to resource partitioning; exploitation of the same resource by similar species without driving the other to extinction, rather than niche complementarity; utilisation of the resource by many species resulting in higher productivity. High forage species diversity increases the chances of deeper rooting species being present due to phenotypic plasticity; morphological adaptation to the particular environment (Steinbeiss *et al.*, 2008).

In De Deyn *et al.* (2009) monoculture pot experiments, grass species (*Lolium perenne* and *Anthoxanthum odoratum*) showed a net loss of soil carbon, while legume monocultures (*Trifolium repens* and *Lotus corniculatus*) accumulated it. Fluxes in carbon pools were due to specific species rather than plant species richness diversity. However, issues with this study include the short two-year duration and restricted rooting and sampling depth (14cm), which is an important condition when measuring soil carbon.

Cong *et al.* (2014) showed that soil carbon accumulation increased with increasing plant species richness, measuring to a depth of 15cm. The larger carbon soil stocks at higher species richness were put down to greater carbon input from increased plant productivity (Cong *et al.*, 2014). Similar results were seen with Skinner and Dell (2016), showing the most significant

difference in carbon accumulation in 10 – 30cm soil depth between a two-species mixture and a five-species grassland mixture over a nine-year experimental period. The IPCC methodology for soil depth measurement of carbon is 0 – 30 cm; justifying this depth due to turnover of carbon being greatest in topsoil, this layer is also the most active for soil biota measurements (UN, 1998; IPCC, 2006; Smith *et al.*, 2014). However, various studies have shown significant amounts of soil carbon deeper than 30cm (Batjes, 1996; Bradley *et al.*, 2005; Rumpel and Kögel-Knabner, 2011). With root exudates being a source of increase in soil carbon and several grassland species found in diverse mixtures of roots reaching 200cm in depth, following the IPCC methodology may not be an appropriate method. Deep-rooting grassland species include yarrow (*Achillea millefolium*), lucerne (*Medicago sativa*), sainfoin (*Onobrychi*s spp.) and sheeps parsley (*Petroselenium crispum*). As a result, methodologies vary amongst soil depth measures for soil chemical analysis in diverse grassland systems. These include 100cm (Fornara and Tilman, 2008; Skinner and Dell, 2016), 30cm (Steinbeiss *et al.*, 2008; Lange *et al.*, 2015) and 15cm depths (Cong *et al.*, 2014). All studies at these varying depths concluded that more carbon accumulated in soil with greater species richness. Skinner and Dell (2016) then conclude that changes in soil organic carbon are highly dependent on sampling depth over time.

1.6. Soil biota, grassland, and livestock

Aside from future-proofing forage production to contend with climate change conditions and aiding soil quality, forage mixture production must be palatable to their grazers. Grazing intensity, however, its frequency and severity, and herbivore diversity can affect soil biota composition by modifying soil processes such as nutrient cycling and grassland productivity (Bardgett and Wardle, 2003; Wang *et al.*, 2019). This change has subsequent cascade effects on the whole soil food web (Bardgett, Wardle and Yeates, 1998; Bardgett and

Wardle, 2003). For example, in heavily grazed grasslands, soil microbial community becomes dominated by bacteria, and in less intensively grazed pastures, fungi dominate the decomposition pathways (Bardgett, Wardle and Yeates, 1998). Soil microbial biomass succession moves from a more bacterial-dominated to a fungal-dominated system (Van der Putten *et al.*, 2009). The balance between microbial zymogenous and autochthonous strategists will affect the soil's ability to recover after stress events. Zymogenous strategists are typically bacteria, opportunistic in responding to the fresh substrate and multiplying quickly. Autochthonous strategists are typically fungi, where they have a slow growth rate and greater population stability. Typical soils contain a large inactive population pool of autochthonous strategists with a smaller population pool of zymogenous strategists.

Long-term aboveground grazing can reduce root biomass, impacting infection potential for AMF. Aboveground grazing can also alter the quality of plant litter input thus affecting the availability and quality of food resources for soil invertebrates such as earthworms (Bardgett, Wardle and Yeates, 1998). A typical forage mixture of perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) shows defoliation increases microbial biomass even after the grazing caused the reduction of root biomass (Bardgett, Wardle and Yeates, 1998). Long-term grazing also decreases soil carbon sequestration but stimulates net nitrogen mineralisation and encourages nitrogen plant uptake, thus altering chemical cycling in the soil (Bardgett, Wardle and Yeates, 1998).

1.7. Summary and Knowledge gaps

Forage systems must be able to maintain productivity under new climes. Diverse forages may be better suited to this challenge; however, they do not offer a simple answer. Due

to the inbuilt diversity, forage management suitable for monocultures may not be applicable to these systems. Optimising the relationship between soil function and forage productivity is thus a key challenge.

Increasing the diversity of forage mixtures has shown environmental benefits (Spehn *et al*., 2000; Tilman, Reich, *et al*., 2001; Fornara and Tilman, 2008), as well as both positive and negative impacts on agricultural production (Hammond *et al.*, 2014; Skinner and Dell, 2016; Boeraeve *et al.*, 2020; Bezner Kerr *et al.*, 2023). As a result, farmers are unlikely to take up suggestions for environmentally improved grassland production systems if it is detrimental to their farming business (Hammond *et al.*, 2014). Improving and sustaining soil health for the benefit of both environment and agriculture to meet sustainable intensification is required. Soils provide critical ecosystem services, and with increased pressures on food production to meet demand, focussed area research and results are required (de Graaff *et al.*, 2019).

Effects of diverse forages on soil chemistry and biota cannot be studied in isolation due to the complex and interactive soil environment (Bünemann *et al.*, 2018a). The microbial loop is just one example that affects all trophic levels, with the response of one trophic level feeding into the reaction of another. With the decline in soil functionality, the ENVironmental ASsessment of Soil for mOnitoring project (ENVASSO) defined important soil indicators for monitoring European soil systems (Table 1.2; Huber *et al.*, 2008). Two key issues to help monitor the decline in soil biodiversity (species diversity and biological functions) has measurement indicators across three levels of importance, from monitoring the soil indicator at all times, to monitoring if relevant to specific issues.

Using the suggestions of the ENVASSO monitoring and reviewing the literature, it is clear that **commercially viable diverse forages short term interaction with earthworms, mesofauna and fungal diversity and activity should all be measured within one system and data modelled to show the benefits these soil biotas have on aboveground biomass yield.** The knowledge gap identified here would cover the ENVASSO monitoring suggestions across all important soil monitoring levels, key issues, and key groups (microflora, mesofauna and macro fauna; Table 1.2).

*Table 1.2. Key issues in the decline in soil biodiversity to monitor which incorporates all functional groups and importance of monitoring. Items in **bold** researched for this thesis. Adapted from ENVASSO report (Huber et al., 2008)*

		Monitoring level of importance		
Key Issue	**Group**	**1 - always**	**2 – if relevant**	**3 - optional**
Species diversity	**Macrofauna**	**Earthworm**	All macrofauna	
	Mesofauna	**Collembola**		Activity based on bait lamina (proxy for invertebrate activity based on soil decomposition (Kratz, 1998))
		Enchytraeids	Acari sub orders	
	Microfauna		Nematode	Proctista
	Microflora		Bacteria and **Fungi based on DNA**	
	Plants			**For grassland and pastures**
Biological functions	**Macrofauna**			Activity
	Mesofauna			Activity
	Microflora	**Soil respiration**	**Bacterial and fungal activity**	

Caution should be taken with all studies due to experimental management best practice being different from management best practice at farm scale. For example, experimental variation includes: variants in nitrogen application rates; variants in the legume content of the experiment; continued species composition through the duration of the experiment (Crews and Peoples, 2004; Sanderso*n et al*, 2005; Skinner, 2008; Skinner and Dell, 2016), and variants in environmental conditions such as soil type and climatic area. This ultimate combination of species mixture and soil is very climatic and type-specific, which lengthens the time required to promote industry standard recommendations and is a limitation of this study.

1.8. Thesis outline

This thesis research was superimposed onto the established collaborative project, The Diverse Forages Project. Their aim was to achieve acceptable yields of good quality forage for livestock production whilst positively affecting the environment. This thesis aim was to **further identify which of the commercially viable grasslands within the Diverse Forages Project had the most positive effect on the soil biota (earthworms, mesofauna and AMF) and whether changes in belowground biota affected aboveground biomass productivity**; complementing the Diverse Forages aim of achieving acceptable yields and positive environmental effects. The thesis consists of three manuscript research chapters which address the soil biodiversity research gaps detailed above by comparing a conventionally fertilised forage pasture to three commercially available diverse grasslands (objectives summarised in Table 1.3). Chapter 2 covers the changes in soil invertebrates, namely earthworms, a selection of soil mesofauna under grazed systems, and measuring AMF activity through trap plant root

colonisation. Chapter 3 looks at overall fungal community diversity and microbial functional diversity, with modelling effects on provisioning ecosystem service delivery of aboveground biomass. And finally, Chapter 4 focuses solely on AMF community's contribution to the commercially available diverse grasslands in dry, stressed environments, suggestive of future climate change conditions. The experimental set up, methodologies and data used for all three research chapters are understandably interlinked, with sampling campaign breakdown identified in Table 1.4; as such, some sections of the methodology description are italicised to highlight repeated information.

Table 1.3 Objective per chapter and knowledge gap objectives covered

Objective	**Chapter**	**Knowledge gap**
• Identify earthworm and mesofauna abundance and diversity under the forage mixtures • Measure rates of AMF colonisation under the forage mixtures across two sites mimicking 'business as usual' and 'future climate dry stress' conditions	2	Commercially viable diverse forages short term interaction with earthworm and mesofauna diversity and fungi activity measured within one system
• Identify fungal community composition measured by DNA extraction • Measure soil microbial diversity and functionality through multiple substrate induced respiration • Model all data collected against aboveground biomass production	3	Commercially viable diverse forages short term interaction with fungal diversity and soil microbial diversity and activity measured within one system. Benefits soil biotas have on aboveground biomass yield**.**
• Identify AMF community composition, and model which variables correlate with AMF colonisation rates	4	Commercially viable diverse forages short term interaction with AMF diversity and community composition.

Table 1.4 Sampling campaigns for experimental Chapters 2, 3 and 4. Site column identifies the three sites used in this thesis research (Crops Research Unit (CRU): Dry site or Well-wetted site (WW), or the Centre for Dairy Research (CEDAR)). Project column identifies sample collector (DivForages: Diverse forages project team, or Sarah Shepperd (SS)).

Sample date	**Site**	**Project collector**	**Sample collected**	**Analysis conducted**	**Chapter**
September 2016		DivForages	Plots sown: PRG, SG, Bio, Her	-	2, 3, 4 (only SG, Bio, Her)
March-October 2019	CRU – Dry and WW	DivForages	Aboveground biomass	-	3, 4
April 2019	CEDAR	SS	Earthworms	Adult earthworms identified Adult and juvenile earthworms weighed	2
April 2019	CEDAR	SS	Soil chemistry	Soil carbon and soil nitrogen (inhouse)	2
April 2019	CEDAR	SS	Mesofauna	Tullgren funnel. Mesofauna identified down to phylum, class or order depending on taxa	2
October 2019	CRU – WW only	SS	Root soil cores	Root biomass – chapter 2 and 3 Root surface area – chapter 3	2, 3
March 2020	CRU – Dry and WW	DivForages	Soil chemistry	Sent to NRM for pH, P, N, K, Mg, OM, C	2, 3, 4
March 2020	CRU – Dry and WW	SS and DivForages	Earthworms	Earthworm biomass	3, 4
March 2020	CRU – Dry and WW	SS	Ryegrass AMF trap plants planted	-	2, 3, 4
September 2020	CRU – Dry and WW	SS	Soil samples for DNA	Bulk soil fungal DNA extracted	3
October 2020	CRU – Dry and WW	SS	Ryegrass AMF trap plants removed	AMF colonisation determined via root staining (Dry and WW) AMF root DNA (Dry site only, Chapter 4 only) Rhizosphere soil AMF DNA (Dry site only, Chapter 4 only)	2, 3, 4
July 2021	CRU – Dry and WW	SS	Soil sampling for chemistry & Microresp experiment	Soil chemistry sent to NRM laboratories for total C and N (Chapters 3 and 4). Microresp data for multiple substrate induced respiration and microbial functional diversity (Chapter 3 only)	3, 4

Chapter 2. Rapid changes in soil invertebrates and mycorrhizal activity when comparing conventional forage pastures to diverse grasslands

Author initials: Sarah Shepperd[1] (SS), Zoe Barker[1] (ZB), Chris Reynolds[1] (CR), Mark Tibbett[1] (MT), Martin Lukac[1] (ML), Deborah Beaumont[2] (DB), Tom Misselbrook[2] (TM), Hannah Jones[3] (HJ)

Author contribution: ML, CR, DB, TM and HJ conceived and implemented the field experiment. SS conceived the study, conducted data gathering, with supplementary data collected by ZB. SS performed statistical analysis and wrote the article, all authors commented on the manuscript.

[1]*University of Reading*
[2]*Rothamsted Research*
[3]*Duchy College*

2.1. Abstract

Conventional ryegrass monocultures used for livestock production require external inputs (e.g., nitrogen fertiliser) to maintain productivity, thus contributing to environmental degradation. Improving the environmental impact of food production systems will be an important contributor to net zero targets that have been set both nationally and globally. The enhancement of aboveground ecosystem service delivery by diverse forage mixtures has been shown, however, little is known about how these effects cascade to the soil and affect its biota. Here we compare the effects of a conventional ryegrass monoculture and three differing diverse grasslands (6, 12 and 17 plant species) on important soil biota indicators (earthworms, soil mesofauna and arbuscular mycorrhizal fungi (AMF)) up to three years from sowing. The conventional monoculture (*Lolium perenne* L.) received nitrogen fertiliser (250kg/ha N) and represented "business-as-usual", while the three diverse grasslands received no nitrogen

fertiliser. Monoculture ryegrass had lower earthworm densities compared to the 12 species diverse grassland only ($F_{1,37}$=4.61, p<0.05), and harboured significantly higher abundances of collembola ($F_{1,37}$=6.51, p=0.018). All diverse grassland mixtures had higher AMF colonisation rates compared to the monoculture (6 species: z =6.37±0.05, p<0.001; 12 species: z=5.72±0.05, p<0.001; 17 species: z=6.66±0.05, p<0.001). Diverse grasslands clearly enhanced the abundance of ecosystem engineers such as earthworms and AMF, a finding important for future-proofing forage production and enhancing ecosystem services. Further research into the microbial community structures affected by ecosystem engineers is required to understand soil food web dynamics in these grassland communities fully.

2.2. Introduction

Modern grassland systems in many countries are dominated by ryegrass (*Lolium* spp.) monocultures requiring the supply of costly and unsustainable external inputs to maintain productivity (Crews and Peoples, 2004). The main purpose of managed grasslands is to provide feed for ruminants, but they can also provide a wealth of ecosystem services if managed for multiple purposes (Hopkins and Wilkins, 2006; Milne *et al.*, 2015). Agricultural intensification, aided by the availability of synthetic nitrogen fertiliser, has reduced grassland diversity due to the dominance of ryegrass, considered the most profitable species in these systems (Hopkins and Wilkins, 2006). Currently, the management of ryegrass monocultures requires intensive interventions to maintain productivity, which often involves an unsustainable investment of energy, fertilisers and herbicides, causing environmental damage and biodiversity decline (Tilman, Fargione, *et al.*, 2001; Crews and Peoples, 2004; Foley *et al.*, 2005; Schindler, 2006; Cordell, Drangert and White, 2009; Elser, 2012). The decline in grassland diversity reduces the ecosystem services they can supply such as carbon sequestration, creating long-term natural capital costs (Fillery, 2001; Hopkins and Wilkins, 2006; Cong *et al.*, 2014; Wagg *et al.*, 2014).

Environmental concerns have intensified discussions to move from highly intensive conventional monoculture grasslands towards diversifying pastures (DEFRA, 2018; Harris and Ratnieks, 2021).

Global and national sustainability targets have been adopted worldwide, many likely to impact current ruminant production systems as we seek to step away from harming the environment further. For example, a United Nations (UN) sustainable development goal of promoting sustainable food production systems by maintaining ecosystems that are adaptive to climate change while improving soil quality is to be achieved by 2030 (UN, 2019). Global pledges from signatories to reduce carbon dioxide emissions by 45% by 2030 relative to 2010 levels, necessitate changes in land-use practices as it is achievable in part by increasing carbon sequestration (UN, 1998, 2015, 2021). At the national level in England, moving away from the current agricultural policy and towards payments for environmental benefits will target the twin goals of improved productivity and ecological enhancement (DEFRA, 2018). The British National Farming Union (NFU) is asking its members to achieve net zero by 2040, likely affecting all farmers managing livestock production systems (NFU, 2019). Forage-producing systems must achieve these goals while facing an increasing frequency of adverse weather events driven by climate change, such as drier and warmer summers or unseasonal floods (Lowe *et al.*, 2019). One of the methods of achieving these goals is the regenerative agricultural practice of producing livestock on diverse forages (Hammond *et al*., 2014; Tilman *et al*., 2001). This approach aims to benefit topsoil by regenerating soil health and increasing biodiversity, eventually leading to enhanced ecosystem service delivery (Schreefel *et al.*, 2020). These benefits collectively increase the resilience of farming systems to climate change (Bardgett & Caruso, 2020; Tilman *et al*., 2006).

Linkages between above- and belowground systems are well established (Bardgett *et al*., 1998; Hooper *et al*., 2000). Diverse forage mixtures contain plant species varying in key traits, such as different rooting strategies, beneficial for both the above- and belowground components of grassland systems (Spehn *et al.*, 2000; Brockwell *et al.*, 2005; Sanderson *et al.*, 2005; Skinner, 2008; Steinbeiss *et al.*, 2008; Hammond *et al.*, 2014; Van Groenigen *et al.*, 2014; Wagg *et al.*, 2014; Skinner and Dell, 2016). Aboveground benefits of a diverse grassland include an extended grazing season due to the different growth habits of the plant species sown (Hammond *et al*., 2014; Tilman *et al*., 2001), and increased aboveground biomass compared to lower diverse mixtures, even in dry years (Hector *et al*., 1999; Hooper *et al*., 2005; Sanderson *et al*., 2005; Tilman *et al*., 2001; Tilman *et al*., 1996b; Tilman & Downing, 1994). Aboveground functional diversity improves ecosystem stability (Bardgett and Caruso, 2020). Higher diversity systems are 70% more stable than monocultures (Tilman, Reich and Knops, 2006), a crucial requirement for future-proofing forage production.

Belowground benefits for ecosystem services achieved in diverse grasslands include the increased carbon sequestration potential, with higher plant species-rich systems accumulating more soil carbon compared to a lower diverse forage mixture (Fornara and Tilman, 2008; Steinbeiss *et al.*, 2008; Cong *et al.*, 2014; Lange *et al.*, 2015; Skinner and Dell, 2016; X. Chen *et al.*, 2019). Other ecosystem services a functional biodiverse soil can provide are water retention, release and purification, and effective nutrient cycling (Milne *et al.*, 2015; Tilman *et al*., 2002). Including legumes in a diverse grassland reduces the need for nitrogen fertiliser (Lüscher *et al.*,2014). Diverse grasslands also reduce nitrogen leaching due to the utilisation of mineral nitrogen from increased species richness (Tilman *et al*., 1996a). A reduced

diversity of soil biota reduces the soils ecosystem function of nutrient cycling as soil with a suitable trophic diversity modifies the release and regulation of nutrient availability to plants (Wagg *et al.*, 2014; Milne *et al.*, 2015)

Soil biota essential for nutrient cycling include earthworms, which are often referred to as ecosystem engineers. Through the egestion of soil aggregates by earthworms, organic nitrogen previously inaccessible in soil residues becomes released, promoting aboveground production and increasing aboveground biomass by 23% (Van Groenigen *et al.*, 2014). Low plant species diversity decreases organic matter (OM) input, decreasing earthworm abundance, density, and biomass (Spehn *et al.,* 2000). A decrease in OM input affects the dynamic of the soil food web, including microbial populations, microbial consumers such as collembola, and subsequently, their predators (Clarholm, 1985). In diverse grasslands, arbuscular mycorrhizal fungi (AMF) represent an additional type of soil biota critical to enhancing and stabilizing system productivity. AMF can double grassland productivity by increasing phosphorus supply to plants (van der Heijden *et al.*, 1998; Vogelsang, Reynolds and Bever, 2006) and by reducing plant competition (Grime *et al.*, 1987). The ability to extend the rooting systems of forage pastures through mutualism with AMF is a crucial requirement for their productivity in drier and warmer summers (Lowe *et al.*, 2019). Soils rich in AMF were shown to be more stable (Gordon, Haygarth and Bardgett, 2008) and better able to resist droughts (Acosta-Martínez *et al.*, 2014). Further, AMF hyphae are a crucial food resource for many soil mesofauna. In a positive feedback loop, the benefits of an abundant and biodiverse soil food web stimulated and supported by aboveground biodiversity supports aboveground productivity (van der Heijden *et al.*, 1998; Vogelsang, Reynolds and Bever, 2006; Van Groenigen *et al.*, 2014; Tsiafouli *et al.*, 2015; Kariman, Barker and Tibbett, 2018).

Many studies show the benefits of diverse grassland for belowground biota in long-term grassland systems (Bardgett, Wardle and Yeates, 1998; Spehn *et al.*, 2000; Orwin and Wardle, 2005; Steinbeiss *et al.*, 2008; Cong *et al.*, 2014; Lange *et al.*, 2015; Skinner and Dell, 2016; X. Chen *et al.*, 2019). In a situation where diverse grasslands are quickly gaining prominence as a sustainable alternative to grass monocultures, we need to understand the short-term dynamics of soil biota as affected by plant diversity owing to grasslands' utilisation in crop rotations. Here we look at the effects of conventional monoculture, and commercially available unfertilised diverse grassland forage mixtures on soil biota. We investigate earthworms and mesofauna at a location suitable for destructive soil sampling and AMF at two sites to compare 'business as usual' scenario AMF colonisation against future drier climate change conditions. These taxa were chosen as key representatives of the soil biome important in sustaining system productivity. We hypothesise that (H1) non-nitrogen fertilised diverse forage pastures differ in soil fauna composition and AMF activity compared to a conventionally nitrogen fertilised ryegrass monoculture. Further, we posit that (H2) increasing aboveground species diversity positively affects soil biota composition and AMF activity. We also hypothesise (H3) that AMF root colonisation will be higher in drier conditions.

2.3. Methods

2.3.1. Experimental set up

Forage mixture

Forage mixtures were sown in September 2016 at two farms belonging to the University of Reading: Centre for Dairy Research (CEDAR) and Crops Research Unit (CRU). Mixtures ranged from a single species perennial ryegrass (*Lolium perenne*) to three diverse forage

mixtures of increasing species richness: SmartGrass (6), Biomix (12), and Herbal (17 species; Table 2.1 and 2.2). Perennial ryegrass plots received the recommended 250kg/ha N (as ammonium nitrate), divided into four application timings annually: two at 75kg/ha N and two at 50kg/ha N. Diverse species mixture plots received no nitrogen fertiliser.

Table 2.1 Diverse forage mixture species selection list (PRG: Ryegrass; SG: SmartGrass; Bio: Biomix; Her: Herbal) sown September 2016 at the University of Reading Centre for Dairy Research, Berkshire and University of Reading Crops Research Unit Farm, Berkshire. Ryegrass received 250kg N/ha (as ammonium nitrate), divided into four application timings across the year: two at 75kg/ha N and two at 50kg/ha N. Diverse mixtures receive no nitrogen fertiliser

	Species	**PRG**	**SG**	**Bio**	**Her**
Perennial Ryegrass	*Lolium perenne* L.	✓	✓	✓	✓
Timothy	*Phleum pratense* L.		✓	✓	✓
Cocksfoot	*Dactylis glomerata* L.			✓	✓
Festulolium	-			✓	✓
Tall Fescue	*Festuca arundinacea* Schreb.				✓
Meadow Fescue	*Festuca pratensis* Huds.			✓	✓
Red Clover	*Trifolium pratense* L.		✓	✓	✓
White Clover	*Trifolium repens* L.		✓	✓	✓
Alsike Clover	*Trifolium hybridum* L.			✓	✓
Sweet Clover	*Melilotus* spp.				✓
Black Medick	*Medicago lupulina* L.			✓	
Lucerne	*Medicago sativa* L.			✓	
Sainfoin	*Onobrychis spp.*				✓
Birdsfoot Trefoil	*Lotus corniculatus* L.				✓
Plantain	*Plantago lanceolata* L.		✓	✓	✓
Chicory	*Cichorium intybus* L.		✓	✓	✓
Yarrow	*Achillea millefolium* L.				✓
Burnet	*Sanguisorba minor* Scop.				✓
Sheep's Parsley	*Petroselenium crispum* Mill.				✓

Table 2.2 Percentage seed mass contribution per forage family per forage mixture at time of sowing September 2016

		Treatment			
	%	**Ryegrass**	**Smart Grass**	**Biomix**	**Herbal**
Forage	**Grass**	100	86	69	40.5
	Legume	-	8.5	22.5	38.5
	Herb	-	5.5	8.5	21

Centre for Dairy Research Farm (CEDAR)

Twenty 1ha paddocks representing the four forage mixtures repeated 5 times were sown at the CEDAR farm in Shinfield, Berkshire (51º24’43”N, 000º54’30”W). The paddocks were located on freely draining, slightly acid (pH 6.1) loamy soils (Cranfield University, 2019; Figure 2.1). Management of the paddocks consisted of rotational strip grazing by 40 growing dairy cattle between March and November, starting two years after paddock establishment. Surplus herbage was cut to prescribed sward heights (7cm) and removed to maintain forage quality across replicates. Land use prior to the establishment of the paddocks was Italian ryegrass (*Lolium multiflorum*) in paddocks 1-10 and perennial ryegrass (*Lolium perenne*) in paddocks 11-20 (Figure 2.1).

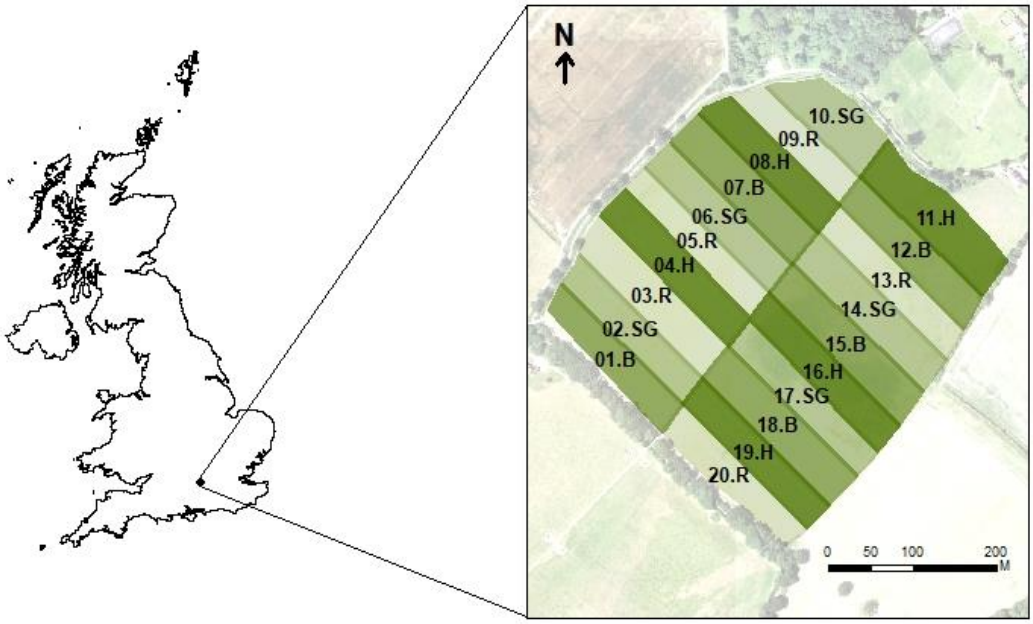

Figure 2.1 Location of experimental paddocks at the University of Reading CEDAR farm, Berkshire. Each paddock is ~1ha and sown with either R – Ryegrass (1 species); SG – SmartGrass (6 species); B – Biomix (12 species); or H – Herbal (17 species). Paddock management includes forty growing dairy cattle rotationally strip grazing March to November, which started in 2018. Surplus herbage cut to prescribed sward heights and conserved. Ryegrass plots fertilised with 250kg N/ha, spread across four applications yearly: two 75kg N/ha and two 50kg N/ha. Image source: High Resolution (25cm) Vertical Aerial Imagery [JPG geospatial data], Scale 1:500, Tiles: su7568, su7569, su7668, su7669, updated: 29 October 2018, Getmapping, using: EDINA Aerial Digimap Service, <https://digimap.edina.ac.uk>, Downloaded: 2019-10-10 12:52:24.759

Crops Research Unit Farm (CRU)

Two locations within the CRU farm in Sonning, Berkshire (51º28'22.4" N 0º54'15.3" W) were selected for their varying soil types. One site was an excessively drained shallow light sandy loam on gravel bed, while the other was a free-draining loamy coarse sand close to the river Thames (Cranfield University, 2019). The first site usually experiences severe drought in the summer (2% soil moisture as of June 2018), hereafter referred to as the dry site. The second site can typically support crop growth throughout the growing season without irrigation (7% soil moisture as of June 2018), hereafter referred to as the well-watered site. Both sites were under arable management prior to the establishment of this experiment. At both CRU sites, four replicate plots 4.2 x 5m in size of the four forage mixtures were sown in a 4x4 Latin square

design. The management regime of all plots simulated animal grazing by hand cutting and removal when biomass reached 2500 kg/ha dry matter from May until September, leaving 7cm height residual forage. Cutting occurred 2 to 5 times per season (three times per year on average) over 3 growing seasons (2017 to 2019).

2.3.2. Soil sampling

Earthworms

Two random 20x20x20cm soil pits per paddock were dug in April 2019 at CEDAR, two and a half years after sward establishment. The CEDAR site was chosen for the destructive sampling due to the larger paddock size compared to the smaller CRU plots. Five litres of mustard flour solution (6g/l mustard flour prepared 24hr before sampling) was poured down each pit and allowed 1 hour to soak away. Destructive soil pit sampling and hand sorting ensure epigeic and endogeic earthworms are observed; chemical extraction ensures the anecic ecological earthworm group is also sampled. The mustard solution was chosen as the chemical irritant over formalin vermifuge due to its non-carcinogenic and non-phytotoxic properties (Gunn, 1992; Pelosi *et al.*, 2009). Excavated soil was placed onto a 90cm x 40cm plastic sheet in the field, where the soil cube was gently teased apart to extract earthworms. Extracted earthworms were placed in a large, loosely closed bag to ensure gas exchange and earthworm survival until identification and weighing. Earthworms that emerged as a result of the mustard solution application were collected and washed with deionized water before placing them in the same bag as the earthworms extracted from the excavated soil. Sample bags with earthworms were kept indoors in a darkened area at room temperature before identification. Earthworms were extracted from the sample bags within 48hrs of field sampling. They were placed into a container of deionized water to remove excess soil before being placed onto damp tissue paper to remove excess water and weighed. All earthworms were weighed, including

half earthworms. Adult earthworms were identified using the 'Key to Common British Earthworms' OPAL field guide (Jones and Lowe, 2012), and diversity measured using the Shannon diversity index.

Soil total carbon: total nitrogen

Approximately 600g of soil was collected from each soil pit at CEDAR after being sifted through for earthworms. Sample bags were kept at 4ºC before analysis. Samples were dried in 40cm x 20cm metal containers for one day at 105ºC. Dried samples were ground using a pestle and mortar, then passed through a series of sieves (5.6mm; 1mm; 425µm). ~0.2g of dried 425µm ground sieved soil, as required by the analyser protocol, was placed into a 502-186 tin foil cup, sealed and inserted into a carbon/nitrogen analyser (LECO CHN628; LECO corporation, Saint Joseph, Michigan, USA). Each sample was analysed for 4 minutes for complete combustion at 1000ºC.

Meso fauna

An additional 600g of soil was collected from each soil pit after sifting through for earthworms. Soil was placed into Tullgren funnels with 20watt bulbs within 5 hours of the collection, ethanol was used as the collection fluid. Funnels were heated continuously for 16 days; ethanol traps were checked daily. Mesofauna were identified down to phylum, class or order level depending on taxa using a dissecting stereo microscope and identification guide (Tilling, 2014).

Root biomass

Soil cores (8cm diameter) were taken at the CRU well-watered site only over 4 depths (0-15cm, 15-30cm, 30-45cm, 45-60cm) in the autumn of 2019, three years after plot establishment. Soil cores were emersed in water and soaked for 24 hours to ease the separation of roots from the soil. Roots were washed clean of soil and then dried at 50ºC to constant weight for dry biomass measurements.

AMF colonisation

Sterile sand (Sibelco UK Ltd) absorbent clay substrate was created as 4 parts sand to 1 part Terra Green (Oil Dri UK), with 0.025 g/kg calcium hydrogen orthophosphate and 10% deionised water. The mixture was autoclaved at 105ºC for 1 hour, rested for 24 hours then autoclaved again at 105ºC for 1 hour. Five-centimetre diameter hydroponic pots were placed within the same size closed bottom cups and each was filled with 140g of the sterile substrate. Afterwards, 14ml deionised water and 20mg ryegrass seeds (Cotswold Seeds Ltd, UK) were added at 1cm depth into the substrate.

Five pots per sunbag (Sigma-Aldrich Inc., Germany) were placed in a growth cabinet set to 22ºC 16-hour days and 15ºC 8-hour nights at 75% humidity for 1 month. Sunbags are transparent plastic bags with 0.02µm pores to enable plant growth and prevent contamination from the external environment, in this instance, the sunbags were used to prevent fungal contamination. Pots were fertilised with 1ml Long Ashton solution, a nutrient solution which contains no phosphorus, and 1ml deionised water twice over the 4 weeks the plants were in the growth cabinets. One ryegrass pot per sun bag was checked for mycorrhizal colonisation to

confirm ryegrass plants free from mycorrhiza were planted in the field. All plants were removed from the growth cabinet and given one week to acclimate to ambient conditions in a sheltered outdoor space while remaining in the sun bags to ensure no mycorrhiza colonisation before planting in the field. In late March 2020, three and a half years after establishing the plots, two trap-plants per plot were planted at the two CRU sites only (N=64). Ryegrass trap-plants were removed from the closed-bottom pots but retained in the hydroponic pots and sterile substrate. Plants were given 5ml deionised water upon planting and monitored to ensure field establishment. Monitoring of the trap-plants continued through the spring and summer 2020.

Trap-plants, retained within their hydroponic pots, and an extra 5x5x5cm of soil from below the hydroponic pots to cover new root growth were extracted from the CRU plots in early October 2020 using a trowel sterilised in 1% virucidal disinfectant Virkon S (Lanxess, Cologne, Germany). Trap-plant and associated soil were kept at 4ºC before processing.

Roots were washed free of soil, placed in 10% KOH, and left at room temperature for 3 days. Roots were then rinsed with deionised water and stained with Ink/Vinegar solution (1-part Shaeffer black ink to 19 parts white vinegar) for 1 hour at room temperature. Roots were rinsed again using deionised water and stored in lactoglycerol (1-part lactic acid, 2 parts glycerol, 1 part water; Walker & Vestberg, 1994). Percentage root AMF colonisation was measured using the grid line intersect method (Giovannetti and Mosse, 1980) with a compound microscope.

Soil chemistry

Three 10cm deep soil cores were taken in late spring 2020 from each plot at CRU only and mixed to create a composite sample. For soil moisture, a subsample of fresh soil was weighed, heated at 105ºC for 24 hours and reweighed. Soils were dried, passed through a 2mm sieve, and sent to NRM Laboratories (Cawood Scientific Limited, Bracknell, UK) for full soil chemical analysis. The analysis included pH measured in water [1:2.5], and available phosphorus determined through sodium bicarbonate extractable Olsens and calculated colour-metrically. Ammonium nitrate extractable method was used to determine soil available potassium and magnesium, and determined by ICP-OES. Loss on ignition at 430ºC was used for OM, and total nitrogen and total carbon were determined through the Dumas method.

2.3.3. Statistics

All statistics were performed using R studio statistical software (R Core Team, 2018).

Earthworms, C:N and root biomass

Two mixed models using stepwise regression were used to compare earthworm density (Linear mixed model (LMM)) and total earthworm biomass (Generalised LMM, family quasipoisson) from the CEDAR paddocks. Random effect was added to account for repeated measures using the 'lme4' package (Bates *et al.*, 2015). Fixed parameters were not significant. To assess differences between adult earthworm species abundance with forage mixtures, either a Wilcoxon signed rank or t-test was used as needed.

ANOVAs were performed for earthworm density, total earthworm biomass, root biomass and C:N ratio with forage mixture as fixed treatment. Factor levels were grouped,

collapsed, and tested for significances between models using ANOVA to further investigate which forage mixtures correlated with these variables.

Meso fauna

Generalised LMM with negative binomial function to account for overdispersion with extension package 'lme4' (Bates *et al.*, 2015) was applied. The add1() command was used to find the minimum adequate model, with family quasipoisson and random effect added to account for repeated measures. Model assumptions were checked by plotting residuals against fitted values, and a likelihood ratio test was also performed. Extension package 'effects' (Fox and Weisberg, 2019) and 'gridExtra' (Auguie, 2017) were used to display model results. Further GLMMs were performed using stepwise regression, including the above Tullgren funnel model criteria on the three most abundant faunal groups; enchytraeidae, beetle larvae, and collembola. A post hoc Tukey test using the 'multcomp' package (Hothorn, Bretz and Westfall, 2008) was used to identify which forage mixtures correlated with collembola abundance from the CEDAR paddocks.

AMF colonisation

A binomial logistic regression model was fitted using a success/failure matrix of AMF colonisation of the roots on ryegrass trap-plants against a two-way interaction with site (CRU dry and WW) and forage mixture treatment. A Chi-squared ANOVA was then performed on this model. Post-hoc Tukey test, using the 'multcomp' package (Hothorn, Bretz and Westfall, 2008), was performed on unique identifier site and forage mixture treatments to test forage mixture site combinations. Logistic regression modelling was preferred over arcsine

transformation to meet ANOVA model assumptions due to the higher power and greater interpretability of logistic regression model fits (Warton and Hui, 2011).

Among all soil chemistry and soil moisture data, phosphorus was the most strongly correlated to AMF colonisation. This was the first parameter fitted to the GLMM against the success/failure AMF matrix, alongside a fixed effect of site and a random effect of forage mixture. Stepwise regression was used to build the minimum adequate model using the add1() command, which added pH, OM and C:N ratio as fixed effects. Soil moisture, potassium, and magnesium showed no significant correlations during the stepwise regression phase, this model was therefore excluded. The final GLMM model parameters include the family defined as binomial and the integral scalar nAGQ equalling zero with extension package 'lme4' (Bates *et al.*, 2015). Model assumptions were checked against plotting residuals and fitted values. Several likelihood ratio tests were performed with the fitted model using ANOVA, comparing the full model against a model minus the parameter being looked at. Extension packages 'effects' (Fox and Weisberg, 2019) and 'gridEXTRA' (Auguie, 2017) was used to produce graphs. GLM with interaction terms were used to test for the effects of soil chemistry parameters used in the GLMM.

2.4. Results

2.4.1. Earthworms and mesofauna

In all, 29 adult earthworms were identified out of a total of 275 whole earthworms collected. We did not see any difference in adult earthworm abundance, richness or species diversity as a result of forage plant diversity (Figure 2.2; Table 2.3). Monoculture ryegrass paddocks had lower earthworm densities (adult and juvenile) compared to Biomix paddocks

($F_{1,37}$=4.61, p<0.05). No difference was seen in combined diverse paddocks earthworm densities compared to ryegrass-only paddocks ($F_{1,38}$=3.49, p=0.069; Figure 2.3).

We identified 338 individuals representing soil mesofauna down into phylum, class or order depending on taxa groups. Perennial ryegrass paddocks showed higher abundances of mesofauna (M=24.4, SD=12.4) compared to the SmartGrass mixture only (M=9.4, SD=5.9; *t*(5.74)=2.47, p=0.0501; Figure 2.4, Table 2.3). Forage mixtures did not correlate with mesofauna richness or diversity (Figure 2.4, Table 2.3). Perennial ryegrass contained significantly more collembola than SmartGrass ($F_{1,37}$=11.58, p<0.001) and Biomix mixtures ($F_{1,37}$=6.51, p=0.018; Figure 2.4, Table 2.4).

Total mesofauna abundance significantly correlated with soil C:N ratio ($\chi^2(1)$=6.00, p=0.014); soil fauna abundance increased at higher C:N (Figure 2.5), as did enchytraeidae abundance (χ2(1)=7.47, p=0.006; Figure 2.5). SmartGrass paddocks had a lower C:N ratio compared to ryegrass ($F_{1,37}$=4.34, p<0.05) and Herbal paddocks ($F_{1,37}$=6.09, p<0.05; Figure 2.6). Perennial ryegrass plots at the CRU WW site had higher root biomass in the 0-30cm depth than the Biomix mixture ($F_{1,13}$=5.17, p=0.042; Figure 2.7).

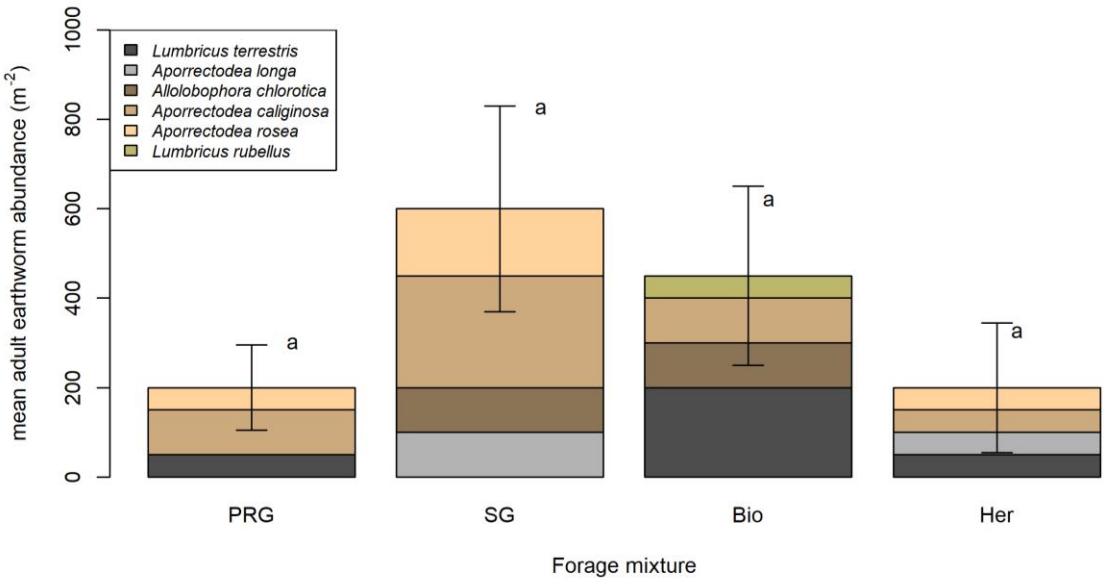

Figure 2.2 Mean adult earthworm abundance sampled at CEDAR spring 2019, 2.5 years after sowing, per forage mixture: PRG – perennial ryegrass (1 species); SG – SmartGrass (6 species); Bio – Biomix (12 species); Her – Herbal (17 species). Error bars show ±1SE of total mean earthworm abundance per forage mixture. Earthworm types: grey– anecic; brown– endogeic; green – epigeic earthworms. Letters denote significant difference at p<0.05.

Table 2.3 Adult earthworm species richness, mesofauna group richness, and adult earthworm and mesofauna abundance and diversity calculated using the Shannon diversity index per forage mixture: PRG – perennial ryegrass (1 species); SG – SmartGrass (6 species); Bio – Biomix (12 species); Her – Herbal (17 species)

	Species/Group richness		**Abundance**		**Shannon Diversity Index**	
	Earthworm	Mesofauna	Earthworm	Mesofauna	Earthworm	Mesofauna
PRG	3	5	4	122	1.05	1.36
SG	4	5	12	47	1.32	1.49
Bio	4	6	9	57	1.26	1.44
Her	4	6	4	112	1.40	1.33

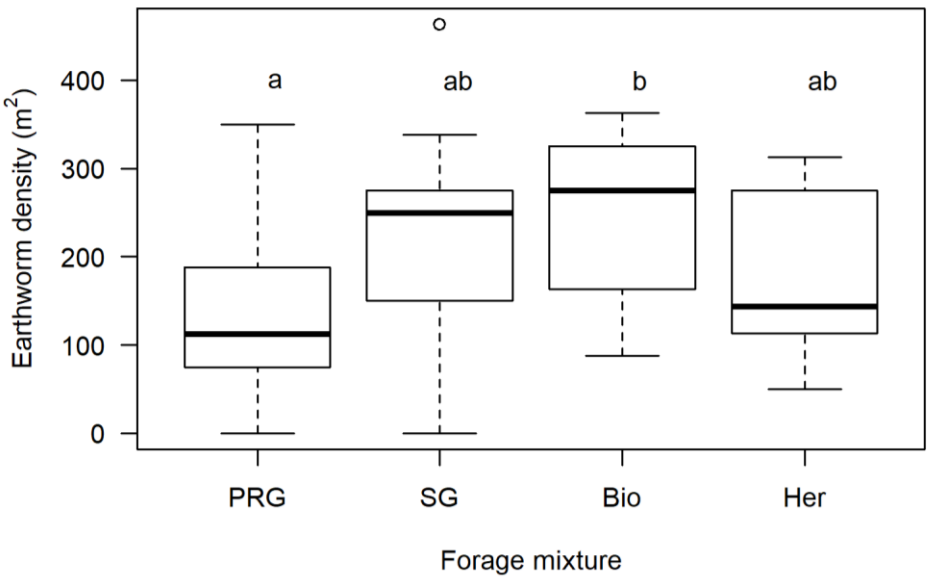

Figure 2.3 Earthworm densities in spring 2019 in four different forage mixture: PRG – perennial ryegrass (1 species); SG – SmartGrass (6 species); Bio – Biomix (12 species); Her – Herbal (17 species). Boxplots show median, middle 50% of data and upper and lower quartile data range. Letters denote significant difference between forage mixture at p<0.05.

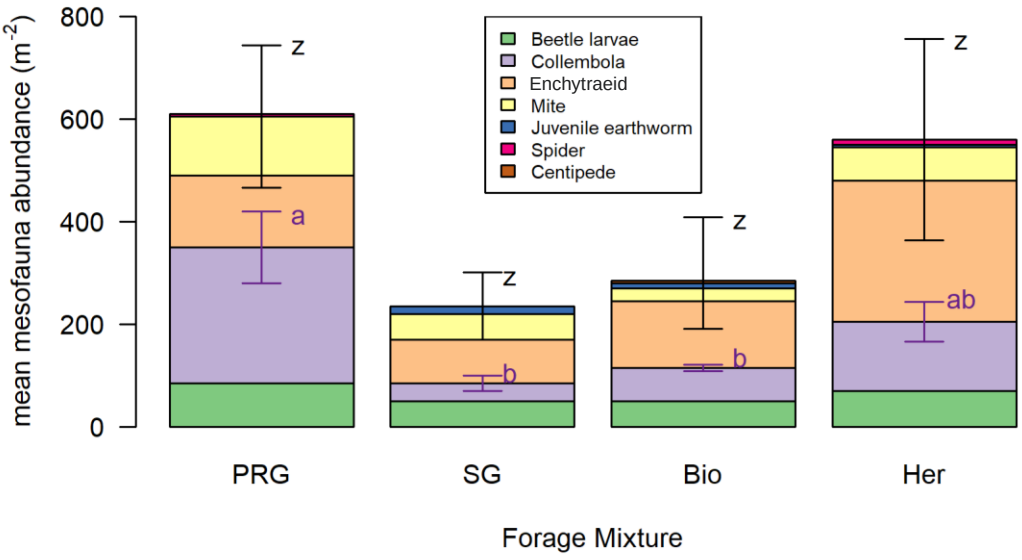

Figure 2.4 Mesofauna abundance collected from Tullgren funnels in spring 2019, 2.5 years after sowing, in the following forage mixtures: PRG – perennial ryegrass (1 species); SG – SmartGrass (6 species); Bio – Biomix (12 species); Her – Herbal (17 species). Black error bars show ±1SE of total mean mesofauna abundance per forage mixture. Purple error bars show ±1SE of collembola abundance per forage mixture, letters denote significant difference at p<0.05

Table 2.4 Post hoc Tukey test results from collembola GLMM. Forage mixture: PRG – perennial ryegrass (1 species); SG – SmartGrass (6 species); Bio – Biomix (12 species); Her – Herbal (17 species)

	estimate	**Std. error**	**Z values**	**P values**
PRG-SG	-2.024	0.545	-3.712	0.001
PRG-Bio	-1.405	0.481	-2.921	0.018
PRG-Her	-0.675	0.434	-1.541	0.410
SG-Bio	0.619	0.596	1.038	0.724
SG-Her	1.350	0.562	2.403	0.075
Bio-Her	0.731	0.500	1.463	0.457

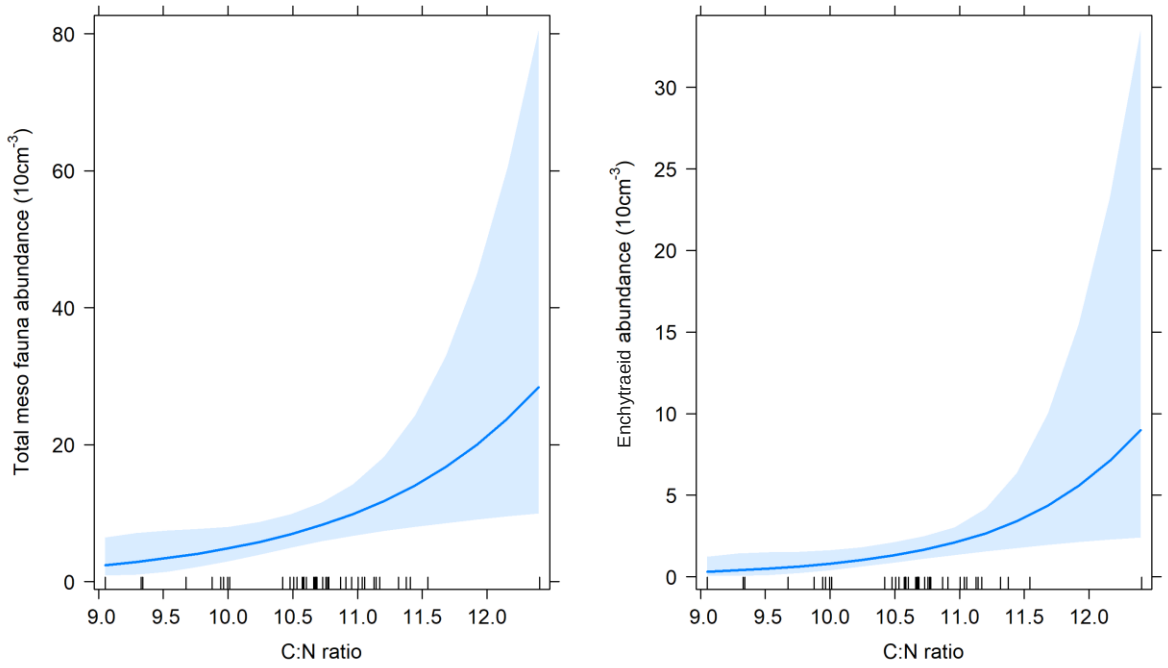

Figure 2.5 GLM model results for C:N ratio fixed effect on total meso fauna abundance and enchytraeidae abundance. Blue line represents trend line, blue shading represents 95% confidence interval, black lines represent data points.

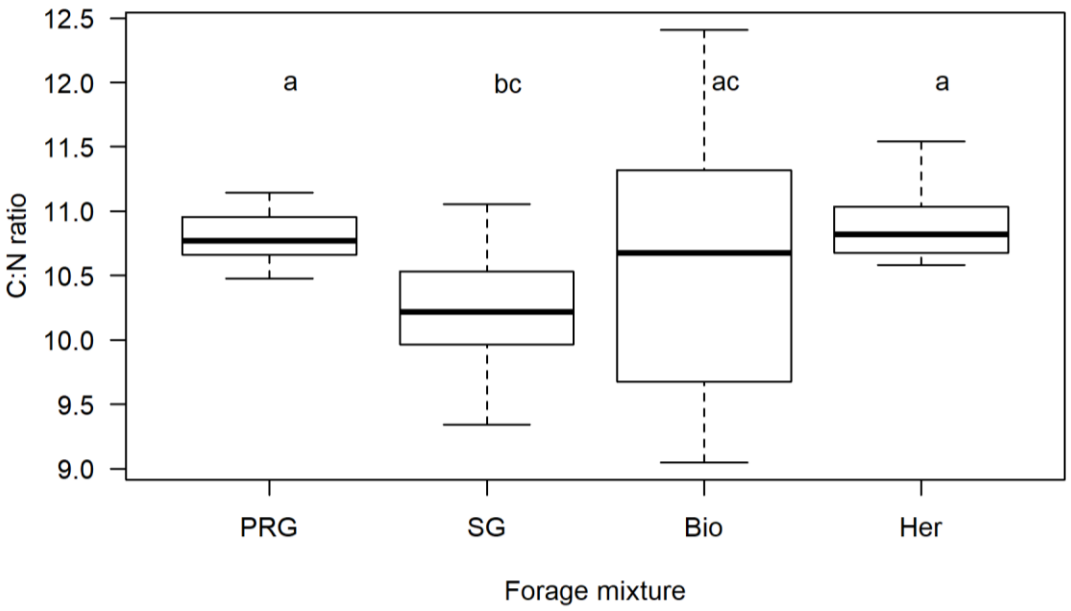

Figure 2.6 C:N ratio measured in spring 2019 per forage mixture: PRG – perennial ryegrass (1 species); SG – SmartGrass (6 species); Bio – Biomix (12 species); Her – Herbal (17 species). Boxes show median, middle 50% of data, and upper and lower quartile data range. Letters denote significant difference between forage mixtures at $p<0.05$.

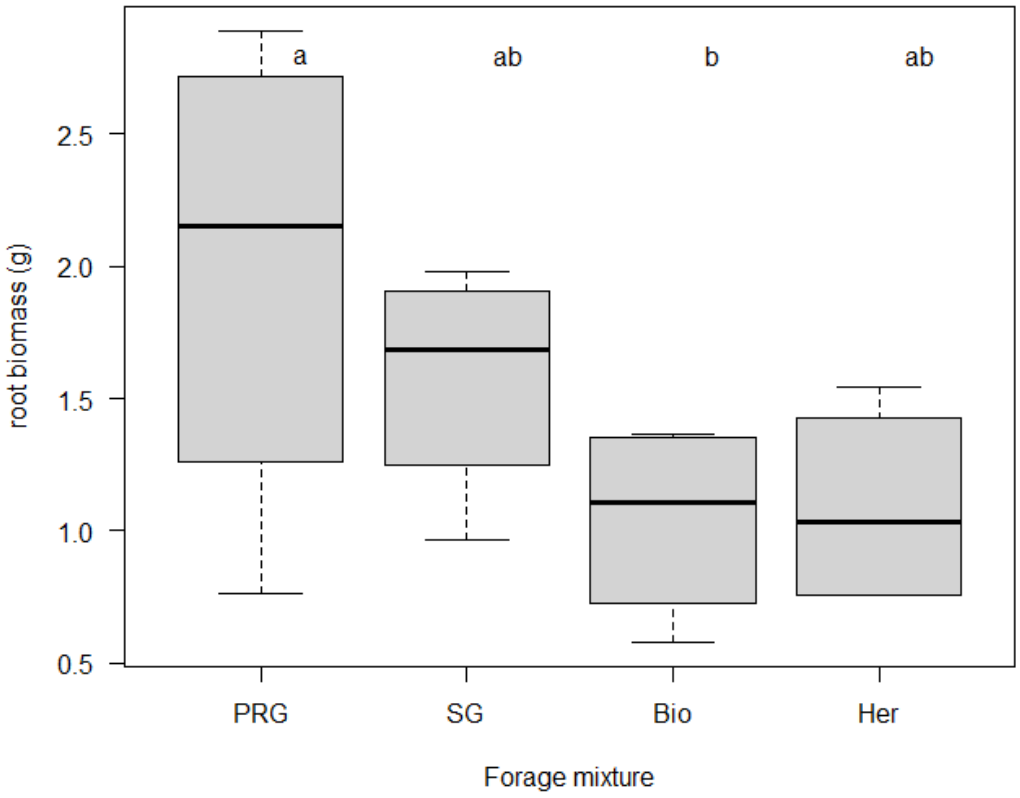

Figure 2.7 dry root biomass (0-30cm depth) from autumn 2019 per forage mixture: PRG – perennial ryegrass (1 species); SG – SmartGrass (6 species); Bio – Biomix (12 species); Her – Herbal (17 species). Boxes show median, middle 50% of data, and upper and lower quartile data range. Letters denote significant difference between forage mixtures at p<0.05.

2.4.2. AMF colonisation

Our model shows that 60% of the variability of AMF colonisation is explained by site (CRU WW and CRU dry) and forage mixture, with significant differences between sites, forage mixtures and their interaction (Table 2.5). The percentage of roots colonised by AMF increases with increasing plant species diversity, also, a higher AMF colonisation was found at the well-watered site than at the dry site (Figure 2.8). Post hoc Tukey tests show that the two most diverse mixtures (Biomix and Herbal) supported higher AMF colonisation of trap-plants than the less diverse forage treatments (Perennial ryegrass and SmartGrass, Table 2.6). All diverse mixtures at the well-watered site had significantly more AMF colonisation compared to the ryegrass mixture (SmartGrass z =6.37±0.05, $p<0.001$; Biomix z=5.72±0.05, $p<0.001$; Herbal z=6.66±0.05, $p<0.001$; Figure 2.8).

Table 2.5 Two-way ANOVA, including interaction term of site and forage mixture, on AMF colonisation. Site (Dry and Well-watered) and forage mixture (perennial ryegrass (1 species); SmartGrass (6 species); Biomix (12 species); Herbal (17 species))

	Df	Deviance	Resid. Df	Resid. Dev	P
Site	1	996.95	67	893.26	<0.001
Forage mixture	3	108.23	64	785.03	<0.001
Site:Forage mixture	3	34.49	61	750.53	<0.001

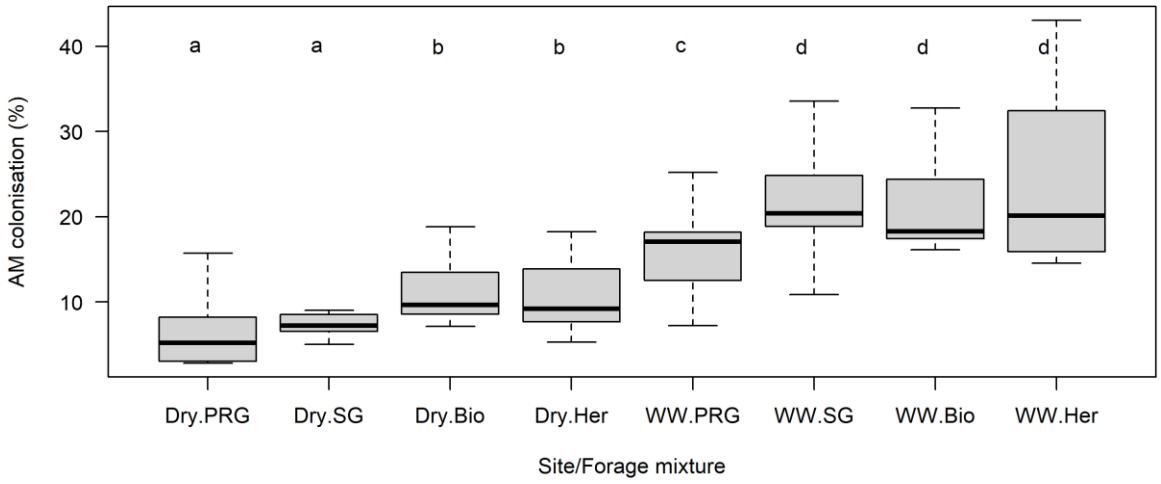

Figure 2.8 AMF colonisation of ryegrass trap-plant roots after 6-month field exposure at two differing sites (Dry and Well-watered (WW)) and four plant diversity forage mixtures (PRG – perennial ryegrass (1 species); SG – SmartGrass (6 species); Bio – Biomix (12 species); Her – Herbal (17 species)). Letters denote significant differences at p<0.05 identified by post hoc Tukey test analysis of a logistic regression model.

Table 2.6 Post hoc Tukey test results from logistic regression model of AMF colonisation of ryegrass trap-plants not taking site into account. Forage mixture treatments: PRG – perennial ryegrass (1 species); SG – SmartGrass (6 species); Bio – Biomix (12 species); Her – Herbal (17 species).

	estimate	**Std. error**	**Z value**	**P value**
PRG-SG	0.10897	0.04434	2.458	0.0663
PRG-Bio	0.27778	0.04251	6.534	<0.001
PRG-Her	0.22013	0.04227	5.208	<0.001
SG-Bio	0.16880	0.04090	4.127	<0.001
SG-Her	0.11116	0.04065	2.735	0.0315
Bio-Her	-0.05764	0.03864	-1.492	0.4418

Phosphorus, pH, OM and the C:N ratio all correlated with AMF colonisation significantly (summary model outputs Table 2.7). An increase in phosphorus availability decreased AMF colonisation ($\chi^2(1)=4.51$, $p=0.034$). An increase in OM correlated with AMF colonisation ($\chi^2(1)=5.279$, $p=0.022$), increasing colonisation by 0.252% ±0.110% standard error (Figure 2.9). Although pH and the C:N ratio were significant factors added to the GLMM, when compared against the likelihood ratio test, neither significantly correlated with AMF colonisation (pH: $\chi^2(1)=1.31$, $p=0.253$; C:N: $\chi^2(1)=3.593$, $p=0.058$).

Soil phosphorus showed significant interaction with the Herbal mixture at the well-watered site ($t=-2.20\pm0.11$, $p<0.05$) and the PRG at the dry site ($t=-2.11\pm0.03$, $p<0.05$), correlating with AMF colonisation.

Table 2.7 GLMM summary output of AMF colonisation success/failure matrix against fixed effects of site, phosphorus, pH, OM and C:N ratio and random effect of forage mixture. Family defined as binomial and integral scalar nAGQ set to zero.

	Estimate	**Standard error**	**Z value**	**Pr(>|z|)**
Intercept	-4.309	0.668	-6.450	1.12e-10
Phosphorus	-0.008	0.004	-2.119	0.034
pH	0.089	0.078	1.145	0.252
Organic matter	0.252	0.110	2.290	0.022
C:N ratio	0.077	0.040	1.901	0.057
Site – well-watered	1.206	0.364	3.310	0.001

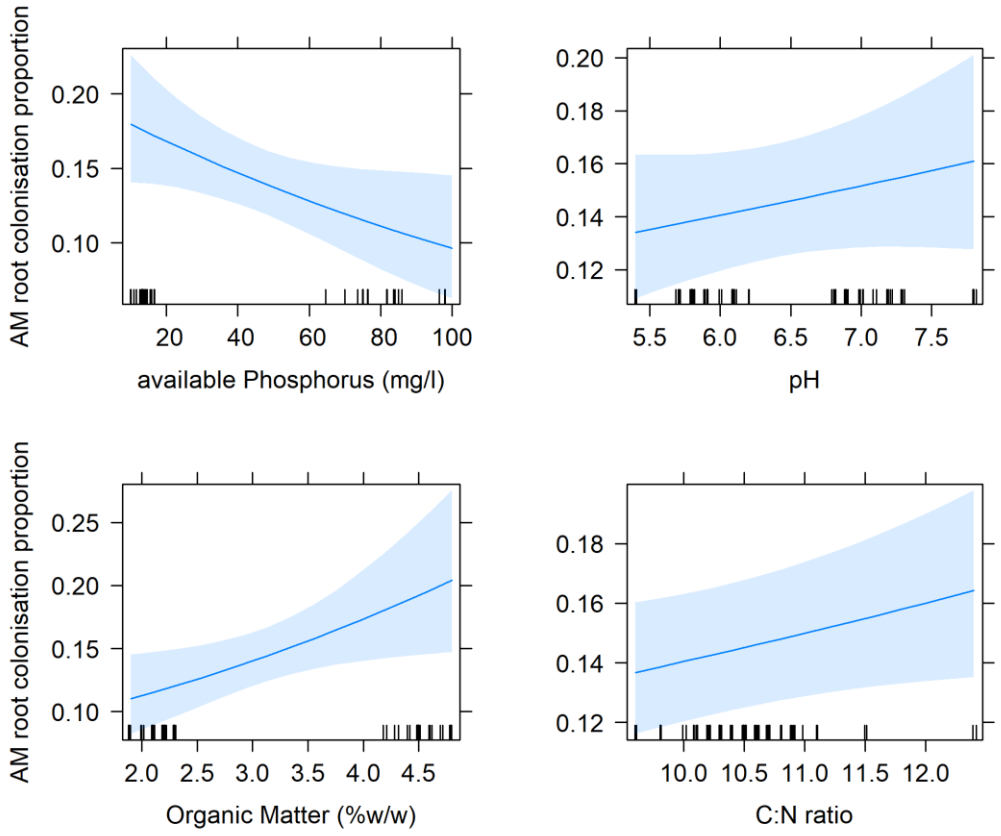

Figure 2.9 GLMM fits of fixed effects model of phosphorus, pH, organic matter and C:N ratio against AMF colonisation. Blue line represents the trend line; blue shading represents 95% confidence interval; vertical black lines on the x-axis indicate data points.

2.5. Discussion

2.5.1. Earthworms and mesofauna

Perennial ryegrass paddocks contained lower earthworm densities than the 12 species diverse forage mixture only. Several published studies on earthworms in long-term diverse grasslands have already suggested this effect (Spehn *et al.*, 2000; Eisenhauer and Scheu, 2008; Eisenhauer *et al.*, 2009). Diverse grasslands and their soils contain a higher variety of root exudates compared to monoculture, increasing OM input, which feeds the belowground microbial loop (Clarholm, 1985; Moore *et al.*, 2003). No difference was seen in earthworm species richness or adult earthworm abundance across the treatments, in contrast to the findings of Tsiafouli *et al*. (2015), who reported that earthworm and collembola richness was negatively

affected by land-use intensity. However, Edwards and Lofty (1982) showed nitrogen fertilisation increased earthworm populations, perhaps balancing out the non-effect seen between the earthworm results of the monoculture versus the diverse mixtures here.

A large proportion of European grasslands are grazed. The intensity and diversity of herbivore grazing have been shown to affect soil biota through the modification of soil processes such as nutrient cycling and grassland productivity (Bardgett & Wardle, 2003; Wang *et al*., 2019). This change has subsequent cascade effects on the whole soil food web (Bardgett, Wardle and Yeates, 1998; Bardgett and Wardle, 2003). Our study is of interest due to the sampling of soil biota under grazing cattle in the paddocks at CEDAR. The interaction between grazer activity and soil biota has not been included in most previous studies and, again, is not used as an experimental variable here. For example, both Eisenhauer *et al*. (2009) and Spehn *et al*. (2000) report on earthworms in grasslands mown for hay. However, our results of earthworms under grazing cattle showed little difference between the diverse treatments and the perennial ryegrass. This lack of effect of aboveground grazers on earthworms is important for the future of regenerative agricultural grassland systems as highly diverse forages may not necessarily be required to maintain these ecosystem engineers for benefits such as aerating and moving nutrients through the soil under grazed systems (Van Groenigen *et al.*, 2014).

Collembola abundance was significantly greater in the ryegrass monoculture compared to the 6 and 12-species grasslands. This was an unexpected result, as previous studies showed increased soil fauna activity under species-rich grasslands where diverse vegetation creates increased resource availability (Birkhofer *et al.*, 2011). Collembolas are opportunistic feeders whose main food resource is fungal hyphae; however, they are also known to eat roots and

nematodes (Hopkin, 1997). There was significantly greater root biomass in the 0-30cm section in the ryegrass monoculture compared to the 12 species Biomix. There was no difference in root biomass at that depth for the 6 species SmartGrass or 17 species Herbal mixture. Increased root biomass availability for collembolas' could explain why perennial ryegrass contains a higher collembola abundance than the Biomix mixture. The C:N ratio was higher in the perennial ryegrass monoculture compared to the SmartGrass 6 species grassland at the CEDAR paddocks. This potentially indicates a greater food resource availability for the collembola due to C:N ratio correlating with soil microbial biomass composition, with fungi dominating when C:N ratio is high and bacteria dominating when C:N is lower (van der Heijden *et al.*, 1998).

2.5.2. AMF colonisation

The colonisation of trap-plant roots by indigenous AMF increased with higher plant species richness. The C:N ratio of soil seen in this study shows that the monoculture plots may contain similar amounts of fungi as the two most diverse mixtures as no significant differences in the C:N ratios were seen between perennial ryegrass, Biomix and Herbal. Soil C:N ratios are known to be associated with fungal or bacterial dominance (van der Heijden *et al.*, 1998). However, the perennial ryegrass plots contained significantly lower colonisation rates than the two most diverse species mixtures at both sites, which may indicate lower fungal biomass in the monoculture plots. Donnison *et al*. (2000) showed nitrogen enrichment, which occurred only on the monoculture plots here, directly reduced fungal biomass. Low colonisation in the monoculture could be explained by perennial ryegrass forming selection preferences of fungal associations (Gollotte, Van Tuinen and Atkinson, 2004). The susceptibility of perennial ryegrass trap-plants to colonisation in the diverse mixtures could be due to a combination of interspecific plant competition and infection potential (Elton, 1958; Grime *et al.*, 1987). Briefly, ryegrass plants in diverse mixtures are in interspecific competition with other plant

species for resources. Allowing colonisation with beneficial fungi to increase rooting surface area to increase nitrogen, phosphorus and water uptake in a highly competitive environment is beneficial to the ryegrass trap-plant (van der Heijden *et al.*, 1998; Vogelsang, Reynolds and Bever, 2006). AMF associations could also benefit from the fact that no nitrogen fertiliser was applied in the diverse mixture plots. Unfertilised forages would increase plant competition for nitrogen acquisition, thus forming more symbiosis. Smaller and patchy nitrogen availability under the diverse mixtures may have required more mining for by the roots/AMF, benefitting AMF colonisation. However, our study's limitation is that the ryegrass monoculture is fertilised. Separating the effect of fertilisation and/or species diversity is not possible here.

More trap-plant AMF colonisation occurred at the well-watered site compared to the dry site. Again, several factors could be at play here. Soil moisture, OM and phosphorus differed between the sites, all shown to correlate with colonisation rate (Hepper and Warner, 1983; Smith and Smith, 2011; Jayne and Quigley, 2014). An increase in soil phosphorus significantly decreased AMF colonisation, easily explainable by more phosphorus in the soil, making it easier for the roots to access the nutrient themselves (Smith and Smith, 2011). There was a higher colonisation rate in the higher soil moisture site; this goes against what might have been expected with water being a limited resource (Jayne and Quigley, 2014). More interspecific competition between the plants at the well-watered site due to higher biomass production could favour AMF establishment (Elton, 1958; Grime *et al.*, 1987). Recent research showed that increased soil microbial diversity increased AMF colonisation (Ferreira *et al.*, 2021). Microbial analysis of the soils to decipher if the different mixtures contribute to fungal composition change to test whether this affects soil biota food resource availability would further the holistic understanding of this grassland system.

2.6. Conclusion

We show that earthworms were not affected by plant species diversity in grazed grassland paddocks in the short-term. In addition, higher mesofauna abundance was seen in a fertilised ryegrass monoculture than in diverse mixtures, both results rejecting our initial hypothesis. AMF ryegrass colonisation increased under diverse mixtures compared to monoculture; increasing rooting surface area is an important function required for future forage production under drier conditions we are already experiencing in the UK. With the future of pasture production aligning towards regenerative agriculture, it is important to research the effects of aboveground diversity on soil food web dynamics to ensure the maintenance of or, indeed, the improvement in ecosystem services soils provide us.

2.7. Supplementary material

Table S 2.1 Percentage dry matter weight contributions per forage family per forage mixture for the Spring 2019 aboveground biomass cut for the CRU plots (dry site and well-watered (WW) site)

		Forage mixture treatment							
	%	**Ryegrass**		**SmartGrass**		**Biomix**		**Herbal**	
		Dry	WW	Dry	WW	Dry	WW	Dry	WW
Forage	**Grass**	95	92	39	53	83	64	48	56
	Legume	-	-	0	36	2	30	3	37
	Herb	-	-	61	11	15	6	49	7
	Other	5	8	-	-	-	-	-	-

Chapter 3. Fungal community diversity and activity critical in future-proofing grassland forage production

Author initials: Sarah Shepperd[1] (SS), Rodica Pena[1] (RP), Zoe Barker[1] (ZB), Chris Reynolds[1] (CR), Mark Tibbett[1] (MT), Martin Lukac[1] (ML), Deborah Beaumont[2] (DB), Tom Misselbrook[2] (TM), Hannah Jones[3] (HJ)

Author contribution: ML, CR, DB, TM and HJ conceived and implemented the field experiment. SS conceived the study, conducted data gathering, with supplementary data collected by ZB. SS performed statistical analysis and wrote the article. RP, MT and ML commented on the manuscript.

[1]*University of Reading*
[2]*Rothamsted Research*
[3]*Duchy College*

3.1. Abstract

Ryegrass monocultures contribute to environmental degradation by their substantial need for external inputs to maintain productivity. Diverse grasslands, however, can provide forage at a lower environmental cost whilst also supplying many ecosystem services. Ample aboveground biomass in diverse grasslands is partly due to the stimulation of soil fungal communities. Here we compare a conventional ryegrass monoculture to three differing diverse grasslands (6, 12 and 17 plant species) in their effect on fungal diversity, functionality and ecosystem service delivery of aboveground biomass production in situ under a business as usual ('well-watered' site) and a future climate change scenario ('dry' site). Our conventional ryegrass monoculture (*Lolium perenne* L.) received nitrogen fertiliser (250kg/ha N), while the

three diverse grasslands received no fertiliser. Fungal community composition at both sites showed differences between the ryegrass monoculture and diverse mixtures (dry: R^2=0.254, $F_{3,12}$=1.359, p = 0.005; well-watered: R^2=0.243, $F_{3,12}$=1.285, p=0.015), along with differences in catabolic function at both sites. Fungal Shannon diversity increased due to higher forage diversity at the dry site only ($F_{3,12}$ = 12.12, p=0.0006), possibly indicating larger dependence of plant growth on a symbiotic fungal community under dry, stressed conditions. AMF colonisation was the only factor explaining the increase in aboveground forage production at the dry site ($\chi^2(1)$=5.3129, p=0.037), whereas, at the well-watered site, plant species diversity ($\chi^2(1)$=46.604, p<0.0001) and soil chemistry were significant factors (pH: $\chi^2(1)$=13.998, p=0.0028; OM: $\chi^2(1)$=6.5304, p=0.02521). Diverse grasslands enhanced the diversity of fungal communities under dry, stressed conditions, a finding important for future-proofing forage production. This result enhances ecosystem services through the increasing potential of AMF colonisation extending plant root surface area, enabling plants to increase access to water and nutrients, encouraging continued forage growth in drier conditions.

3.2. Introduction

Ryegrass (*Lolium* spp.) monocultures require unsustainable external inputs to maintain productivity yet dominate modern grassland systems (Crews and Peoples, 2004). Alongside providing feed for ruminants, managed grasslands can also offer a wealth of ecosystem services if managed for such, for example, carbon sequestration and nutrient cycling(Hopkins and Wilkins, 2006; Milne *et al.*, 2015). Continuous investment of energy and mineral resources in the form of fertilisers and herbicides needed in ryegrass monocultures causes environmental damage and biodiversity decline (Tilman, Fargione, *et al.*, 2001; Crews and Peoples, 2004; Foley *et al.*, 2005; Schindler, 2006; Cordell, Drangert and White, 2009; Elser, 2012). In addition, it reduces grassland and its associated soils' ecosystem services and increases long-

term natural capital costs (Fillery, 2001; Hopkins and Wilkins, 2006; Cong *et al.*, 2014; Wagg *et al.*, 2014). Discussions about moving away from high-intensity farming practices to systems with more environmental benefits are gaining prominence (DEFRA, 2018; Harris and Ratnieks, 2021). However, these new systems must achieve environmental and food system goals while facing extreme weather events such as drier, warmer summers (Lowe *et al.*, 2019). Regenerative agriculture contributes to these goals by producing livestock on diverse forages (Tilman, Reich, *et al.*, 2001; Hammond *et al.*, 2014; Teague and Kreuter, 2020). The approach aims to improve soil health by increasing biodiversity and enhancing ecosystem service delivery (Schreefel *et al.*, 2020), which has already been shown to increase farming system resilience to climate change (Tilman, Reich and Knops, 2006; Bardgett and Caruso, 2020).

Diverse forage mixtures are beneficial for both above and belowground grassland components, the link between the two parts is well-established (Bardgett, Wardle and Yeates, 1998; Hooper *et al.*, 2000; Spehn *et al.*, 2000; Brockwell *et al.*, 2005; Sanderson *et al.*, 2005; Skinner, 2008; Steinbeiss *et al.*, 2008; Wagg *et al.*, 2014; Hammond *et al.*, 2014; Van Groenigen *et al.*, 2014; Skinner and Dell, 2016). Benefits of diverse grasslands include increased aboveground forage production compared to lower diverse mixtures, even in dry years, as well as an extended grazing season (Tilman and Downing, 1994; Tilman, Wedin and J. Knops, 1996; Hector *et al.*, 1999; Tilman, Reich, *et al.*, 2001; Hooper *et al.*, 2005; Sanderson *et al.*, 2005; Hammond *et al.*, 2014). As a result of the presence of specific plant species or plant functional groups, e.g. legumes, their inclusion increases soil nitrogen availability and plant productivity (De Deyn *et al.*, 2009). Belowground benefits of increased plant species diversity include increased organic matter (Abbott and Manning, 2015), which benefits the soil microbial population (Clarholm, 1985; Spehn *et al.*, 2000). Such soil microbial populations include fungi, which were shown to enhance and stabilise grassland system productivity (Gordon, Haygarth

and Bardgett, 2008). Arbuscular mycorrhizal fungi (AMF) associate with 80% of terrestrial plants, the benefits of this association include increased plant nutrient supply and resistance to drought (Smith and Read, 2008; Acosta-Martínez *et al.*, 2014). AMF can double grassland aboveground biomass by increasing plant phosphorus supply (van der Heijden et al., 1998; Vogelsang, Reynolds and Bever, 2006) and reducing plant competition (Grime *et al.*, 1987). Aboveground biomass production is also increased in the presence of legumes and AMF as nitrogen is transferred from legumes to grasses through fungal hyphae (Haystead, Malajczuk and Grove, 1988). The ability of forage pastures to extend their rooting systems through symbiosis with fungi is an essential requirement for maintaining forage productivity in drier, warmer summers (Lowe *et al.*, 2019). Increasing plant root surface area increases the ability of the plant to obtain water, enabling continued plant growth.

Many studies show the benefits diverse grasslands have on belowground biodiversity under long-term grassland systems (Bardgett, Wardle and Yeates, 1998; Spehn *et al.*, 2000; Orwin and Wardle, 2005; Steinbeiss *et al.*, 2008; Cong *et al.*, 2014; Lange *et al.*, 2015; Skinner and Dell, 2016; W. Chen *et al.*, 2019). In a positive feedback loop, biodiverse soils enhance aboveground biodiversity and productivity (van der Heijden *et al.*, 1998; Vogelsang, Reynolds and Bever, 2006; Van Groenigen *et al.*, 2014; Tsiafouli *et al.*, 2015; Kariman, Barker and Tibbett, 2018). For example, increased fungal diversity increases plant diversity by increasing the abundance of subordinate herbs (Grime *et al*., 1987). With grassland diversity gaining prominence as an alternative to monocultures, understanding fungal community changes in situ and under future climate change scenarios in the short term is an important research requirement. Here we look at the effects of a short-term conventional monoculture and commercially available diverse grassland forage production on fungal community composition, functionality, and diversity at two sites. We hypothesise that (H1) unfertilised

diverse forage pastures differ in soil fungal composition, fungal species diversity and microbial functional diversity, from a conventional ryegrass monoculture fertilised with nitrogen. Further, we posit that (H2) increasing aboveground species diversity positively affects soil fungal composition and diversity (species and functionality), increasing ecosystem service provision of aboveground biomass, particularly under more dry stressed environments as plants are more likely to invest in a fungal symbiosis to help improve their water and nutrient uptake in highly competitive settings.

3.3. Methods

3.3.1. Experimental set up

Two locations within the University of Reading Crops Research Unit (CRU) farm in Sonning, Berkshire (51º28'22.4"N 0º54'15.3"W) were selected for their varying soil types. One site was an excessively drained shallow light sandy loam on a gravel bed, while the other was a free-draining loamy coarse sand close to the river Thames (Cranfield University, 2019). The first site usually experiences severe drought in the summer (2% soil moisture as of June 2018), hereafter referred to as the dry site. The second site can typically support crop growth throughout the growing season without irrigation (7% soil moisture as of June 2018), hereafter referred to as the well-watered site. Both sites were under arable management prior to the establishment of this experiment. Forage mixtures were sown in September 2016 and ranged from a single species perennial ryegrass (Lolium perenne) control to three diverse forage mixtures of increasing species richness: SmartGrass (6), Biomix (12), and Herbal (17 species; Table 3.1 and Table 3.2). Perennial ryegrass plots received 250kg/ha N (as ammonium nitrate), divided into four applications: two at 75kg/ha N and two at 50kg/ha N. Diverse species mixture plots received no nitrogen fertiliser. Four replicate plots 4.2 x 5m in size of the four forage

mixtures were sown in a 4x4 Latin square design at both sites. The management regime of all plots simulated animal grazing by hand cutting when biomass reached 2500 kg/ha dry matter from May until September, leaving 7cm height residual forage three times per year on average.

Table 3.1. Diverse forage mixture species selection list (PRG: Perennial Ryegrass; SG: SmartGrass; Bio: Biomix; Her: Herbal) sown September 2016 at the University of Readings Crops Research Unit Farm, Berkshire (4x4 Latin square). Ryegrass receives 250kg/ha N (as ammonium nitrate), divided into four application timings across the year: two at 75kg/ha N and two at 50kg/ha N. Diverse mixtures receive no nitrogen fertiliser

Species	**Latin**	**PRG**	**SG**	**Bio**	**Her**
Perennial Ryegrass	*Lolium perenne* L.	✓	✓	✓	✓
Timothy	*Phleum pratense* L.		✓	✓	✓
Cocksfoot	*Dactylis glomerata* L.			✓	✓
Festulolium	-			✓	✓
Tall Fescue	*Festuca arundinacea* Schreb.				✓
Meadow Fescue	*Festuca pratensis* Huds.			✓	✓
Red Clover	*Trifolium pratense* L.		✓	✓	✓
White Clover	*Trifolium repens* L.		✓	✓	✓
Alsike Clover	*Trifolium hybridum* L.			✓	✓
Sweet Clover	*Melilotus* spp.				✓
Black Medick	*Medicago lupulina* L.			✓	
Lucerne	*Medicago sativa* L.			✓	
Sainfoin	*Onobrychis spp.*				✓
Birdsfoot Trefoil	*Lotus corniculatus* L.				✓
Plantain	*Plantago lanceolata* L.		✓	✓	✓
Chicory	*Cichorium intybus* L.		✓	✓	✓
Yarrow	*Achillea millefolium* L.				✓
Burnet	*Sanguisorba minor* Scop.				✓
Sheep’s Parsley	*Petroselenium crispum* Mill.				✓

Table 3.2 Percentage seed mass contribution per forage family per forage mixture at time of sowing September 2016

		Treatment			
	%	**Ryegrass**	**SmartGrass**	**Biomix**	**Herbal**
Forage	Grass	100	86	69	40.5
	Legume	-	8.5	22.5	38.5
	Herb	-	5.5	8.5	21

3.3.2. Sampling

Soil sampling

Five 10cm deep soil cores were taken in a W pattern per plot and mixed to create a composite sample; this was repeated three times for the three different analyses required. The soil corer was sprayed with a 1% virucidal disinfectant Virkon (Lanxess, Cologne, Germany) and rinsed with deionised water between plots for aseptic sampling. Soil samples destined for DNA analysis were taken in September 2020, four years after plot establishment, and frozen at -20ºC before further analysis. Samples for the MicroResp™ experiment to measure soil microbial functional diversity and chemical analysis were taken in July 2021. Samples destined for the MicroResp™ were 2mm sieved for homogenisation and stored at 4ºC for 72 hours. Subsamples were taken for water holding capacity (WHC) and soil moisture measurements. Briefly, a subsample of fresh soil was saturated in water overnight and allowed to drain for 8 hours the next day. A weighted subsample of soil was placed in an oven set to 105ºC until no further water loss occurred (24 hours). Dried soil was reweighed to work out WHC. For soil moisture, a subsample of fresh soil was weighed, heated at 105ºC for 24 hours and reweighed. Samples destined for chemical analysis had roots and stones removed, and soil was dried and ground before being sent to NRM Laboratories (Cawood Scientific Limited, Bracknell, UK).

Additional soil sampling occurred where three 10cm deep soil cores were taken in late spring 2020 from each plot and mixed to create a composite sample. Samples were dried, passed through a 2mm sieve, and sent to NRM Laboratories (Cawood Scientific Limited, Bracknell, UK) for complete chemical analysis.

Earthworms

One random 20x20x20cm soil pit per plot was excavated in March 2020 at the dry and well-watered site three and a half years after sward establishment. Excavated soil was placed onto a 90cm x 40cm plastic sheet in the field, where the soil cube was gently teased apart for 15 minutes per pit to extract earthworms. Extracted earthworms were weighed, including half earthworms.

Roots

Soil cores (8cm diameter) were taken at the well-watered site at 4 depths (0-15cm, 15-30cm, 30-45cm, 45-60cm) in the autumn of 2019, three years after plot establishment. Soil cores were submerged in water and soaked for 24 hours to ease the separation of roots from the soil. Roots were washed clean of soil, suspended and spread in clean water in a clear plastic tray and scanned. Roots were dried at 50ºC to constant weight for dry biomass measurements.

WINRHIZO (Regent Instruments Canada Inc) software was used to process scanned images. Calibration using the object of known dimensions method was used to obtain accurate readings for the root surface area data outcomes. Batch image analyses was used with settings detailed in Table 3.3.

Table 3.3 criteria settings for the WINRHIZO software (Regent Instruments Canada Inc) for root samples taken in the autumn of 2019 at the well-watered site

Criteria	**Setting**
Greyscale	Yes
Root and background	Automatic
Rough edges	Off
Debris	1.5
L:W	7.5
Lagarde	64 px

Aboveground biomass

Three 50x50cm quadrat cuts per plot were taken three times during the growing season, each time leaving a residual height of 7cm. The material was dried at 60ºC for 72 hours. Aboveground biomass results are shown as the total of all three cuts and converted into tonnes per dry matter weight per hectare (t DM/ha).

Arbuscular Mycorrhizal colonisation

Sterile sand (Sibelco UK Ltd) absorbent clay substrate was created as 4 parts sand to 1 part Terra Green (Oil Dri UK), with 0.025 g/kg calcium hydrogen orthophosphate and 10% deionised water. The mixture was autoclaved at 105ºC for 1 hour, rested for 24 hours, and then autoclaved again at 105ºC for 1 hour. Five-centimetre diameter hydroponic pots were placed within the same size closed bottom cups, and each was filled with 140g of the sterile substrate. Afterwards, 14ml deionised water and 20mg ryegrass seeds (Cotswold Seeds Ltd, UK) were added at 1cm depth into the substrate.

Five pots per sunbag (Sigma-Aldrich Inc., Germany) were placed in a growth cabinet set to 22°C 16-hour days and 15°C 8-hour nights at 75% humidity for 1 month. Sunbags are transparent plastic bags with 0.02µm pores to enable plant growth and prevent contamination from the external environment, in this instance, the sunbags were used to prevent fungal contamination. Pots were fertilised with 1ml Long Ashton solution, a nutrient solution which contains no phosphorus, and 1ml deionised water twice over the 4 weeks the plants were in the growth cabinets.

One ryegrass pot per sun bag was checked for mycorrhizal colonisation to confirm ryegrass plants planted in the field were free from mycorrhiza. All plants were removed from the growth cabinet and given one week to acclimatise to ambient conditions in a sheltered outdoor space while remaining in the sun bags to ensure no mycorrhiza colonisation prior to planting in the field.

In late March 2020, three and a half years after establishing the plots, two trap plants were planted per plot at the two sites (N=64). Ryegrass trap plants were removed from the closed-bottom pots but retained in the hydroponic pots and sterile substrate. Plants were given 5ml of deionised water upon planting and monitored to ensure field establishment. Monitoring of the trap plants continued through the spring and summer 2020.

Trap plants, retained within their hydroponic pots, and an extra 5x5x5cm of soil from below the hydroponic pots to capture new root growth were extracted from the plots in early October 2020 using a trowel sterilised in 1% virucidal disinfectant Virkon S (Lanxess, Cologne, Germany). Trap plants and associated soil were kept at 4°C before processing.

Roots were washed free of soil, placed in 10% KOH, and left at room temperature for 3 days. Roots were then rinsed with deionised water and stained with Ink/Vinegar solution (1-part Shaeffer black ink to 19 parts white vinegar) for 1 hour at room temperature. Roots were rinsed again using deionised water and stored in lactoglycerol (1-part lactic acid, 2 parts glycerol, 1 part water; Walker and Vestberg, 1994). Percentage root AMF colonisation was measured using the grid line intersect method (Giovannetti and Mosse, 1980) with a compound microscope.

Soil chemical analysis

Soil chemical analyses were conducted by NRM Laboratories (Cawood Scientific Limited, Bracknell, UK). Chemical analysis on soil samples from July 2021 included total carbon and total nitrogen determined through combustion in pure oxygen at 1200ºC. The resultant gas mixture led through a splitter with the carbon dioxide gas measured by Infra-Red (IR) detector. Nitrogen oxide gas was reduced to nitrogen by passing through a copper reduction oven, and nitrogen was determined by a thermal conductivity detector, the Dumas method (AOAC Official Method, 1997). *Chemical analysis of soil samples taken in spring 2020 included pH measured in water [1:2.5], available phosphorus following Olsen and Sommers (1982) and Prokopy (1995). Ammonium nitrate extractable method was used to produce soil available potassium and magnesium and determined by ICP-OES (Knudsen, D., Peterson, G. A. and Pratt, 1982; Soltanpour, Benton Jones, Jr. and Workman, 1982). Loss on ignition at 430ºC was used for OM.*

DNA extraction and amplification

DNeasy powersoil pro kit (QIAGEN LLC, Hilden, Germany) was used to extract DNA from the dry and well-watered soil samples at the University of Reading: 0.25g of soil was weighed, and the DNeasy powersoil pro kit extraction protocol followed, using the Tissuelyser II (QIAGEN, Hilden, Germany). Samples were checked for DNA yields using 1.5µl of the sample on the NanoDrop 2000/2000c spectrophotometer (ThermoFisher Scientific, Waltham, Massachusetts, US). Extracted DNA samples were adjusted to 10ng/µl using elution buffer and stored at -18ºC before amplification.

Fungal community analysis was performed at LGC Group (Teddington, UK) for ITS data generated from amplicon sequencing on 32 DNA samples using a two-step PCR approach with primer pair ITS1FKyo2 (TAGAGGAAGTAAAAGTCGTAA) and ITS86R (TTCAAAGATTCGATGATTCA) run on illumina Inc (San Diego, CA, US) MiSeq v3 pair-end sequencing of 2x300bp. The second amplification used i7- and i5- sequencing adaptors. First amplification settings were 1-minute 96ºC pre-denaturation followed with 30 15s cycles at 96ºC denaturation, 58ºC 30s annealing, 68ºC 90s extension, 70ºC 2-minute final extension and 8ºC final hold. The second amplification followed the first amplification process with annealing changed to 3 cycles at 50ºC followed by 7 cycles at 58ºC. The process involved demultiplexing, clipping, primer detection and forward and reverse reads using BBMerge. Amplicon pre-processing included chimera removal, resulting in high-quality reads clustered into operational taxonomic units (OTU) picked using Mothur at the 97% identity level. The total number of reads came to 638,574, an average of 19955.438 ± 22.404 sd per sample (Table 3.4). Taxonomic classification of the fungi was processed against the UNITE database, totalling 626,983 fungal sequences obtained from the 32 samples.

Table 3.4 OTU counts per sample for the two CRU sites per forage mixture (PRG: perennial ryegrass; SG: SmartGrass; Bio: Biomix; Her:Herbal)

	Dry site				**Well-watered site**			
	Sample number							
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**
PRG	19947	19961	19965	19968	19941	19953	19954	19956
SG	19953	19954	19970	19967	19894	19962	19968	19967
Bio	19931	19952	19958	19965	19954	19960	19964	19971
Her	19863	19961	19975	19966	19956	19976	19973	19969

MicroResp™

Soil samples were wetted to the same moisture content (40% WHC as per Campbell *et al.*, 2003). Each soil sample was used to fill 24 deepwells per plate, with the plate weighed after each filling. Fully filled and parafilmed deepwell plates were placed into a container with soda lime and wet paper towel for 3 days at 25ºC to allow the soil to settle after the initial disturbance.

Indicator solution was made with 18.75mg cresol red, 16.77g KCl and 0.315g $NaHCO_3$ dissolved by heating in 900ml water. Agar gel was made with 3g agar dissolved in 100ml water. Indicator gel solution was created in a 1:2 agar gel to indicator solution ratio. 150µl of the indicator gel was pipetted into each well of a microplate. Microplates were covered with parafilm and placed in a desiccator with soda lime and a beaker of water in the dark until use.

Carbon substrates chosen were malic acid, citric acid, D-(+)-glucose, D-(+)-galactose, α-ketoglutaric acid, ϒ-amino butyric acid, L-arginine and a water blank (Campbell *et al.*, 2003;

Creamer *et al.*, 2009, 2016; Andersen *et al.*, 2013; Moscatelli *et al.*, 2018). Substrates were prepared as 30mg g^{-1} of soil water calculated from the prior weighing of the deepwell plates during soil addition.

25µl of each substrate was pipetted into each of the soil containing deepwells (3 replicates per substrate). Initial measurement for time zero was taken with the microplate filled with indicator gel on the SpectraMax i3x (Molecular Devices Limited, San Jose, CA, US) with absorbance set to 570nm. The read microplate was clamped together and divided with a rubber seal with the substrate/soil filled deepwell plate for 6 hours at 25^{o}C. The microplate was reread after the 6 hour incubation to measure final absorbance, again set to 570nm.

Multiple substrate-induced respiration (MSIR) was calculated by subtracting the water blank basal rate from the substrate-induced respiration values recorded as µg C-CO_2 g^{-1} hr^{-1} (formula in section 3.7). Microbial functional diversity values were calculated from the Shannon-Weiner biodiversity (H_{mic}) derived from substrate use (Klimek *et al.*, 2016).

3.3.3. Statistics

All statistics were performed using R studio statistical software (R Core Team, 2018) with ggplot2 package (Wickham, 2016) to create graphs.

A non-metric multidimensional scaling (NMDS) graph using Bray-Curtis dissimilarity with the metaMDS function in the vegan package (Oksanen *et al.*, 2020) was used to visualise fungal community structures across the forage treatments and sites separately (k=2 at both

sites). The permutational multivariate analysis of variance (PERMANOVA) using the Adonis function was used and homogeneity assumptions checked with the betadisper function to test for differences in the fungal community structures. Supporting data correlation effects, including soil chemistry and Microresp data, were plotted on the ordination if $p<0.05$ using the 'envfit' function on 999 permutations.

ANOVAs were performed on the Shannon diversity indexes on the fungal OTU, MSIR and microbial functional diversity against site and forage mixture. Post hoc Tukey tests using the agricolae package (Mendiburu, 2021) was used to identify the significant effects of forage mixtures.

Linear models were performed separately on aboveground biomass production at both sites using extension package lme4 (Bates *et al*., 2015) and lmerTest (Kuznetsova, Brockhoff and Christensen, 2017). Stepwise regression was used to build the minimum adequate models using the add1() command, which added forage mixture, pH and organic matter to the well-watered site model and only AMF colonisation was added to the dry site model. Model assumptions and normality were checked by plotting residuals against fitted values and using the Shapiro test. Interaction terms were checked, none were found significant. Several likelihood ratio tests were performed with the fitted models using ANOVA, comparing the full model against the model minus the parameter being looked at to test for significance. Extension package effects (Fox and Weisberg, 2019) and gridEXTRA (Auguie, 2017) were used to create graphs of the significant variables added to each model.

3.4. Results

3.4.1. All fungi

Bulk soil DNA extraction and amplification resulted in 760 fungal OTU lineages classified into the 5 phylum's (Ascomycota, Basidomycota, Chytridiomycota, Glomeromycota and Zygomycota; Figure 3.1).

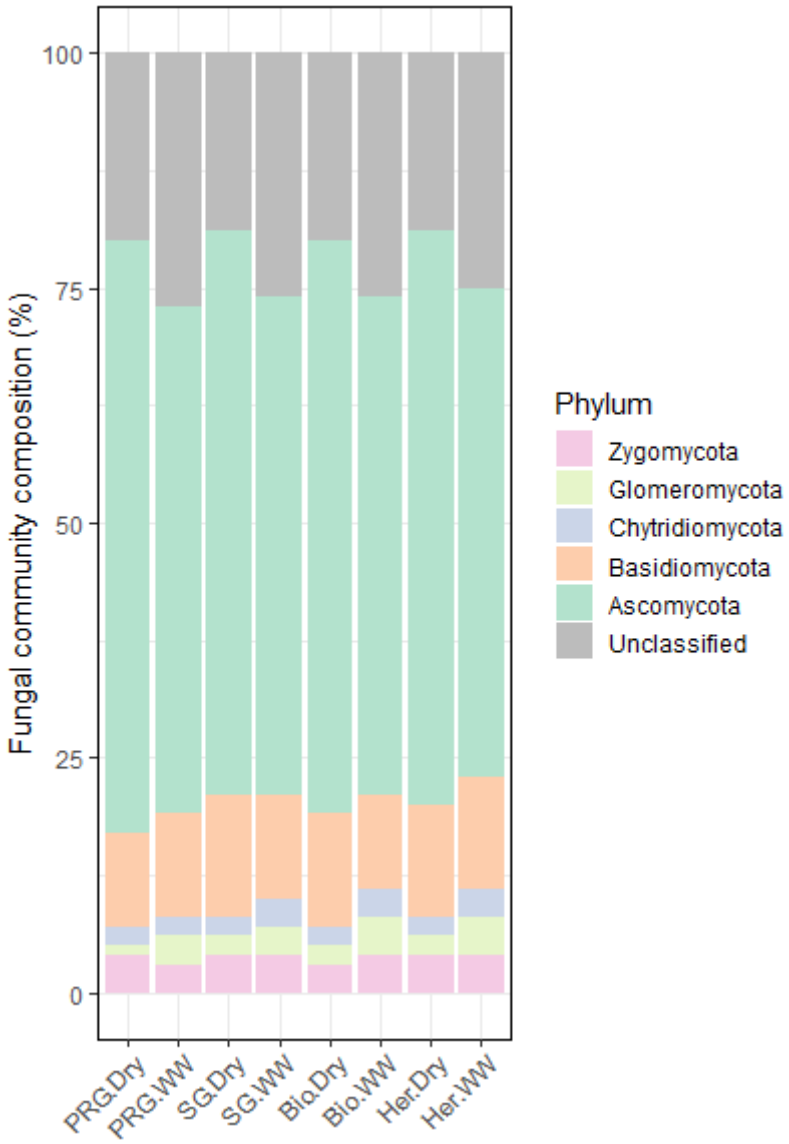

Figure 3.1. Phylum fungal community composition at the two study sites: dry and well-watered (WW). Forage mixture: PRG – perennial ryegrass (1 forage species); SG – SmartGrass (6 species); Bio – Biomix (12 species); and Her – Herbal (17 species).

NMDS plots (Figure 3.2) show clustering of the fungal community composition in the PRG monoculture is distinctly different from the clustering of the two most diverse forage mixtures (Biomix and Herbal) at the dry site (R^2=0.254, $F_{3,12}$=1.359, p = 0.005) and all three diverse forage mixtures at the well-watered site (R^2=0.243, $F_{3,12}$=1.285, p=0.015). Supporting data correlating to the community structures at the dry site include soil moisture (p=0.014) and pH (p=0.001;Table 3.5). Carbon substrate usage from the MicroResp experiment correlating with the fungal community composition at the dry site includes galactose (p=0.031) and malic acid (p=0.005), whereas at the well-watered site only glucose showed a significant correlation (p=0.049;Table 3.5).

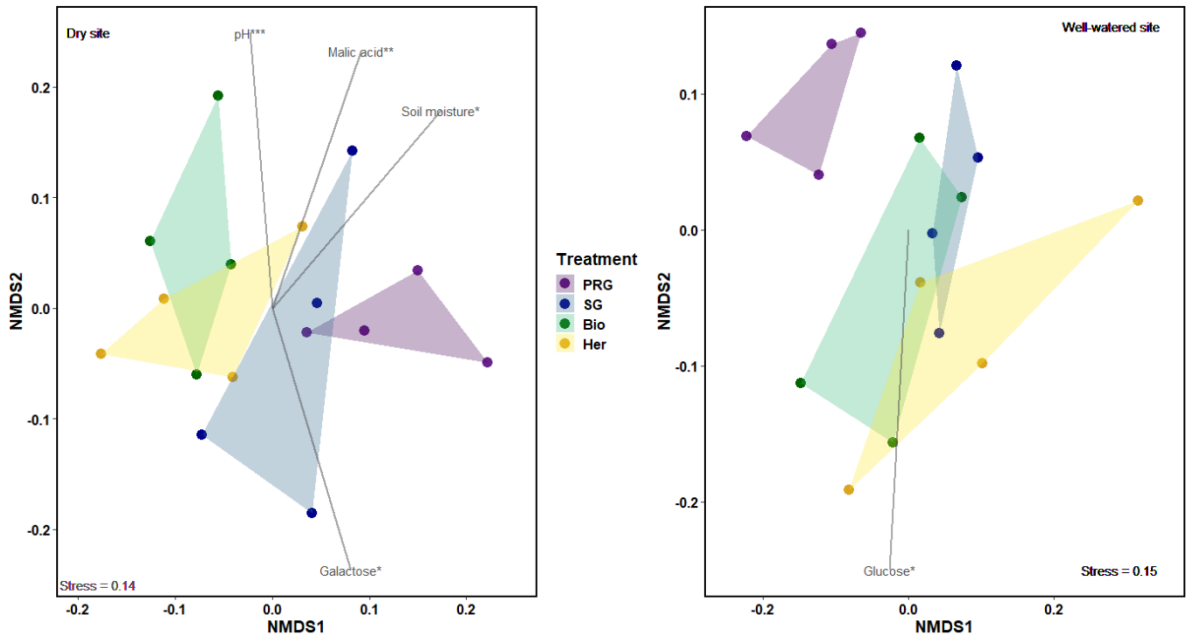

*Figure 3.2 non-metric multidimensional scaling (NMDS) graph of fungal community structures at the two study sites: a. dry site – PERMANOVA Adonis results R^2=0.254, p=0.005, $F_{3,12}$=1.359. b. well-watered site – PERMANOVA Adonis results R^2=0.243 p=0.015, $F_{3,12}$=1.285. Forage mixture treatments: PRG – perennial ryegrass (1 forage species); SG – SmartGrass (6 species); Bio – Biomix (12 species); and Her – Herbal (17 species). Supporting data plotted where p<0.05 (pH, soil moisture). Carbon substrate usage measured from multiple substrate induced respiration include malic acid, galactose and glucose. Significance level p<0.001***; p<0.01**; p<0.05**

Table 3.5 Ordination correlation variables R^2 and p values that showed significance at p<0.1 from NMDS at the two sites dry and well-watered

			Dry site		**Well-watered site**	
			R^2	**p**	**R^2**	**p**
Soil chemistry		AM colonisation	-	-	0.312	0.080
		Soil moisture	0.501	0.014	-	-
		pH	0.675	0.001	-	-
		Phosphorus	0.341	0.086	-	-
MicroResp experiment		H_{mic}	0.366	0.065	0.348	0.069
		Citric acid	-	-	0.317	0.092
		Galactose	0.397	0.031	0.348	0.072
		Glucose	-	-	0.372	0.049
		Malic acid	0.548	0.005	-	-

OTU Shannon diversity index at the dry site differed amongst forage mixtures ($F_{3,12}$ = 12.12, p=0.0006), with all diverse forage mixtures being more diverse than the perennial ryegrass monoculture (Figure 3.3). No differences were seen in the OTU Shannon diversity index at the well-watered site ($F_{3,12}$ = 0.382, p=0.768; Figure 3.3).

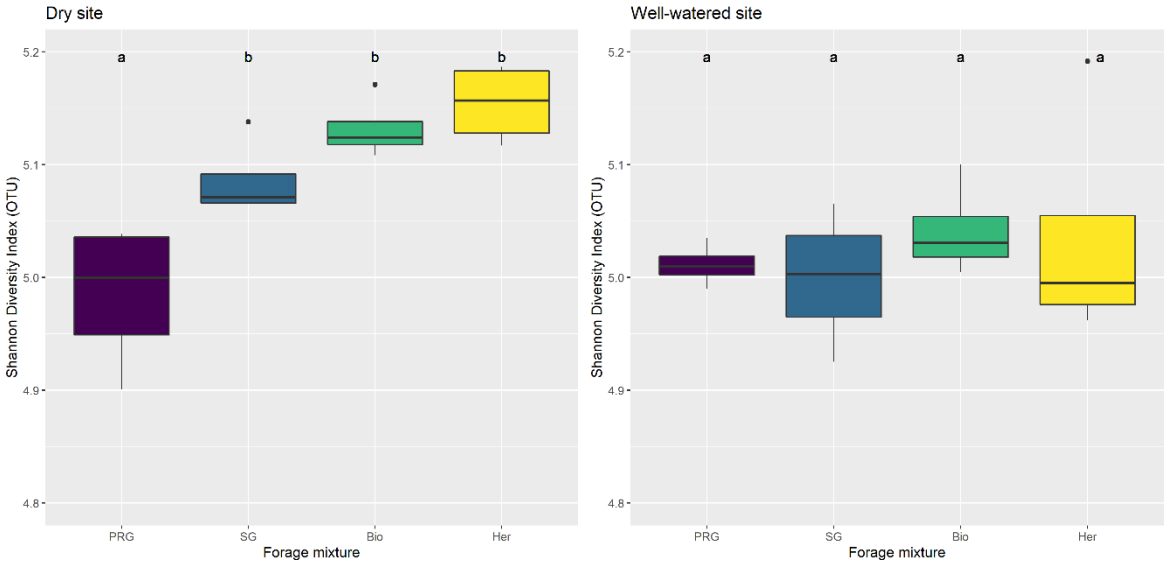

Figure 3.3 Shannon diversity index from fungal OTU at the two sites a. Dry ($F_{3,12}$ = 12.12, p=0.000608) and b. well-watered, ($F_{3,12}$ = 0.382, p=0.768) between the forage mixture. PRG – perennial ryegrass (1 forage species); SG – SmartGrass (6 species); Bio – Biomix (12 species); and Her – Herbal (17 species)).

3.4.2. MSIR/H_{mic}

Microbial functional diversity (H_{mic}) showed no differences amongst treatments or sites. MSIR showed differences in the dry site perennial ryegrass monoculture against the well-watered site ryegrass monoculture (p=0.027) and the well-watered Herbal treatment (p=0.044), with respiration rates being lower at the well-watered site (Figure 3.4).

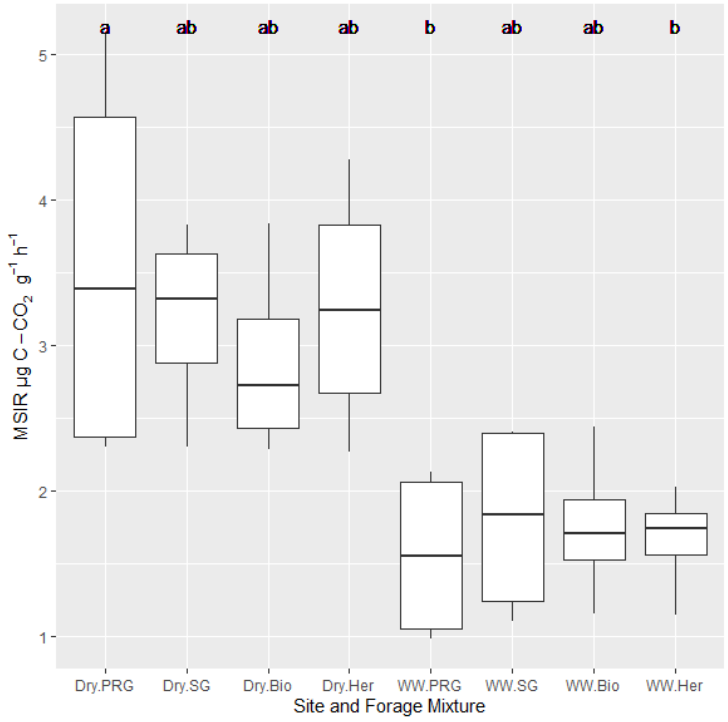

Figure 3.4 multiple substrate induced respiration (MSIR) ANOVA results from two sites (Dry and Well-watered (WW)) and four plant diversity forage mixtures (PRG – perennial ryegrass (1 species); SG – SmartGrass (6 species); Bio – Biomix (12 species); Her – Herbal (17 species)). Letters denote significant differences at p<0.05 identified by post hoc Tukey test

3.4.3. Provisioning ecosystem service aboveground biomass model

Our model for the dry site shows that 28% of the variability of aboveground biomass production was explained by AMF colonisation. The well-watered site model showed that 87% of the variability of aboveground biomass production was explained by forage mixture, pH and organic matter (Table 3.6). Root surface area, H_{mic}, MSIR, soil moisture, P, K, Mg, total N, total C, Labile C, worm biomass, OTU abundance and OTU Shannon diversity showed no significant correlations during stepwise regression phase so they were not added to the models (Table 3.7).

Aboveground biomass production increases with increasing plant species diversity ($\chi^2(1)=46.604$, $p<0.0001$) and increasing pH (from acidic to more neutral) ($\chi^2(1)=13.998$,

p=0.0028) at the well-watered site (Table 3.6; Figure 3.5). Increasing organic matter by 1%w/w increases aboveground biomass production by 1.064 t DM/ha±0.42 SE at the well-watered site ($\chi^2(1)=6.5304$, p=0.02521; Table 3.6; Figure 3.5). At the dry site, aboveground biomass production increases with increasing AMF colonisation ($\chi^2(1)=5.3129$, p=0.037), with a 1% increase in root AMF colonisation increasing aboveground biomass by 0.068 t DM/ha ±0.03 SE (Table 3.6;*Figure* 3.6).

Table 3.6 Well-watered and Dry site LM summary output of aboveground biomass production against significant variables added to the models

		Estimate	Standard error	Z value	Pr(>|z|)	Effect	Correlation coefficient
Well-watered	Intercept	-7.948	2.52	-3.158	0.008		
	Forage mixture	0.097	0.01	6.827	<0.0001	+ve	0.74
	pH	1.627	0.43	3.741	0.0028	+ve	0.13
	Organic matter	1.064	0.42	2.555	0.0252	+ve	0.58
Dry	Intercept	2.394	0.31	7.755	<0.0001		
	AMF colonisation	0.068	0.03	2.305	0.037	+ve	0.52

Table 3.7 Non-significant variables during the stepwise regression phase of the aboveground biomass models for the well-watered and dry site

	Well-watered site		Dry site	
	Sum of square	**P(>Chi)**	**Sum of square**	**P(>Chi)**
Forage mixture	-	-	0.110	0.499
Root surface area	0.006	0.764	-	-
H_{mic}	0.074	0.267	0.671	0.082
MSIR	0.045	0.390	0.024	0.753
Soil moisture	0.014	0.631	0.101	0.516
pH	-	-	0.006	0.871
P	0.045	0.390	0.000	0.975
K	0.035	0.446	0.234	0.319
Mg	0.014	0.635	0.409	0.183
Total N	0.269	0.255	-	-
Total C	0.176	0.079	-	-
Labile C	0.176	0.079	0.051	0.644
OM	-	-	0.004	0.892
Earthworm biomass	0.017	0.599	0.034	0.708
OTU abundance	0.099	0.197	0.017	0.794
OTU Shannon diversity	0.153	0.103	0.713	0.072
AMF colonisation	0.001	0.883	-	-

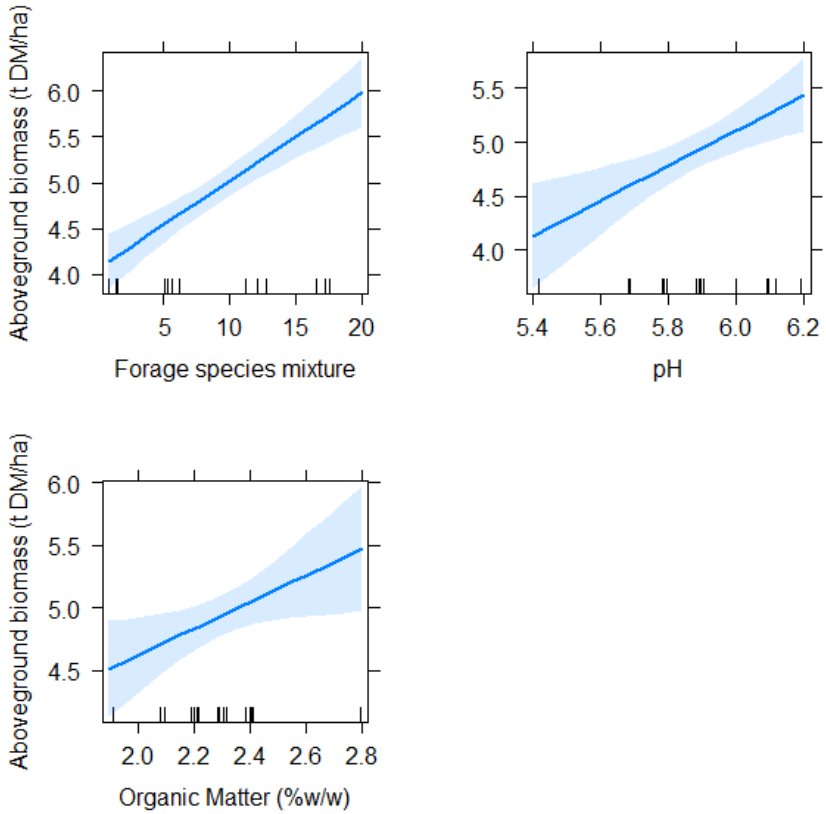

Figure 3.5 Linear model of a) forage species mixture, b) pH and c) organic matter against aboveground biomass production at the well-watered site. Blue line represents the trend line, blue shading represents 95% confidence interval, vertical black lines on the x-axis indicate data points

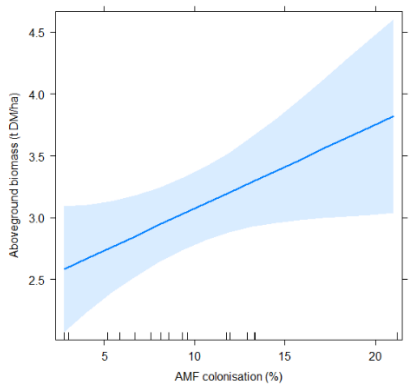

Figure 3.6 Linear model of AMF colonisation against aboveground biomass production at the dry site. Blue line represents the trend line, blue shading represents 95% confidence interval, vertical black lines on the x-axis indicate data points

3.5. Discussion

Fungal community composition differed between the perennial ryegrass monoculture and the diverse forage mixtures at both sites. At the dry site, pH correlated with the community

composition changes. Many previous studies show that pH is the main factor in soil microbial composition under different land use types (Wakelin *et al.*, 2008; Zhalnina *et al.*, 2015; Creamer *et al.*, 2016). Soil pH affects microbial activity and soil function (Creamer *et al.*, 2016). Our catabolic function observations from the dry site follow that from Creamer *et al.* (2016), where pH shows a negative correlation to galactose usage, with malic acid usage optimised in soils with more neutral soil pH. Soil moisture was also a significant factor in shaping the fungal community composition at the dry site only as shown by the supporting data plotted on the NMDS graph. This result is supported by findings from Meisner *et al.* (2018), who showed in a mesocosm experiment that droughting events influenced microbial community composition the most. No measured environmental factors correlated with the fungal community composition at the well-watered site, indicating that the fungal community compositional differences between the monoculture and diverse forage mixtures are likely due to plant species richness only. This result conflicts with previous work from Zhalnina *et al.*, (2015) where plant species richness of a long-term grassland experiment showed no relationship to microbial community composition, but soil properties influenced the microbial community. However, our Shannon diversity OTU observation at the well-watered site supports Zhalnina *et al.* (2015) findings that plant species richness showed no relationship to soil microbial diversity in a grassland system. However, our dry site results show that Shannon OTU diversity increased in the diverse forage mixtures. This indicates that under dry stressed environments plants invest more in their microbial community, intensifying microbial diversity and functionality.

Soil carbon content did not influence fungal community composition at either site. This observation is in disagreement with many studies which show that soil labile carbon, along with soil pH, is the main soil property affecting microbial composition, diversity and catabolic

function (Lagomarsino, Grego and Kandeler, 2012; Murugan *et al.*, 2014; Creamer *et al.*, 2016), with higher labile carbon promoting microbial diversity (Murugan *et al.*, 2014). Soil carbon content is shown to be greater under diverse grasslands (X. Chen *et al.*, 2019) 5 years after changing land use (Skinner and Dell, 2016), a result not seen here.

Future-proofing forage systems to maintain productivity under the weather events such as those we are already experiencing is required to satisfy the predicted increase in meat and dairy consumption (Crist, Mora and Engelman, 2017). Pasture production, controlled by nutrient availability, results from the interaction of top-down and bottom-up controls within the decomposer subsystem (Clarholm, 1985; Wardle, 2002). Our aboveground biomass model (section 3.4.3) showed that at the well-watered site, forage mixture type and soil chemistry of pH and organic matter were components in understanding the provisioning ecosystem service. These results further support previous research on diversity-productivity, where increasing plant species diversity results in resource partitioning, allowing for a longer growing season and greater aboveground biomass production with greater plant species diversity (Tilman and Downing, 1994; Tilman, Wedin and J. M. H. Knops, 1996; Hector *et al.*, 1999; Tilman, Reich, *et al.*, 2001; Hooper *et al.*, 2005; Sanderson *et al.*, 2005; Hammond *et al.*, 2014). pH was shown to impact aboveground biomass of forage production, with pH at either end of the scale negatively impacting production, with more neutral soils being amongst the most productive (Brady, 1990). Organic matter is also a well-established soil property contributing to soil health and thus ecosystem service provisioning (Abbott and Manning, 2015), where low plant species diversity decreases OM input (Spehn *et al.*, 2000).

Our experimental set-up was designed with the well-watered site as a business-as-usual scenario, whereas the dry site indicated future conditions. AMF colonisation was the only variable measured in this study that significantly correlated with aboveground biomass production modelled at the dry site. Forage mixture composition not being a factor in the model at the dry site contradicting established diversity-productivity studies is an interesting component for the future-proofing of our forage systems under drier and warmer summers (Lowe *et al.*, 2019). The inclusion of specific grassland plant species or functional groups contributes to ecosystem services, for example, nitrogen cycling from legumes (De Deyn *et al.*, 2009). In our study, all diverse forage mixtures contained legumes which increase soil nitrogen availability, typically enhancing plant productivity until soil phosphorus becomes the limiting factor (De Deyn *et al.*, 2009). The limitation of soil phosphorus availability increases AM inoculum potential and stimulates AM colonisation, driving further plant growth. This cycle heavily relies on the growth rate of the legume hosts, taxonomy and plant density, as these can all affect nitrogen fixation rates and therefore directly affect the AM community (Brockwell *et al.*, 2005).

Fungal diversity, particularly the presence of AMF, is a known driver of grassland community composition; higher fungal diversity increases plant diversity due to the increased exploitation of soil phosphorus (van der Heijden *et al.*, 1998), increasing the abundance of subordinate herbs over competitive grasses (Grime *et al.*, 1987). Plant productivity has been shown to double if associations with AMF are made, chiefly due to increased phosphorus uptake (van der Heijden *et al.*, 1998; Vogelsang, Reynolds and Bever, 2006). Fungal presence is further shown to contribute towards productivity in diverse grasslands as Haystead, Malajczuk and Grove, (1988) showed that nitrogen is transferred from legumes to grasses through fungal hyphae. Our result of increased aboveground biomass correlating with the

higher rates of AMF colonisation at the dry site is suggestive that these mechanisms of increased phosphorus and nitrogen uptake are taking place. Plant productivity was also shown to increase due to fungal functional complementarity (Maherali and Klironomos, 2007), with Jonsson *et al.*, (2001) showing plant productivity was led by fungal species identity, not fungal species richness. However, within a microcosm fungal diversity manipulation experiment, six species AM soil mixture did not produce greater plant biomass than the most productive single species mycorrhizal soil system, suggesting no functional complementarity existed (Vogelsang, Reynolds and Bever, 2006). Our results from Shannon OTU diversity increasing at the dry site under diverse grasslands indicate that increased plant diversity invest in the fungal community under dry stressed environments. Having shown the benefits fungi can bring to increasing aboveground biomass production, grassland systems more supportive of fungal communities are going to be an important requirement with future-proofing our forage systems.

3.6. Conclusion

Our results show that fungal community diversity and activity are enhanced under dry stress. This suggests a higher plant investment in the fungal community may enable it to cope with dry stress. However, plant investment is at least partially dependent on plant diversity. With the required move towards a more sustainable forage production system whilst maintaining forage productivity under drier, warmer summers, our research shows that fungal symbiosis is critical in maintaining aboveground biomass. Further research into which diverse forage mixture nurtures the critical fungal indicator taxa driving aboveground biomass under diverse grasslands is essential to fully comprehend our food systems' future-proofing.

3.7. Supplementary material

Table S 3.1 Percentage dry matter weight contributions per forage family per forage mixture for the Spring 2019 aboveground biomass cut for the CRU plots (dry site and well-watered (WW) site)

		Forage mixture treatment							
	%	Ryegrass		SmartGrass		Biomix		Herbal	
		Dry	WW	Dry	WW	Dry	WW	Dry	WW
Forage	**Grass**	95	92	39	53	83	64	48	56
	Legume	-	-	0	36	2	30	3	37
	Herb	-	-	61	11	15	6	49	7
	Other	5	8	-	-	-	-	-	-

Calculation for converting Microresp absorbance into respiration rate:

- Normalise the absorbance data:
  - Calculate the mean of t=0 readings for all the wells on the plate
  - For each well divide the t=6 reading by the t=0 reading and then multiply by the mean of t=0 readings.
- Convert absorbance to %CO2
  - Use the formula: $\%CO2 = \frac{A+B}{1+D \times Normalised\ Absorbance}$
  - Where A = -0.2265, B = -1.606, D = -6.771
  - These constants are based on the calibration done by the manufacturers. We need to calibrate ourselves (see Appendix 2)
- Convert %CO2 to to CO2 respiration rate
  - Use the formula: $\frac{\left(\frac{\%CO2}{100}\right) \times vol \times \left(\frac{44}{22.4}\right) \times \left(\frac{12}{44}\right) \times \left(\frac{273}{273+T}\right)}{\frac{Soil\ fwt \times \left(\frac{Soil\ \%\ dwt}{100}\right)}{I}}$
  - Where:
    - vol = the volume of the headspace in the deepwell (µl)
    - T = incubation temperature (°C)
    - Soil fwt = Fresh weight of soil per well (g)
    - Soil % dwt = The percentage dry weight of the soil
    - I = the incubation time (hours)

Chapter 4. Plant species diversity does not affect community composition and diversity of arbuscular mycorrhizal fungi in diverse grasslands

Author initials: Sarah Shepperd[1] (SS), Rodica Pena[1] (RP), Zoe Barker[1] (ZB), Chris Reynolds[1] (CR), Mark Tibbett[1] (MT), Martin Lukac[1] (ML), Deborah Beaumont[2] (DB), Tom Misselbrook[2] (TM), Hannah Jones[3] (HJ)

Author contribution: ML, CR, DB, TM and HJ conceived and implemented the field experiment. SS conceived the study, conducted data gathering, with supplementary data collected by ZB. SS performed statistical analysis and wrote the article. RP, MT and ML commented on the manuscript.

[1]*University of Reading*
[2]*Rothamsted Research*
[3]*Duchy College*

4.1. Abstract

Diverse grasslands used for livestock production could answer the current sustainability challenge in food production of net zero targets by 2040 and the necessary shift toward more environmentally responsible agriculture. Increasing plant diversity of grasslands shows environmental benefits and positively impacts agricultural production. The enhancement of aboveground biomass by diverse forage mixtures is widely acknowledged, however, little is known about how these affect the soil microbiome in situ. Arbuscular mycorrhizal fungi (AMF) are obligate symbionts, aiding plant nutrient uptake in exchange for carbon, contributing to aboveground production and climate change resilience by increasing plants rooting surface area. Here we compare the effects of three different commercially viable

unfertilised diverse grasslands (6, 12 and 17 plant species) on AMF community structure and diversity in both the soil rhizosphere and ryegrass trap plant roots. Operational taxonomic units were generated from amplicon sequencing processed against the UNITE and SILVA databases and the FUNGuildR package. No differences between AMF community composition and AMF Shannon diversity were seen between the diverse mixtures (R^2=0.27374, $F_{5,18}$=1.3569, p = 0.077). AMF Shannon diversity was higher in the roots than in the soil ($F_{1,22}$=6.178, p=0.021), indicating differences in AMF life history strategies. Our results suggest that the benefits achieved by AMF presence, such as increased forage productivity and resilience to drought, is seen equally under a 6-species grassland mixture and a 17-species mixture. This result is informative for land managers who need an option of a cost-effective, diverse forage mixture, balancing the seed cost and soil health benefits.

4.2. Introduction

Forage grassland management needs to be adapted to continue to provide feed for ruminants in the drier, warmer summers the UK is already experiencing and the predicted increase in meat and dairy consumption (Crist, Mora and Engelman, 2017; Lowe *et al.*, 2019). Increasing plant species richness in grassland systems could be an answer; many studies show increasing plant species richness increases aboveground biomass, even in dry years (Tilman and Downing, 1994; Tilman, Wedin and J. M. H. Knops, 1996; Hector *et al.*, 1999; Tilman, Reich, *et al.*, 2001; Hooper *et al.*, 2005; Sanderson *et al.*, 2005; Hammond *et al.*, 2014; Xu *et al.*, 2021). Plant species mixtures in diverse grassland provide additional benefits that increase farming systems' resilience to climate change (Tilman, Reich and Knops, 2006; Bardgett and Caruso, 2020).

Aboveground biomass production relies on maintaining nutrient availability at the right time and in the correct quantities. Enhanced aboveground productivity resulting from higher plant species richness is partly due to specific plant species or functional groups, such as legumes, which increase soil nitrogen availability (De Deyn *et al.*, 2009). Diverse grassland plant species, through phenotypic plasticity, have increased rooting depth and biomass (Steinbeiss *et al.*, 2008), increasing their ability to maintain or even enhance water uptake and nutrients, further aiding aboveground production. Higher soil biodiversity that dilutes species-specific pathogens can also maintain productivity in highly diverse plant communities (Scherber *et al.*, 2010). Overall, high above- and belowground biodiversity has economic and environmental benefits, for example, the ability to exploit soil phosphorus more efficiently (Oelmann *et al.*, 2021; Chen, Chen and Chang, 2022).

Plant species diversity impacts the belowground system and vice versa, with linkages and benefits well established, although sometimes not fully understood (Bardgett, Wardle and Yeates, 1998; Hooper *et al.*, 2000; Spehn *et al.*, 2000; Brockwell *et al.*, 2005; Sanderson *et al.*, 2005; Skinner, 2008; Steinbeiss *et al.*, 2008; Van Groenigen *et al.*, 2014; Wagg *et al.*, 2014; Hammond *et al.*, 2014; Skinner and Dell, 2016; Oelmann *et al.*, 2021). For example, increasing plant species richness provides more niches for beneficial arbuscular mycorrhizal fungi (AMF) (Waldrop *et al.*, 2006; Antoninka, Reich and Johnson, 2011). AMF are obligate symbionts that associate with 80% of terrestrial plant families aiding plant nutrient uptake in exchange for carbon (Kariman *et al* 2018). As a result of better niche exploitation, AM symbiosis has enhanced and stabilised grassland productivity (Gordon, Haygarth and Bardgett, 2008). Other benefits of AMF supply to plants include enhanced plant resistance to drought and pathogens, increased plant diversity, and reduced nitrogen loss (Jia *et al.*, 2021). These benefits are AMF-specific; for example, some AMF are more influential in their contribution to nutrient use

efficiency than others (Smith and Read, 2008). This ultimately relies on a specific plant and AMF combinations as plant ecological groups have distinct AMF communities, with grasses forming non-specific associations (Jansa, Smith and Smith, 2008; Davison *et al.*, 2020). Increased aboveground biomass production is supported directly through increased nutrient uptake and indirectly through reduced loss of plant species richness, as well as reduced nitrogen leaching (Grime *et al.*, 1987; van der Heijden *et al.*, 1998; Klironomos *et al.*, 2000; de Vries *et al.*, 2006; Vogelsang, Reynolds and Bever, 2006; Acosta-Martínez *et al.*, 2014).

Including legumes in diverse grasslands typically enhances plant productivity in mixtures until phosphorus becomes the limiting factor (De Deyn *et al.*, 2009). This is where AMF may be double beneficial, as they transfer nitrogen and enhance phosphorus recovery from the soil (Sobat and Whalen, 2022). This process relies heavily on the legume growth rate, sward taxonomy and plant density, all factors affecting the rate of nitrogen fixation and overall plant productivity (Brockwell *et al.*, 2005). Some grassland legume species require AMF associations to be successful, e.g. birdsfoot trefoil (*Lotus corniculatus*), black medick (*Medicago lupulina*) and red clover (*Trifolium pratense)*. These legumes support both AMF and nitrogen-fixing bacteria; through fungal hyphae, nitrogen can be transferred directly to grasses (Haystead, Malajczuk and Grove, 1988), boosting forage production.

AMF can resist various drought and heat conditions (Acosta-Martínez *et al.*, 2014). Warming experiments have shown increases in AMF colonisation (Rillig *et al.*, 2002), increases in AMF diversity (Kim *et al.*, 2014), and changes in certain AMF taxa abundances (Cao *et al.*, 2020). Kokkoris *et al.* (2020) predict increased co-dependency between plant and AMF communities in more stressed environments. This demonstrates AMFs' major

importance in ecosystem service provisioning under predicted shifts towards drier vegetative periods in some locations due to climate change (Zhao *et al.*, 2017). Increasing environmental stress drives changes in plant communities (Li and Shipley, 2018), which is also seen for AMF communities (Shi *et al.*, 2014). Tedersoo *et al.* (2014) showed that AMF and plant richness are partially driven by environmental variation, while Schappe *et al*. (2017) suggested that abiotic variables were stronger drivers for AMF communities than plant host identity. Soil carbon availability and fungal competition significantly affect AMF richness, as do species and stocking densities of grazers (Waldrop *et al.*, 2006; Antoninka, Reich and Johnson, 2011; Mendoza *et al.*, 2011).

The benefits of grassland diversity for aboveground forage production, together with the role of AMF in this process, are well established in the long term. We are missing a detailed description of these effects in productive systems in field conditions in the short term. Here we consider three commercially available forage mixtures of contrasting diversity under dry, stressed conditions *in situ* and observe their effects on the community structure and diversity of AMF in roots and rhizosphere soil. We hypothesise that (H1) the three diverse forage mixtures support different AMF communities within their soils and roots, (H2) increasing plant species diversity increases AMF diversity, and (H3) plant root colonisation by AMF increases with higher AMF soil diversity.

4.3. Methods

4.3.1. Experimental setup

n.b. experimental setup same as Chapters 2 and 3 but no perennial ryegrass monoculture plot data used for this Chapter and only CRU dry site

Forage mixtures were sown in September 2016 at the University of Reading Crops Research Unit (CRU) farm in Sonning, Berkshire (51º28'22.4''N 0º54'15.3''W). The excessively drained shallow light sandy loam site on a gravel bed (Cranfield University, 2019) usually experiences severe drought in the summer (2% soil moisture as of June 2018) was under arable management prior to this experiment. Three diverse forage species mixtures were established: SmartGrass (6 species), Biomix (12 species), and Herbal (17 species, (Table 4.1; Table 4.2)). Four replicate plots 4.2 x 5m in size of the 3 forage mixtures were sown in a Latin square design. The management regime of all plots simulated animal grazing by hand cutting when biomass reached about 2500 kg/ha dry matter, from May until September. The swards were typically cut three times per year, leaving 7cm height residual forage. Plots received no fertiliser addition due to legume inclusion (Table 4.1; Table 4.2).

Table 4.1 Diverse forage mixture species selection list (SG: SmartGrass; B: Biomix; H: Herbal) sown September 2016 at the University of Readings Crops Research Unit Farm, Berkshire. Diverse mixtures receive no nitrogen fertiliser

Species	**Latin**	**SG**	**B**	**H**
Perennial Ryegrass	*Lolium perenne* L.	✓	✓	✓
Timothy	*Phleum pratense* L.	✓	✓	✓
Cocksfoot	*Dactylis glomerata* L.		✓	✓
Festulolium	-		✓	✓
Tall Fescue	*Festuca arundinacea* Schreb.			✓
Meadow Fescue	*Festuca pratensis* Huds.		✓	✓
Red Clover	*Trifolium pratense* L.	✓	✓	✓
White Clover	*Trifolium repens* L.	✓	✓	✓
Alsike Clover	*Trifolium hybridum* L.		✓	✓
Sweet Clover	*Melilotus* spp.			✓
Black Medick	*Medicago lupulina* L.		✓	
Lucerne	*Medicago sativa* L.		✓	
Sainfoin	*Onobrychis spp.*			✓
Birdsfoot Trefoil	*Lotus corniculatus* L.			✓
Plantain	*Plantago lanceolata* L.	✓	✓	✓
Chicory	*Cichorium intybus* L.	✓	✓	✓
Yarrow	*Achillea millefolium* L.			✓
Burnet	*Sanguisorba minor* Scop.			✓
Sheep's Parsley	*Petroselenium crispum* Mill.			✓

Table 4.2 Percentage seed mass contribution per forage family per forage mixture at time of sowing September 2016

		Treatment		
	%	SmartGrass	Biomix	Herbal
Forage	Grass	86	69	40.5
	Legume	8.5	22.5	38.5
	Herb	5.5	8.5	21

4.3.2. Trap plants

Sterile sand (Sibelco UK Ltd) absorbent clay substrate was created as 4 parts sand to 1 part Terra Green (Oil Dri UK), with 0.025 g/kg calcium hydrogen orthophosphate and 10%

deionised water. The mixture was autoclaved at 105°C for 1 hour, rested for 24 hours then autoclaved again at 105°C for 1 hour. Five-centimetre diameter hydroponic pots were placed within the same size closed bottom cups and filled with 140g sterile substrate each. Afterwards, 14ml deionised water and 20mg of ryegrass seeds (Cotswold Seeds Ltd, UK) were added at 1cm depth into the substrate.

Five pots per sunbag (Sigma-Aldrich Inc., Germany) were placed in a growth cabinet set to 22°C 16-hour days and 15°C 8-hour nights at 75% humidity for 1 month. Sunbags are transparent plastic bags with 0.02µm pores to enable plant growth and prevent contamination from the external environment. In this instance, the sunbags were used to prevent fungal contamination. Pots were fertilised with 1ml Long Ashton solution, a nutrient solution which contains no phosphorus, and 1ml deionised water twice over the 4 weeks the plants were in the growth cabinets. One ryegrass pot per sunbag was checked for mycorrhizal colonisation to confirm the plants were free from mycorrhiza. All plants were removed from the growth cabinet and given one week to acclimate to ambient conditions in a sheltered outdoor space while remaining in the sunbags to ensure no mycorrhiza colonisation before planting in the field. In late March 2020, three and a half years after the establishment of the plots, one trap plant per plot was planted in the field. Ryegrass trap plants were removed from the closed bottom pots but retained in the hydroponic pots and sterile substrate. Plants were given 5ml deionised water upon planting and monitored to ensure field establishment. Monitoring of the trap plants continued through the spring and summer 2020.

Trap plants, retained within their hydroponic pots and an extra 5x5x5cm of soil from below the hydroponic pots to cover new root growth were dug out from the CRU plots in early

October 2020 using a trowel sterilised with 1% virucidal disinfectant Virkon S (Lanxess, Cologne, Germany). Trap plant and associated soil were kept at 4°C before processing.

Trap plant roots and soil were separated, with the soil and a subsample of root frozen to -20°C for further DNA analysis. The remaining roots were stained for arbuscular mycorrhizal (AM) colonisation measurements. Briefly, roots were placed in 10% KOH and left at room temperature for 3 days. Roots were then rinsed with deionised water and stained with Ink/Vinegar solution (1-part Shaeffer black ink to 19 parts white vinegar) for 1 hour at room temperature. Roots were rinsed again using deionised water and stored in lactoglycerol (1-part lactic acid, 2 parts glycerol, 1 part water; Walker and Vestberg, 1994). Using the grid line intersect method, a compound microscope was used to measure the percentage root AMF colonisation (Giovannetti and Mosse, 1980).

4.3.3. DNA extraction and amplification

DNeasy powersoil pro kit (QIAGEN LLC, Hilden, Germany) was used to extract soil DNA. 0.25g of soil was weighed, and the protocol was followed using the Tissuelyser II (QIAGEN, Hilden, Germany). DNeasy plant mini kit (QIAGEN LLC, Hilden, Germany) was used to extract DNA from the ryegrass trap-plant roots. 0.12g of root sample was ground using a pestle and mortar with liquid nitrogen. Samples were checked for DNA yields using 1.5µl of the sample on the NanoDrop 2000/2000c spectrophotometer (ThermoFisher Scientific, Waltham, Massachusetts, US). Extracted DNA samples were adjusted to 10ng/µl using elution buffer and stored at -18°C before amplification.

Fungal community analysis was performed using ITS data generated from amplicon sequencing on 24 DNA samples (12 roots and 12 soil) using a two-step PCR approach with primer pair ITS1FKyo2 (TAGAGGAAGTAAAAGTCGTAA) and ITS86R (TTCAAAGATTCGATGATTCA) run on Illumina Inc (San Diego, CA, US) MiSeq v3 pair-end sequencing of 2x300bp at LGC Group Ltd (Teddington, UK). The second amplification used i7- and i5- sequencing adaptors. First amplification settings were 1-minute 96°C pre-denaturation followed with 30 15s cycles at 96°C denaturation, 58°C 30s annealing, 68°C 90s extension, 70°C 2-minute final extension and 8°C final hold. The second amplification followed the first amplification process with annealing changed to 3 cycles at 50°C followed by 7 cycles at 58°C. The process involved demultiplexing, clipping, primer detection and forward and reverse reads using BBMerge. Amplicon pre-processing included chimaera removal, resulting in high-quality reads clustered into operational taxonomic units (OTU) picked using Mothur at the 97% identity level. Taxonomic classification of the extracted DNA using Quantitative Insights into Microbial Ecology (QIIME 1.8; Caporaso et al., 2010) was processed against the UNITE v8.2 database (Kõljalg et al., 2013) and the SILVA database (Quast et al., 2013), classifying 1350 fungal OTU sequences.

4.3.4. Additional sampling

Soil sampling

Five 10cm deep soil cores were taken in a W pattern per plot and pooled to create a composite sample. For aseptic sampling, the soil corer was sprayed with a 1% virucidal disinfectant Virkon (Lanxess, Cologne, Germany) and rinsed with deionised water between plot sampling. Samples had roots and stones removed before being sent to NRM Laboratories (Cawood Scientific Limited, Bracknell, UK).

Additional soil sampling occurred where three 10cm deep soil cores were taken in late spring 2020 from each plot and mixed to create a composite sample. Samples were dried, passed through a 2mm sieve, and sent to NRM Laboratories (Cawood Scientific Limited, Bracknell, UK) for full chemical analysis.

Soil chemical analysis

NRM Laboratories conducted soil chemical analysis (Cawood Scientific Limited, Bracknell, UK), samples from July 2021 were tested for total carbon and total nitrogen through combustion in pure oxygen at 1200^{o}C (AOAC Official Method, 1997). Organic and inorganic carbon was determined by combustion of dried ground soil, acidified with orthophosphoric acid and sparged at 150^{o}C. Organic carbon was calculated as the difference between total carbon and inorganic carbon. Organic matter was calculated using the Van Bemmelen factor of 0.58 from organic carbon. Labile carbon was determined using the dried ground soil mixed with permanganate with the absorbance of the supernatant measured by a spectrometer. Chemical analysis on the soil samples taken in spring 2020 includes pH measured in water [1:2.5], available phosphorus (Olsen P) determined through sodium bicarbonate extractable and determined colourmetrically as described in Olsen and Sommers (1982) and Prokopy (1995). Ammonium nitrate extractable method was used to produce soil-available potassium and magnesium and determined by ICP-OES (Knudsen, et al., 1982; Soltanpour et al., 1982).

Aboveground biomass

Three 50x50cm quadrat cuts per plot were taken thrice during each growing season, leaving a residual height of 7cm. The material was separated into plant species, dried at 60^{o}C for 72hours and weighed per species group and combined plot total. Results are shown as t DM/ha growing season total (2019).

4.3.5. Statistics

All statistical analyses were performed using R studio statistical software (R Core Team, 2018) with ggplot2 package (Wickham, 2016) to create graphs.

FUNGuildR package (Furneaux and Song, 2021) was used to assign taxonomic trait classification of the soil fungi using the FUNGuild database (Nguyen *et al.*, 2016). Trophic guilds classified as arbuscular mycorrhizal with a confidence level set to highly probable were used for statistical analysis.

A non-metric multidimensional scaling (NMDS) graph using Bray-Curtis dissimilarity with the metaMDS function in the vegan package (Oksanen *et al.*, 2020) was used to visualise AMF community structures across the forage treatments and type (root or soil; k=3). Data were Hellinger-transformed to meet homogeneity assumptions after checking with the betadisper function. The permutational multivariate analysis of variance (PERMANOVA) using the Adonis function was used to test for differences in the fungal community structures. Supporting

data correlation effects, including soil chemistry, were plotted on the ordination if $p<0.05$ using the 'envfit' function on 999 permutations.

Taxonomic heat trees were created using the extension package metacoder (Foster, Sharpton and Grünwald, 2017) for pairwise comparison of root/soil sample OTU presence/absence for each forage mixture. Taxonomic information with low OTU abundance counts of less than 10 were removed, allowing proportion values to be created.

ANOVAs were performed on the Shannon diversity indexes using AMF OTU per treatment, per type and unique identifier of type and treatment. Post hoc Tukey tests using the agricolae package were used (Mendiburu, 2021) to identify significance.

A generalised linear mixed model on a success/failure matrix of AMF colonisation using extension package lme4 (Bates *et al.,* 2015) and lmerTest (Kuznetsova, Brockhoff and Christensen, 2017) was created, with a random effect of treatment. Stepwise regression was used to build the minimum adequate model using the add1() command, which added OTU Shannon diversity of both soil and roots, and labile soil carbon as fixed effects to the model, with family defined as binomial and the integral scalar nAGQ equalling zero. Model assumptions were checked by plotting residuals against fitted values. Several likelihood ratio tests were performed with the fitted model using ANOVA, comparing the full model against the model minus the parameter being looked at to test for significance. Extension package effects (Fox and Weisberg, 2019) and gridEXTRA (Auguie, 2017) were used to create graphs of the variables added to the model.

4.4. Results

The NMDS plot analysis shows the AMF community composition clustering in the SmartGrass mixture. Both root and soil differ from the clustering of the other two more plant-diverse forage mixtures (Biomix and Herbal, Figure 4.1; Adonis R^2=0.27374, $F_{5,18}$=1.3569, p = 0.077). Soil chemistry correlating with AMF community composition includes labile carbon (p=0.044) and organic matter (p=0.017). Other variables used in the analysis but showed no significance in the results include soil nitrogen (p=0.855) and soil available phosphorus (p=0.706; Table 4.3).

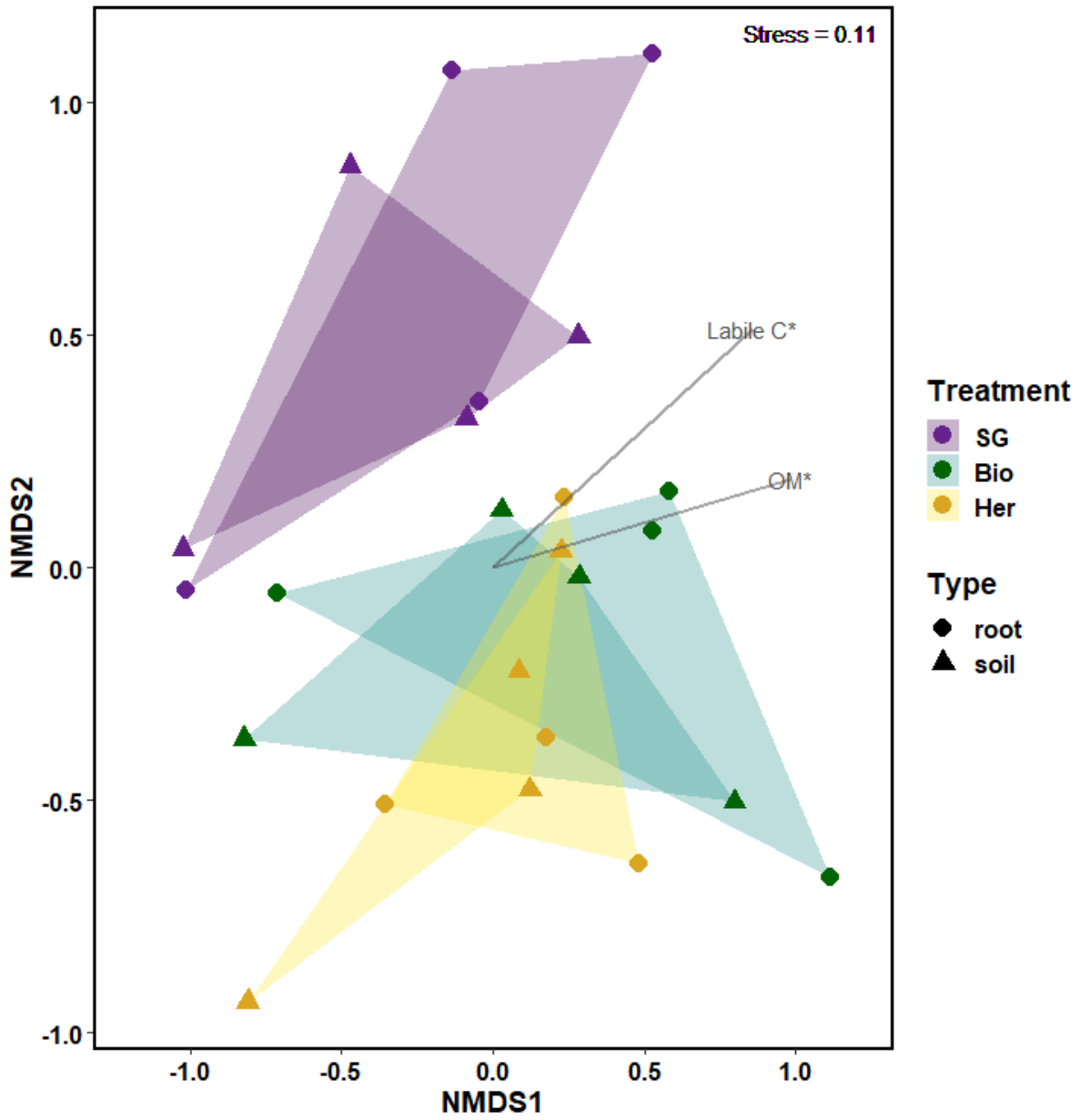

Figure 4.1 non-metric multidimensional scaling (NMDS) graph of AMF community structures. PERMANOVA Adonis results R^2*=0.27374, p=0.077,* $F_{5,18}$*=1.3569. Forage mixture treatments: SG – SmartGrass (6 species); Bio – Biomix (12 species); and Her – Herbal (17 species). Correlated supporting data plotted where p<0.05 (soil chemistry: labile Carbon (C), organic matter (OM). Significance level p<0.01**

Table 4.3 Results of variables used in the NMDS analysis which showed no significance with AMF community composition

	R^2	**Pr(>r)**
Soil moisture	0.1102	0.345
pH	0.1809	0.149
P	0.0321	0.706
K	0.1577	0.174
Mg	0.0644	0.513
N	0.0144	0.855
C	0.1742	0.162
Aboveground biomass	0.1236	0.287
AMF colonisation	0.1924	0.131
H_{mic}	0.2590	0.052
MSIR	0.2460	0.075

AMF taxonomic classification identified that the SmartGrass mixture shows the presence of the Claroideoglomeraceae lineage in roots, a lineage not seen in the roots or soil of the Biomix or Herbal forage mixtures. Ambisporaceae lineage had a higher presence in the roots of Biomix and Herbal forage mixtures but had a higher presence in the soil of the SmartGrass mixture. Archaeosporaceae taxon had a higher presence in the roots of SmartGrass, but was the same in the soils or roots under the Biomix or Herbal mixtures. Acaulosporaceae lineage was found in the soil and roots of the Biomix mixture but not in the SmartGrass or Herbal mixtures (Figure 4.2).

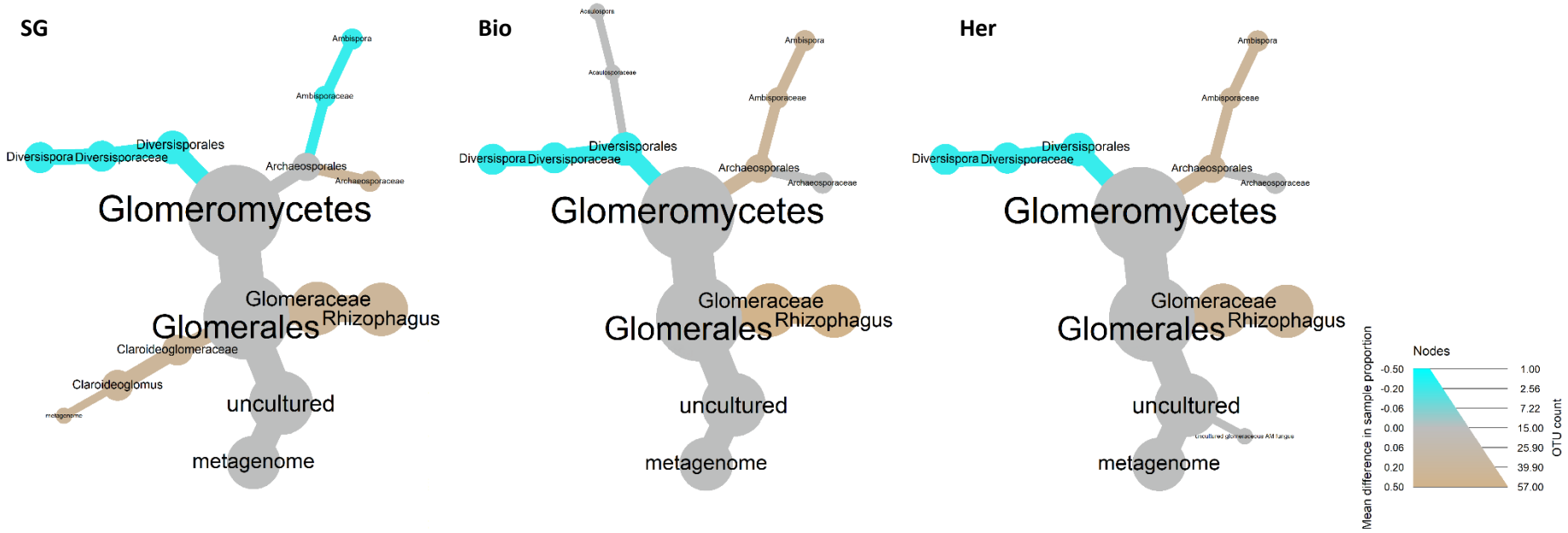

Figure 4.2: AMF presence/absence taxonomic heat trees per forage mixture: SG – SmartGrass (6 species); Bio – Biomix (12 species); and Her – Herbal (17 species). Each node represents a taxon used to classify an OTU; its diameter is proportional to the number of OTUs classified as that taxon. Colours represent the mean difference in sample proportion of presence/absence (min count set to 10) of taxon found in the forage mixture comparing root samples against soil samples. Found in both root and soil equally = grey, Root = brown, soil = blue

No differences were seen in the AMF OTU Shannon diversity index results between treatments or between the unique identifier of treatment and type. AMF OTU Shannon diversity was higher in the roots than in the soil (Figure 4.3; $F_{1,22}$=6.178, p=0.021).

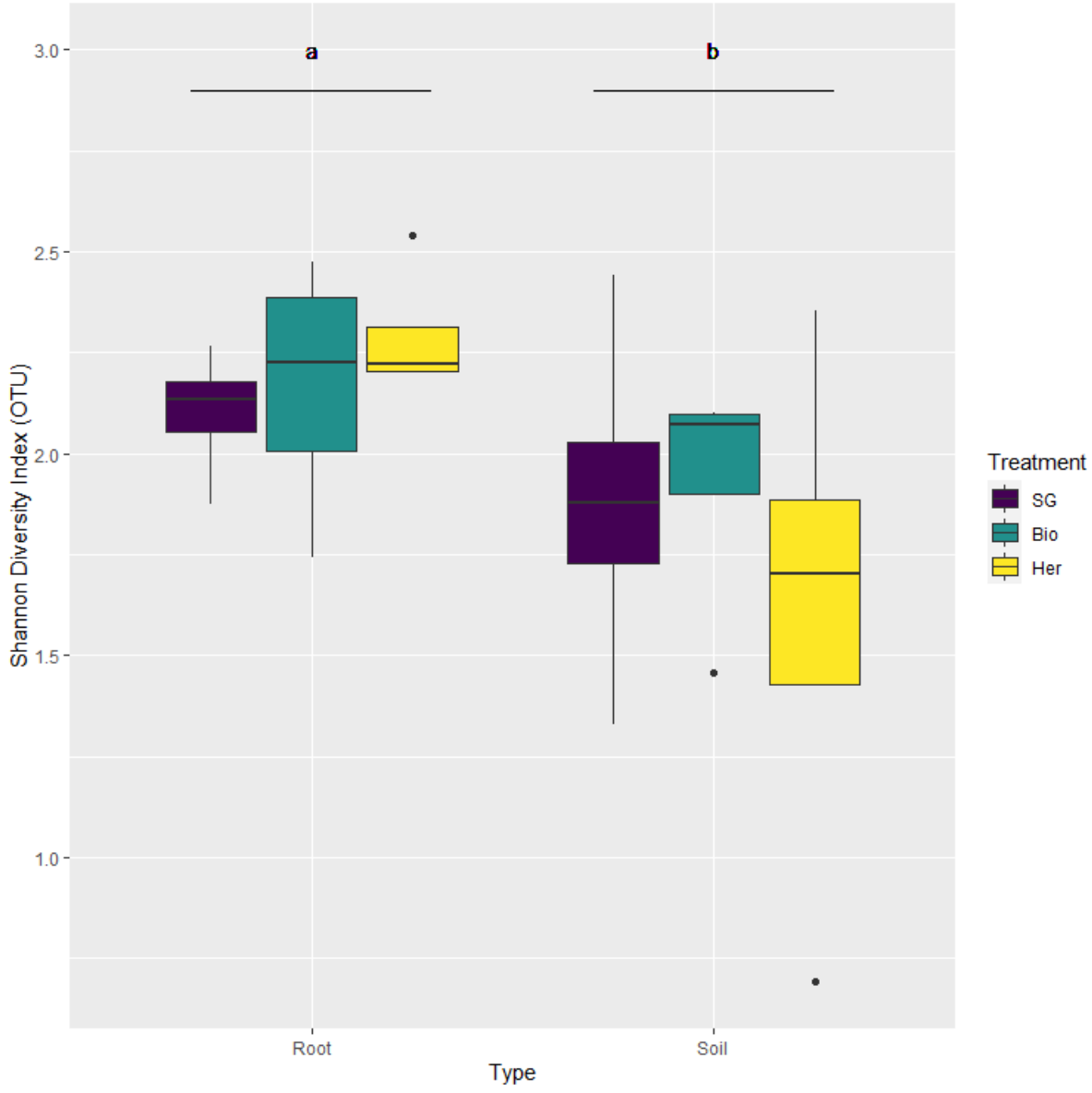

Figure 4.3: Shannon diversity index from AMF OTU between the forage mixtures. SG – SmartGrass (6 species); Bio – Biomix (12 species); and Her – Herbal (17 species) and sample type (root/soil). Letters denote significance between type root v soil $F_{1,22}$=6.178 p=0.021

Our model shows correlations of AMF colonisation increasing by 2.572 % ±0.283 SE with increasing root AMF OTU Shannon diversity ($\chi^2(1)=84.108$, $p<2.2e-16$) and increasing 6.803e-04% ±3.117e-04SE with increasing soil labile carbon ($\chi^2(1)=4.7959$, $p=0.02853$). Although soil AMF OTU Shannon diversity was a significant variable added to the model, post hoc results show only non-significance in increasing AMF colonisation by 0.165 % ± 0.093 SE with increasing soil AMF OTU soil Shannon diversity ($\chi^2(1)=3.136$, $p=0.07657$; Table 4.4; Figure 4.4).

Table 4.4 generalised linear mixed model summary output of AMF colonisation against significant variables added to the model

	Estimate	**Standard error**	**z value**	**Pr(>|z|)**	**Effect**
Intercept	-20.56	2.648	-7.766	8.12e-15	
Root AMF OTU Shannon diversity	2.572	0.283	9.077	<2e-16	+ve
Soil AMF OTU Shannon diversity	0.165	0.093	1.765	0.0776	+ve
Labile C	6.803e-04	3.117e-04	2.183	0.0290	+ve

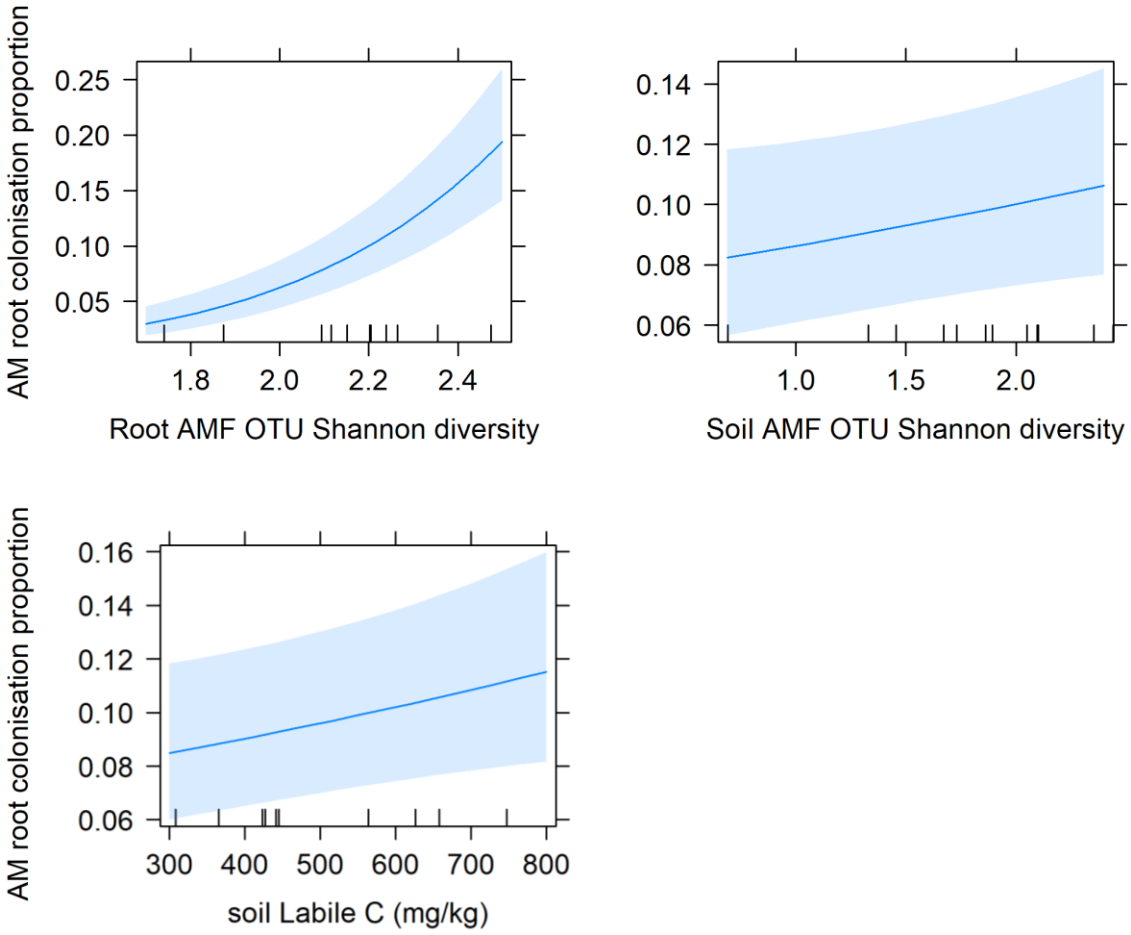

Figure 4.4 Generalised linear mixed model of a) root AMF OTU Shannon diversity; b) soil AMF OTU Shannon diversity, c) soil labile carbon against AMF colonisation. Blue line represents the trend line, blue shading represents 95% confidence interval, vertical black lines on the x-axis indicate data points

4.5. Discussion

AMF OTU community composition showed no differences between the three diverse grasslands, therefore rejecting our H1 hypothesis. It is well established that increasing plant species diversity increases AMF niche availability compared to monocultures (Burrows and Pfleger, 2002; Dietrich *et al.*, 2020; Guzman *et al.*, 2021). The host preference effect suggests that the AMF community is enriched by more dissimilar neighbouring plants (Mony *et al.*, 2021). Our result is suggestive that benefits achieved by AMF presence seen under a 6-species grassland mixture are equal to those seen under a more plant-diverse grassland.

The most abundant AMF taxa were Glomeraceae, with greater abundance in the roots of all three diverse grasslands. Glomeraceae are known to be very adaptive to semiarid environments and produce large numbers of spores enabling them to dominate AMF communities (Zhao *et al.*, 2017). Claroideoglomeraceae were only present in the 6 species grassland mixture SmartGrass, with greater abundance in the roots than in the soil. Claroideoglomus increases plant root length by improving nutrient uptake (Liu, Srivastava and Wu, 2017). Its presence only in the 6 species mixture, together with the benefits of the taxa shown by Liu, Srivastava and Wu, (2017), could indicate that plants in the SmartGrass mixture are under greater stress than the 12 or 17 plant species mixtures. The SmartGrass mixture investing in an AMF that enhances plant root growth could also result from a lower level of legume presence in the SmartGrass mixture.

Soil labile carbon and organic matter correlated with AMF community composition in this experiment. Many studies show that labile carbon is the main soil factor affecting microbial composition and catabolic function (Waldrop *et al.*, 2006; Antoninka, Reich and Johnson, 2011; Lagomarsino, Grego and Kandeler, 2012; Murugan *et al.*, 2014; Creamer *et al.*, 2016). However, an interesting result here is that fungal community composition is not correlated with soil nitrogen or phosphorus. Soil nitrogen typically constrains plant productivity, N fertilisation enhances plant growth until soil phosphorus availability becomes the limiting factor. In turn, phosphorus unavailability increases AMF inoculum potential, compelling the plants to invest in the AMF symbiosis, with a cascade of effects on the AMF community (Brockwell *et al.*, 2005).

No differences were seen in the OTU Shannon diversity results between the grassland mixtures, rejecting our H2 hypothesis. There was, however, higher AMF OTU diversity in the roots compared to the soils across all three diverse grasslands. This result is supported by other research (Öpik *et al.*, 2009; Mahmoudi *et al.*, 2019), but conflicts with Hu *et al.* (2019) and Hempel, Renker and Buscot, (2007), who showed bulk soil had greater AMF species diversity, discerning that AMF may not be fully obligatory symbionts to plants. Conflicting results between AMF diversity in roots versus soils could result from differences in AMF life history strategies and sampling time (López-García *et al.*, 2014; Hart *et al.*, 2015). Here we sampled at the end of the growing season in October, whereas Hempel, Renker and Buscot, (2007) sampled in July. We suggest that there is a high turnover of AMF in the soils at the end of the growing season when the plants are no longer in need of forming symbiosis and root AMF niches are already occupied at this time. The result seen here could also reflect our relative abundance-based method of evaluating diversity, as the soil samples would have more reads taken up with non-AMF fungi, therefore, sampling a lower diversity of AMF in the soil.

Our model showed correlations of AMF colonisation with increased root AMF OTU diversity and soil labile carbon but not with soil AMF OTU diversity as predicted in hypothesis H3. Increasing labile carbon allows for higher biomass and diversity of the fungal community and a higher infection potential (Waldrop *et al.*, 2006; Antoninka, Reich and Johnson, 2011). Increasing root AMF diversity increases niche complementarity; there is evidence for functional complementarity in AMF (Maherali and Klironomos, 2007). However, within a microcosm fungal diversity manipulation experiment, such functional complementarity was not indicated (Vogelsang, Reynolds and Bever, 2006). A six-species AM soil mixture did not produce greater plant biomass than the most productive single-species mycorrhizal soil system (Vogelsang, Reynolds and Bever, 2006). Increasing the diversity of communities, both above-

and belowground, has a two-fold benefit. First, it increases the chances of containing a species that responds differentially to changes in environmental conditions. Second, increased diversity boosts the chances of that species joining an existing functional effect group, allowing for functional replacement to occur during perturbation. If many taxonomically distinct species perform similar ecosystem functions but are different in their response to perturbations, the system will be more resistant to said perturbation, promoting stability (Oliver *et al.*, 2015). Soil is notorious for its high heterogeneity, therefore, it contains considerable functional redundancy (Walker, 1992; Liiri *et al.*, 2002; Wardle *et al.*, 2004). Some soil species are, however, more redundant than others, with some organisms being functionally irreplaceable (Laakso and Setälä, 1999), such as the presence of AMF for forage productivity (Haystead, Malajczuk and Grove, 1988; Gordon, Haygarth and Bardgett, 2008).

4.6. Conclusion

AMF are important soil symbionts that can contribute to future-proofing forage production systems. Encouraging the presence of beneficial AMF by creating desirable niches is important for forage maintenance in climate change extreme weather events. Our study showed no difference in the AMF community composition between three diverse grasslands, suggesting that the benefits achieved by AMF presence seen under a 6-species grassland mixture are equal to those under a 17-species mixture. This result is informative for land managers' decisions in choosing a cost-effective, diverse forage mixture, balancing the seed cost and soil health benefits. Future research into AMF community composition under forage grasslands should include a more holistic approach, with grazers present in situ, as both species and stocking density can affect AMF community composition.

4.7. Supplementary material

Table S 4.1 Percentage dry matter weight contributions per forage family per forage mixture for the Spring 2019 aboveground biomass cut for the CRU plots

		Forage mixture treatment		
	%	**SmartGrass**	**Biomix**	**Herbal**
Forage	**Grass**	39	83	48
	Legume	0	2	3
	Herb	61	15	49
	Other	-	-	-

Chapter 5. General Discussion

5.1. Summary and implications of project findings

Aboveground plant species richness has been linked to changes in soil biota and the functioning of trophic systems and ecosystem services. However, this relationship has been poorly researched in diverse grasslands, especially within the context of actual agricultural systems. Ryegrass monoculture pastures require intensive and likely unsustainable inputs to maintain productivity (Crews and Peoples, 2004). Food producers face increasingly unpredictable conditions such as fertiliser and crop prices or climate change challenges of drier, warmer summers (Lowe *et al.*, 2019). Nitrogen requirements of grassland systems and their resilience to climate change could be met by regenerative agricultural farming techniques designed to increase biodiversity and improve soil health (Bardgett & Caruso, 2020; Tilman *et al*., 2006). In this study, fertilised perennial ryegrass grassland, used as an example of the business as usual, was significantly different from all other diverse mixtures far more often than the mixtures were different to each other, i.e. the three diverse forages were more similar in their results to each other than either one was to the ryegrass monoculture (summary Table 5.1). Key differences in the perennial ryegrass monoculture compared to the diverse mixtures included lower earthworm densitiy, lower AMF root colonisation and lower fungal OTU Shannon diversity. The three diverse forage mixtures were consistently the same, except for AMF root colonisation, where the lowest diversity mixture SmartGrass had the lowest AMF root colonisation compared to the two most diverse forage mixtures (Biomix and Herbal).

Table 5.1 Summary of research findings

Chapter/Research Question	Main findings
2. How do short term grasslands which vary in plant species richness and management affect earthworm/soil mesofauna abundance/diversity?	-Monoculture ryegrass grasslands had lower earthworm densities than the 12 species diverse grassland. This is reflected in current literature under long term studies. We show this result in the short term of 2 years -Monoculture ryegrass grasslands had higher collembola abundances than the 6 species and 12 species diverse grasslands. This is not supported by current literature. We suggest this difference is due to an increased food source (root biomass) availability in the monoculture plots at time of sampling
2. How do short term grasslands which vary in plant species richness and management affect AMF root colonisation activity measured at two sites?	-Percentage of roots colonised by AMF increased with increasing plant species diversity in situ, further supporting current knowledge. Increased soil moisture correlated with higher AMF root colonisation rates, which conflicts literature. This opened the question for researching whether the presence of AMF is improving aboveground biomass production in the grasslands.
3. How do short term grasslands which vary in plant species richness and management affect the soil fungal composition and microbial diversity, and does this affect ecosystem service provisioning of aboveground biomass production across two sites?	-Monoculture ryegrass fungal community composition differs from the 12 and 17 species diverse grasslands at the dry site and all three diverse grasslands at the well-watered site. This result is supported in the literature through mesocosm experiments, however our study shows this in situ under short term grasslands. Soil properties such as pH and moisture correlate to the results at the dry site. No soil properties correlated to the results at the well-watered site. -Soil fungal diversity is higher in the diverse grasslands compared to the ryegrass monoculture at the dry site only, suggesting under dry stressed environments plants invest more in their microbial community due to interspecific competition -Aboveground biomass production at the dry site is correlated only by AMF root colonisation, not plant species diversity conflicting established diversity-productivity literature. However, the result suggests the mechanisms of P and N uptake for plant growth is occurring supporting literature of plant productivity being led by fungal species identity. This opened the question of what specific AMF species are in the rhizosphere and roots under these grasslands.
4. How do short term diverse grasslands which vary in plant species richness grown in situ in a dry stressed environment affect AMF soil and root community composition and AMF root colonisation activity?	-No difference in AMF community composition was seen between the three diverse grassland soils/roots suggestive that the host preference effect benefits are seen equally under a 6 species and 17 species grassland -AMF diversity is higher in roots than in the soil which further adds to the literature where no clear consensus is reached. We suggest here differences are seen due to life history strategies, season the samples were taken and relative abundance-based method of evaluating diversity. -Increased AMF root colonisation correlated with increases in AMF root diversity and soil labile carbon, showing functional complementarity in situ which was not seen in previous literature of a microcosm fungal diversity manipulation experiment

Existing descriptions of key aboveground plant diversity effects on soil biota include earthworms (Eisenhauer and Scheu, 2008; Eisenhauer *et al.*, 2009); with low earthworm abundance under low diversity grasslands altering nutrient availability, which leads to plant species loss (Spehn *et al.*, 2000). This research confirmed that the diverse forage mixtures contained higher densities of earthworms than the ryegrass monoculture, which is likely to be beneficial for ecosystem function. Previous research has shown that the presence of earthworms can increase aboveground biomass by 23% (Van Groenigen *et al.*, 2014). Earthworms increase soil nutrient availability, creating an environment more favourable to plant production (Spehn *et al.*, 2000). Increased earthworm densities in the diverse forage mixtures may increase nutrient availability for plant productivity, which is beneficial to provisioning ecosystem services. Increasing earthworm densities also improve other ecosystem services; earthworm burrowing increases water filtration, alleviating flooding scenarios (Bastardie *et al.*, 2003; Lavelle, 2004), with higher densities of earthworms creating more burrowing, continuing to improve soil structure. Grasses have been shown to invest less in root development with earthworm presence (Alphei, Bonkowski and Scheu, 1996), further reducing their ability to resist drought conditions. However, through the release of phytohormones, earthworms increase AMF colonisation, which expands the surface area for water uptake and, therefore, improves plant drought resistance (Azcon, Azcon-G De Aguilar and Barea, 1978; Zarea *et al.*, 2009).

Higher AMF root colonisation, observed in the more diverse forage mixtures, improves ecosystem service delivery. AMF colonisation advantages include enhancing plant species diversity by reducing plant competition (Grime *et al.*, 1987). Increasing plant species diversity in grasslands would continue the advantages of increased functional diversity, delivering

improved ecosystem services such as soil carbon storage (Pastore, Hobbie and Reich, 2021). Other environmental improvements that increase AMF colonisation include reduced nitrogen leaching (de Vries *et al.*, 2006). Plants associated with AMF extend their rooting surface area, increasing phosphorus and water uptake. A larger root surface area enables continued forage production in the drier, warmer summers we are already experiencing (Lowe *et al.*, 2019). The lowest AMF colonisation rate was seen in perennial ryegrass monoculture grassland, likely due to the lack of interspecific competition occurring and the addition of artificial nitrogen fertilisation, where nitrogen concentrations were up to five-fold higher than the diverse mixtures in the well-watered site for example (Shepperd *et al.,* 2020). A recent study suggests that the cost-benefit of plant and AMF symbiosis depends on the nitrogen source (Savolainen and Kytöviita, 2022). Results from a previous study conducted on the CRU plots used for this thesis showed there were higher average concentrations of ammonium in the fertilised monoculture at the well-watered site. In contrast, at the dry site, there were higher average nitrate concentrations in the fertilised monoculture (Shepperd *et al*., 2020). Govindarajulu *et al.* (2005) showed that AMF take up soil ammonium and nitrate and transforms the nitrogen into arginine without requiring carbon in exchange. Plants forming a symbiosis with AMF yet acquiring nitrate and ammonium without the need to exchange for carbon further shows the benefit of the increased AMF colonisation occurring in the diverse forage mixture ryegrass trap plants.

Shannon fungal OTU diversity was higher for the diverse mixtures than for the perennial ryegrass monoculture at the dry site only. This indicates that plants invest more in their microbial community in dry, stressed environments due to interspecific competition, intensifying microbial diversity and functionality. The benefits to plants investing in their soil microbial community include increased pathogen and drought resistance (Jia *et al*., 2021). High

fungal diversity supports higher plant diversity and a better supply of soil phosphorus (van der Heijden *et al.*, 1998), increasing the competitiveness of subordinate herbs over grasses (Grime *et al.*, 1987). Observations from the Diverse Forages project indicate that in future climate change conditions of drier summers, diverse forage mixtures should maintain their diversity through the presence of AMF and, therefore, not lose the improvements in ecosystem service delivery that biodiversity supplies (Pastore, Hobbie and Reich, 2021).

Many challenges persist in modern farming: maintaining farm profitability, climate change, water quality, biodiversity, antimicrobial and anthelmintic resistance and consumer acceptance. Specific to grazing, global and national discussions about moving towards the diversification of pastures stimulated by environmental concerns have come to the fore (Department for Environment Food & Rural Affairs (DEFRA), 2018; Harris and Ratnieks, 2021). The United Nations Sustainable Development Goals target of achieving sustainable food production systems by 2030 is a good example of this trend (UN, 2019). England, for example, is moving away from the EU agricultural policy and towards payments for environmental benefits in its flagship agriculture support scheme (DEFRA, 2018). Using diverse forages reduces the need for external inputs to maintain forage productivity; diverse mixtures had greater aboveground biomass than the fertilised PRG monoculture (data not published at the time of writing). The result gives a clear link to farm profitability and environmental impact. The regeneration of soil health and biodiversity through diverse grasslands increases earthworm abundance, AMF fungal diversity and AMF colonisation, which enhances ecosystem service delivery and resilience to climate change (Bardgett & Caruso, 2020; Tilman *et al*., 2006). At the time of writing, a sudden global spike in fertiliser prices generated by the geopolitical situation put the PRG monoculture at an even greater disadvantage compared to

the diverse mixtures not requiring fertilisation (Agriculture and Horticulture Development Board, 2021).

This study used agronomically recommended amounts of fertiliser in the perennial ryegrass monoculture to maintain its forage productivity, but the three diverse forage mixtures were not fertilised at all. The aboveground biomass model (Chapter 3) shows that it is not the presence of soil nitrogen that controls aboveground biomass production in our diverse pastures. It was forage mixture, soil pH and soil organic matter at the well-watered site and AMF colonisation at the dry site; all these variables positively impacted aboveground biomass. Nitrogen fertiliser is thus not a critical requirement for aboveground biomass production in diverse grasslands. Aboveground biomass production was higher in the diverse mixtures than in the ryegrass monoculture (data not published at time of writing). Other biotic factors must be nurtured to maintain or improve forage productivity, particularly in drier climate change conditions. This research contributes to the current debate about the sustainability of food production and a shift towards more environmentally responsible agriculture (DEFRA, 2018).

The grassland mixtures used in this study include Cotswolds Seed commercially available Herbal ley, which cost £244/hectare at the time of writing. Our Biomix and SmartGrass mixtures are similar to Cotswold Seed ‘simple herbal ley’, which consists of 10 plant species costing £194/hectare. Cotswolds Seed simple herbal ley mimics our six species SmartGrass mixture more accurately with the percentage contribution of grass, and legume to herb seed (Table 5.2). Cotswolds Seeds ryegrass monoculture costs £174-£199/hectare. The matching of costs between the ryegrass monoculture and the simple herbal ley is clear, however further financial benefits for using the simple herbal ley include less nitrogen fertiliser needing

to be applied, along with biodiversity benefits and ecosystem service delivery, even a simple diverse grassland can achieve.

Table 5.2 Percentage seed mass contribution per forage family per forage mixture from commercially available Cotswold Seeds (CSeeds) or the Diverse Forages project (DForages)

	% seed			
	Grass	**Legume**	**Herb**	**price per hectare (£)**
CSeeds/DForages perennial ryegrass	100	-	-	174-199
DForages SmartGrass	86	8.5	5.5	-
CSeeds 'simple herbal ley'	88.4	7.6	3.8	194
DForages Biomix	69	22.5	8.5	-
CSeeds/DForages Herbal	40.5	38.5	21	244

Summarising the results, it is clear that diverse grasslands benefit the belowground ecosystem and forage productivity. The two most diverse mixtures, Biomix and Herbal, showed the best promise regarding above and belowground ecosystem service delivery; higher earthworm densities, higher AMF colonisation and higher fungal diversity support this. However, the six-species SmartGrass mixture still showed environmental benefits over the ryegrass monoculture. Our results further support that of Pastore *et al*., (2021), who suggested that planting even a low-diversity mixture (i.e. 4 plant species) would increase ecosystem services compared with monoculture grasslands.

The benefits of diverse grasslands are apparent, but above and belowground environments cannot be studied in isolation due to the complexity and interactiveness of the

ecosystem (Bünemann *et al.*, 2018b). This work addresses the knowledge gap in researching commercially viable diverse forage mixtures and their interaction with earthworms, fungi and functional response, all measured within one system.

5.2. Limitations of thesis research and opportunities for future work

This study was carried out in situ in the southeast of England. The benefits of field research include assessing the grassland system more holistically and considering the whole agronomic operation. However, the sampling sites used for this thesis do not represent UK-wide climate and soil type conditions, so recommendations on which forage mixture provides the best ecosystem services may be limited to the unique location.

A larger 5-year UK-wide project enabled this thesis research, The Diverse Forages Project was led by the University of Reading in collaboration with Rothamsted Research, Duchy College and Cotswold seeds. Earlier research indicated agricultural benefits such as annual dry matter production similar to the highly fertilised PRG monoculture, or lambs reared on the six species SmartGrass mixture being heavier and requiring fewer anthelmintic treatments than lambs grown on PRG monoculture or PRG white clover grasslands (Grace *et al*., 2019; Grace *et al*., 2018). Diverse Forages aimed to achieve acceptable yields of good quality forage for livestock production whilst positively affecting the environment and to assess nitrogen use efficiency at the animal and farm-scale. The project consisted of agronomy plots across multiple sites and field-scale sites at several demonstration farms across the UK. Using the data from demonstration farms was outside the scope of this thesis, however, the headline results from the Diverse Forages project show that, after 3 years, the diverse forage mixtures

had significantly higher annual harvest biomass than the PRG monoculture (data not published at the time of writing). The Diverse Forages project also included a digestion trial with steers measuring methane emissions and steer growth rate, with results not available when writing this thesis. Using multiple locations to research grassland mixtures' effect on livestock is important for direct agronomical results. The consideration of the belowground sub-system effects is coming to the fore as agronomically it is becoming equally as important as aboveground measurements (Teague and Kreuter, 2020).

Future research requires a more holistic approach to include the impact of aboveground ruminant grazers in real grazing conditions (stock density, grazer type, etc.) on soil biota dynamics under different grassland systems. Grazers were shown to modulate soil biodiversity, affecting soil processes such as nutrient cycling and grassland productivity (Bardgett and Wardle, 2003). Recent research suggests livestock type and diversity are important in promoting a multi-diverse soil system (Wang *et al.*, 2019). Thus, research into the whole habitat effect on soil functioning and aboveground production under different diversities of forage is an important future research requirement. For example, our results of earthworms under grazing cattle showed no difference between the diverse treatments and the perennial ryegrass for earthworm abundance, richness, or species diversity. However, both Eisenhauer *et al*. (2009) and Spehn *et al*. (2000) report on earthworms in grasslands mown for hay, earthworm density and biomass decrease with plant species richness loss. Future holistic grassland soil biota research should include sampling farms UK-wide where diverse grasslands are used as part of the farm cropping rotation, such as the Diverse Forages project. Researching the short-term results aboveground diversity has on soil biota would create more realistic data and benefit UK farming. Here, some of the soil sampling happened five years after its establishment. This timescale is unlikely to occur in crop rotations on farms; for example, herbal leys are grown

for 3-4 years in a crop rotation as a fertility-building exercise in organic arable production systems, with little soil chemistry changes occurring after 2 years (Shepperd *et al.*, 2020).

Limitations of this study and improvements for future experiments include temporal sampling events to sample mesofauna yearly or seasonally and monitor the abundance as Eisenhauer *et al*., (2009) stated in their study: single data points may be misleading in the complexity of above-belowground systems. The high abundance of collembola seen here could be due to spring sampling when the soil moisture is high, and the ryegrass monoculture is the most productive. It would be interesting to see if the differences in the collembola abundance continue during a dry summer when the ryegrass monoculture suffers, but the diverse mixtures are still productive. A more thorough collembola species identification would have also been interesting in Chapter 2 to identify which feeding guild (fungivores, herbivores, predators, etc.) contributed to the increase in collembola abundance in PRG. The feeding strategy is affected by resource availability and agricultural management type (Ngosong *et al.*, 2009). Temporal sampling would also have been beneficial in the earthworm sampling for a better reflection of earthworm diversity; issues with very few adult earthworms to identify down to species level make diversity calculations inaccurate.

Interesting observations of the fungal community composition concerning grassland diversity were shown (Chapter 3). Future work in this area could include planting each species in a monoculture and assessing which fungal species are found under which plant species monoculture – testing the host discrimination passenger hypothesis (Kokkoris *et al.*, 2020). Briefly, the passenger hypothesis assumes that the presence of specific plant species is required first to enable the presence of specific fungi, i.e., the fungi depend on the plants (Kokkoris *et*

al., 2020). Determining which grassland plant species selectively invest in their fungal community could help formulate the ultimate forage mixture for future-proofing grassland productivity for the drier summers. For example, *Glomeraceae* are known to be adaptive to semi-arid environments, and *Claroideoglomus* increases plant root length (Liu, Srivastava and Wu, 2017; Zhao *et al.*, 2017). If these fungal families are selected explicitly for specific grassland plant species, then encouraging a grassland mixture with higher quantities of the plant species would further improve ecosystem services from the benefits fungal presence achieves.

5.3. Conclusion

Grassland diversity is gaining prominence as an alternative to monocultures for livestock production. Sustainable development goals and net-zero target policies necessitate this progression. Aboveground functional biodiversity improves ecosystem stability, a requirement for agricultural systems in climate change conditions. This thesis research further shows the benefits aboveground biodiversity has on soil biota, enhancing ecosystem services and aboveground biomass productivity and, therefore, agricultural stability. Further work is still required to fully understand the complex plant-soil feedback loop to include aboveground grazers for a more comprehensive holistic ecosystem response.

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