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Assessing earthworm diversity and population dynamics in agroecosystems

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This thesis is dedicated to

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Abstract

Earthworms play a fundamental role in the maintenance of soil health, and managing agroecosystems in ways that help earthworm populations to flourish is increasingly being recognised as a possible way to tackle the global issues of soil degradation and food insecurity. In order for this to become a reality, existing knowledge gaps surrounding the effects of different land use practises on earthworms need to be addressed. In addition, better management of earthworm populations will require more extensive population monitoring, as to date they have been severely under-recorded. This thesis aims to investigate how changes in land use, particularly temporary arable conversion to ley, affect earthworms in agroecosystems. It also aims to demonstrate the feasibility of a novel soil eDNA sampling technique for monitoring their populations, and assess its effectiveness relative to traditional methods. To do this, a novel mesocosm experiment was performed which investigated how earthworm activity and population variables respond to different land use practises, under varying climatic scenarios. Earthworm eDNA sampling in the field was also carried out alongside traditional hand-sorting, and laboratory experiments investigated earthworm population responses and associations with other soil quality indicators. Consistent positive effects of arable to ley conversion on earthworms were found, indicating that ley farming can significantly boost earthworm activity, abundance, biomass and diversity across short time scales. eDNA sampling also proved to be an effective tool for monitoring earthworms in agroecosystems, which found more species per sample when compared with hand-sorting. The eDNA technique was able to detect fine-scale changes in arable earthworm communities bought about by recent conversion to ley, indicating that it could also be a useful tool for monitoring the effectiveness of changes in land use aimed at improving soil health. Taken together, the results in this thesis offer new insights into the protection, restoration and monitoring of earthworms in agroecosystems, whilst also highlighting the need for greater consideration of these important animals and opening up new avenues for future research.

Acknowledgements

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During my time in Animal & Plant Sciences I have been privileged to meet so many interesting people who have helped to shape this work into what it is today. I am extremely grateful to Gavin Thomas and everyone in the Macrobird lab, for taking me under their wing and making me feel welcome. Particular thanks go to Emma, Yichen, Frane, Chris, Alison and Angela for their friendship, and for making the office such a nice place to work. I would also like to thank Rachel Tucker and Katy Maher, who patiently answered endless questions about DNA, sequencing and bioinformatics. Thanks also to Lizzy, Kirsty, Chris and the C57 group for the cuppas and catchups. It is no secret that the past year has been tough for most people, but I am especially grateful to the 'pandemic PhD' writing retreat group and my good friend Roberta, who helped make a very difficult year a little more manageable.

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A special thank you goes to my Mum, whose achievement of simultaneously raising three kids, working multiple jobs and getting through night school dwarfs this one. Thank you for always being there to support me. And to Gemma, my four-years-early twin, it's safe to say I might be a little bit grateful to you too (but don't let that go to your head).

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Declaration

I, Joseph Llanos, confirm that this thesis is my own work. I am aware of the University's Guidance on the Use of Unfair Means (www.sheffield.ac.uk/ssid/unfair-means). This work has not been previously been presented for an award at this, or any other, university.

This is a publication format thesis. Each data chapter (2, 3, 4 and 5) is presented as a stand-alone research article. At the time of submission none of the chapters have been published, but some of the data reported in Chapter 2 (earthworm abundances and biomass) have been published in the following paper, of which I am a co-author:

Berdeni, D., Turner, A., Grayson, R.P., Llanos, J., Holden, J., Firbank, L.G., Lappage, M.G., Hunt, S.P.F., Chapman, P.J., Hodson, M.E., Helgason, T., Watt, P.J., Leake, J.R., (2021). Soil quality regeneration by grass-clover leys in arable rotations compared to permanent grassland: Effects on wheat yield and resilience to drought and flooding. *Soil Tillage Research*. 212.

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Author contributions to the above paper: JRL designed and led the study. LGF and MGL established the ley strips, and DB, MGL, SPFH, MEH, PJC, and AT extracted and transported the monoliths. AT devised the open sided-polytunnel and temporary sealing of boxes to flood monoliths, and assisted DB in data collection. RPG and JH guided the infiltrometry. JL, supervised by PJW, undertook the earthworm population studies. DB collected and analysed the data and wrote the paper with JRL, with comments from the other authors.

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Chapter 1

General Introduction

1.1 Introduction

This chapter aims to provide a general overview of the topics, themes and issues explored in this publication format thesis. It begins by outlining the taxonomic and functional diversity of earthworms, before exploring their role as soil ecosystem engineers and their contributions to soil health and sustainability. The significance of earthworms for agriculture is also discussed, in the context of their populations helping to tackle the rising issues of food insecurity and soil degradation. Following on from this, this chapter then explores how earthworm populations are affected by conventional cropping and other forms of land use management in agriculture, before outlining the sampling approaches that have traditionally been used to monitor them. The chapter concludes with the research questions addressed by this thesis and a brief outline of each subsequent chapter.

1.2 Earthworm taxonomic and functional diversity

In 1881, Charles Darwin published his last scientific book, outlining his ground-breaking research on earthworms and highlighting the crucial role they play in soil formation. The book was an instant success, selling thousands of copies in the first few weeks of its release. Almost 140 years on from the publication of *The formation of the vegetable mould through the action of worms, with observations of their habits*, the importance of earthworms is now widely recognised, but many unanswered questions concerning their diversity, ecology and behaviour remain. Approximately 3700 Megadrile earthworm species (i.e., terrestrial or semi-aquatic) have been described globally (Hendrix et al., 2008), but due to a lack of sampling effort and the prevalence of cryptic speciation the actual number is likely to be more than double this figure (Lavelle and Lapied, 2003; Novo et al., 2010; Reynolds, 1994). Native and exotic earthworm assemblages are found across all continents except Antarctica, with the highest local diversity and abundances typically seen in mid-latitude temperate areas (Phillips et al., 2019). In the UK, where all data collection for the subsequent chapters took place, a total of 31 native species are currently recognised (Sherlock, 2018).

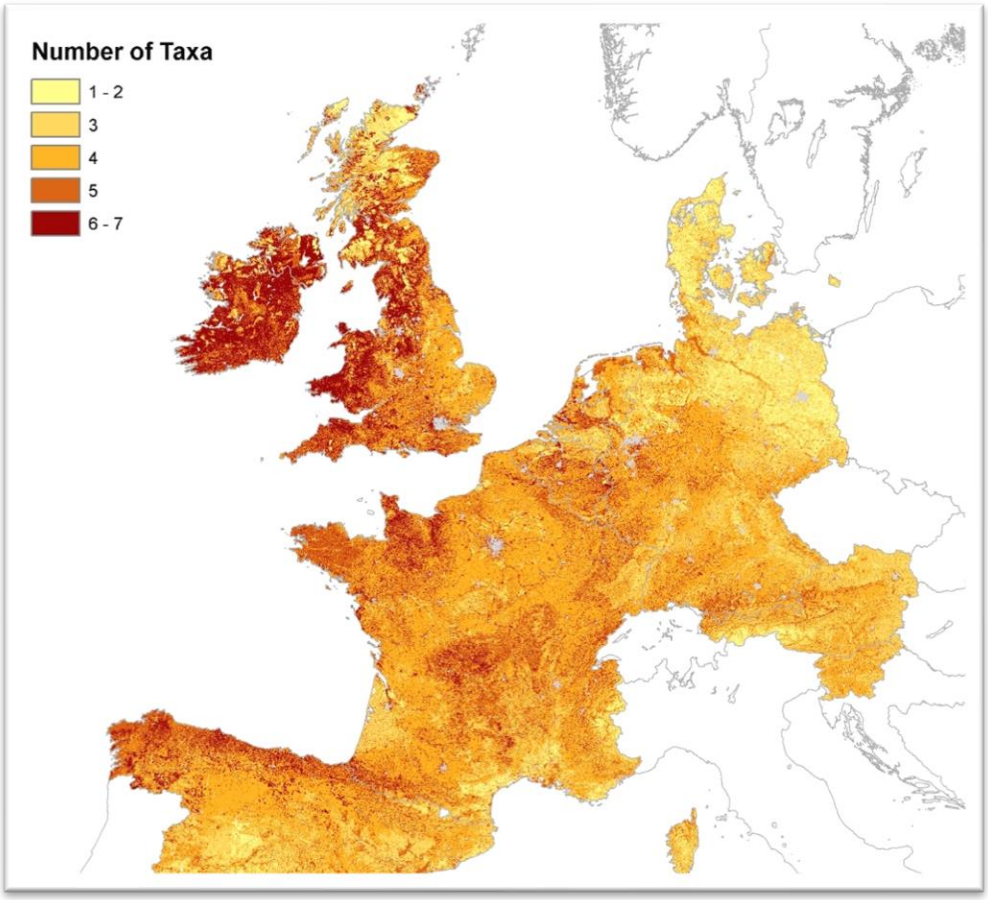

Figure 1.1. Predicted local earthworm diversity across Europe, extrapolated from models built using community datasets originating from eight European countries. (Taken from Rutgers et al., 2016).

Earthworms can be highly variable and exhibit a range of different behaviours, life histories and body sizes, ranging from the Giant Gippsland earthworm that can reach up to three metres in length, to the smallest composting worms whose adults often do not exceed 20 mm (Reynolds, 1994). Traditionally earthworms have been split into three main ecological categories that were first described by Bouché (1972) and often referred to as functional groups (although this practise is debated – see Bottinelli and Capowiez, 2021). The epigeic category describes earthworms that typically live at the soil surface and prefer substrates containing high organic matter, including leaf litter, animal dung or the underside of rotting logs (examples include *Satchellius mammalis* and *Lumbricus rubellus*). In comparison to other ecotypes, epigeic earthworms are often darkly pigmented, with smaller body sizes, high reproductive rates and short lifespans (Ali and Kashem, 2018; Bottinelli et al., 2020). Endogeic earthworms (such as *Allolobophora chlorotica* and *Aporrectodea rosea*) live within the soil, often making temporary horizontal burrows when feeding and moving through it. Endogeics tend to be larger than epigeics, with less colouration, and ingest soil directly to feed on organic matter. Anecic

earthworms (including *Lumbricus terrestris* and *Aporrectodea longa*) are typically large-bodied and feed on organic matter gathered from the soil surface, which is dragged down into permanent vertical burrows (Sims and Gerard, 1985). Anecics often return to the soil surface to cast, with some species producing distinctive cast piles at the burrow entrance known as middens (Butt and Lowe, 2007).

Whilst these ecological categories have been widely applied, there are limitations to their use and critics say they can oversimplify earthworm-soil dynamics and lead to important species-specific behaviours being overlooked (Bottinelli et al., 2020; Bottinelli and Capowiez, 2021). For example, studies have demonstrated that earthworm species belonging to the anecic ecological category can show substantial differences in their feeding, burrowing and morphological characteristics that would otherwise be overlooked if they were grouped together in the same category (Bastardie et al., 2005; Briones and Álvarez-Otero, 2018; Eisenhauer et al., 2008). These differences have led to the suggestion that anecic earthworms should be further subdivided into 'strict-anecic' and 'epi-anecic' groups, particularly as the two sub-groups have been shown to have contrasting effects on key soil processes like litter decomposition (Hoeffner et al., 2019). Despite the debate, the use of ecological categories is still regarded as a useful tool for studying earthworms, but it is recognised by researchers that care must be taken when generalising above the species level.

1.3 Soil health and earthworms

In recent decades the concept of 'soil health' has been gaining increasing attention in both scientific research and wider policy circles, as the importance of soil for nature and human society becomes more widely recognised (Bonfante et al., 2020; Liu et al., 2020). First mentioned in the literature over 100 years ago by Wallace (1910), the concept of soil health has evolved considerably and is now commonly defined as "the continued capacity of soil to function as a vital living system, within ecosystem and land-use boundaries, to sustain biological productivity, promote the quality of air and water environments, and maintain plant, animal, and human health" (Doran, 1996; Doran and Zeiss, 2000). The closely related term 'soil quality', which became popular in the 1990s, is often used synonymously with soil health (Liu et al., 2020). There is ongoing debate on whether or not this is appropriate, as some consider soil quality to focus more on specific soil functions and chemical/physical attributes, whereas soil health also encompasses wider biological interactions and considers soil a dynamic living resource (Laishram et al., 2012; Lal, 2016; Liu et al., 2020; Sherwood and Uphoff, 2000). In this thesis the term soil health is used as standard, but soil quality is occasionally

featured when discussing more specific soil functions or attributes (in a similar way to Doran and Zeiss, 2000).

Many studies have shown that earthworms play a fundamental role in the promotion and maintenance of soil health. Earthworms make up the majority of animal biomass found within most soils (Edwards, 2004) and are often described as ecosystem engineers, due to the significant impacts they have on the physical, chemical and biological properties of soils (Le Bayon et al., 2017). Many of these impacts arise as a result of bioturbation, which refers to the process through which feeding and burrowing earthworms mix the soil layers and distribute organic matter throughout the soil profile (Piron et al., 2017). It is worth noting that when invasive earthworms colonise soils that have developed without natural earthworm assemblages the resulting bioturbation can have negative consequences for the wider ecosystem (Blume-Werry et al., 2020; Bohlen et al., 2004; Migge-Kleian et al., 2006). However, in places where earthworms are naturalised, bioturbation is considered an integral process for the maintenance of soil health. Some of the key ways in which earthworms effect soil properties and contribute to soil health, either as a result of bioturbation or otherwise, are explored further below.

Firstly, by feeding on organic matter at the surface of the soil, breaking it into smaller units and transferring it to lower soil layers, earthworms prevent the build-up of dense mats of decaying matter (Hoogerkamp et al., 1983), which reduces nutrient leaching and makes organic matter more accessible for organisms residing below the soil surface (Edwards, 2004). As they move through the soil, earthworms also create burrows either by ingesting the soil directly or physically moving it aside (Jégou et al., 2000). These burrows act as macropores and increase soil porosity, which can increase soil water holding capacity and facilitate gas exchange (Hallam et al., 2021; Hallam and Hodson, 2020; Lubbers et al., 2013; Ma et al., 2021). Another earthworm behaviour that affects the physical structure of soils is cast production, which as well as contributing to soil pedogenesis (Cunha et al., 2016), also leads to the formation of soil macroaggregates (Bossuyt et al., 2006). The resulting packing voids between these macroaggregates can further increase water retention and provide habitat that other soil animals, roots and microbes can occupy (Bossuyt et al., 2006; Castellanos-Navarrete et al., 2012; Piron et al., 2017; Zangerlé et al., 2011). Earthworms have also been shown to initiate the formation of soil microaggregates, which again alters the soil architecture and can have important consequences for the long-term storage of soil carbon (Bossuyt et al., 2005; Pulleman et al., 2005; Totsche et al., 2018).

As well as its physical structure, earthworms can affect soil chemistry in numerous ways. The activity of these animals can heavily influence carbon and nitrogen cycling and increase the concentration of

other trace elements in the soil. Earthworms have been shown to interact with the soil carbon pool in multiple ways and their overall contribution to the carbon cycle is still under debate (Zhang et al., 2013). By feeding on organic matter, earthworms break it down and the resulting carbon can be locked away in stable microaggregates below the surface, which increases carbon stabilisation (Bossuyt et al., 2006; Fonte et al., 2007; Zhang et al., 2013). However, the same feeding behaviour also facilitates further decomposition of organic matter by soil microbes ('priming'), which in turn can increase carbon mineralisation and lead to soils becoming a net source of carbon dioxide (Kamau et al., 2020; Lubbers et al., 2013). Which mechanism comes to dominate is likely to be highly context specific, and calls have been made for further research that looks at the consequences of earthworms for long-term carbon storage and climate change mitigation (Lubbers et al., 2017; Zhang et al., 2013). Complex and context-specific interactions are likewise seen for earthworms and soil nitrogen (Dong and Yin, 2007; Fonte et al., 2007), but increased nitrogen mineralisation in the presence of earthworms has consistently been observed (Medina-Sauza et al., 2019). Alongside nitrogen and carbon, other trace elements considered important for plant growth such as phosphorus, potassium, magnesium and calcium, have been found to occur in higher concentrations in the casts produced by earthworms (Boonchamni et al., 2019; Clause et al., 2014; Jouquet et al., 2008; Van Groenigen et al., 2019).

The biological properties of soils are also greatly affected by earthworms, particularly as a result of their interactions with the soil microbiota. When earthworms feed, the resulting decomposition of organic matter and nitrogen mineralisation can increase microbial diversity, stimulate microbial activity and lead to casts and burrows becoming 'hotspots' for microbe proliferation (Hoang et al., 2016; Hoeffner et al., 2018; Kuzyakov and Blagodatskaya, 2015; Medina-Sauza et al., 2019). Earthworm mucus has also been shown to be particularly important for rapidly increasing the abundance and activity of soil microbes, giving rise to a mutualistic interaction between the two that has been named the "Sleeping Beauty Paradox" (Lavelle et al., 1995). This paradox describes how energy-rich mucus ('the kiss') laid down by the earthworm ('Prince Charming') awakens the dormant microbiota ('Sleeping beauties'), which then proceed to proliferate and further accelerate the breakdown of organic matter (Brown et al., 2000). Alongside a similar mutualism known as the priming effect (Bernard et al., 2012; Medina-Sauza et al., 2019), these earthworm-microbe interactions can have significant implications for wider soil processes and help to boost soil health (Brown, 1995). However, the relationships between earthworms and the microbiota are not all mutualistic, with studies showing that earthworms can cause shifts in microbial diversity as a result of selective grazing and predation of certain fungi and protozoa (Bonkowski and Schaefer, 1997; Curry and Schmidt, 2007; Shan et al., 2013). In addition to interactions with the microbiota, earthworms also shape the biological properties of the soil through their direct and indirect relationships with other soil meso

and macrofauna, often resulting in a general increase in wider soil biodiversity (Decaëns et al., 1999; Gunadi et al., 2002; Monroy et al., 2011).

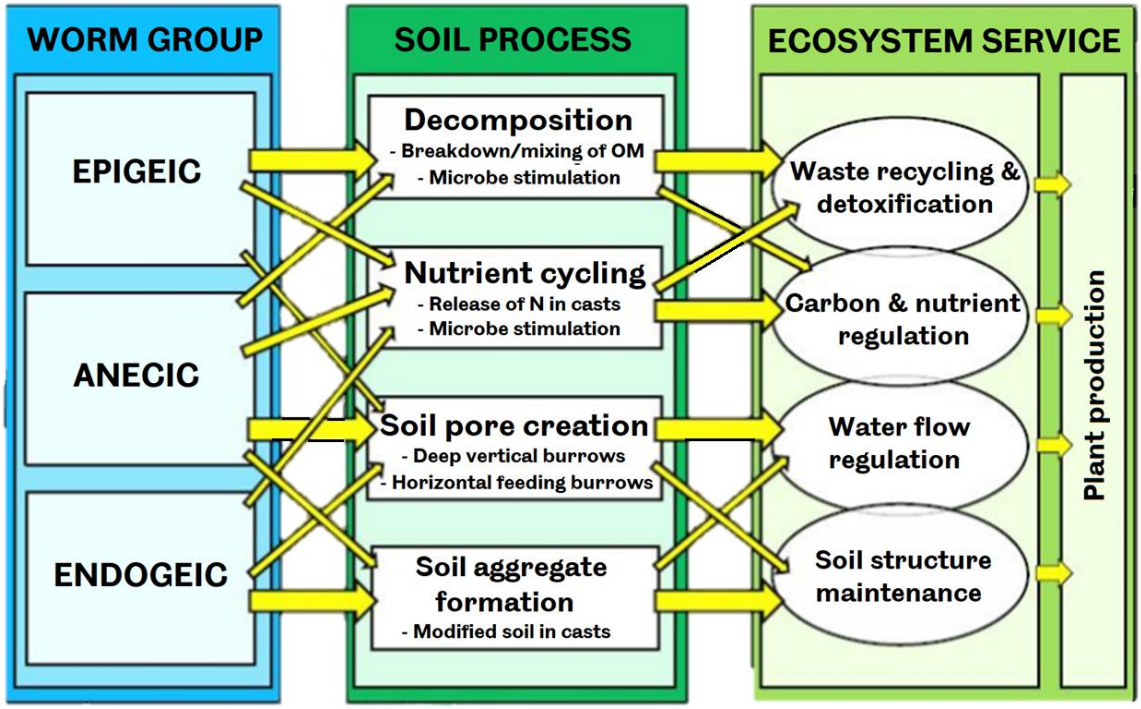

Figure 1.2. A summary of the earthworm ecological categories and their interactions with soil processes and ecosystem services. The thickness of the arrow indicates the relative size of the contribution. (Adapted from Keith & Robinson, 2012).

1.4 Earthworms in agriculture: improving food security and tackling soil degradation

Forecasts suggest that as the global human population continues to rise, by 2050 agricultural calorie production will need to increase by 56% in order to provide everyone with sufficient food (Ranganathan et al., 2018). To further compound this issue, there are a number of additional threats that are already putting the resilience and sustainability of current food systems at risk, including climate change, geopolitical instabilities and waste (Llanos and Border, 2020). This has led the United Nations to prioritise ending hunger and achieving food security as a key objective in the UN Sustainable Development Goals (United Nations, 2019). In the past, the intensification of production systems has allowed great strides to be made with regards to tackling food insecurity and reducing

calorie deficits (Godfray et al., 2010; Tilman et al., 2002). However, there are growing concerns that conventional intensive farming is also having unintended negative consequences, that if left unchecked could threaten the long-term sustainability of agriculture (Kopittke et al., 2019; Tscharntke et al., 2012).

Soil degradation is one unintended consequence of agricultural intensification that is causing particular concern amongst scientists (Jie et al., 2002; Tamene et al., 2019). Although multiple drivers of soil degradation have been identified, including climate and land use change (Muñoz-Rojas et al., 2017; Zhao et al., 2005), studies have shown that it can be particularly exacerbated by intensive farming practises (Kopittke et al., 2019; Lal, 2013; Tilman et al., 2002). Across the globe, an estimated 25% of the total land surface is classed as 'highly degraded or experiencing high degradation', 8% as 'moderately degraded or experiencing moderate degradation' and 36% as stable but 'slightly or moderately degraded' (FAO, 2011). Despite only accounting for 11.2% of the land area, 50.5% of global soil erosion in 2012 occurred in croplands, which experienced a degradation rate that was estimated to be 77 times higher than that seen in forests (Borrelli et al., 2017). The total amount of soil lost in 2012 was thought to be around 35.9 gigatons. The social and economic costs of these losses are profound, especially as 1.5-3 billion people are dependent on soils that are classed as degrading (Bai et al., 2008; Nkonya et al., 2016). In a recent study by Sartori et al. (2019), it was estimated that accelerating soil degradation was reducing global food yields by 33.7 million tonnes every year, with an associated economic cost of eight billion US dollars. Significant costs of soil degradation are also seen in the European Union (Borrelli et al., 2018; Panagos et al., 2018) and in England and Wales, where £1.2 billion is lost each year primarily as a result of declines in soil organic matter and compaction (Graves et al., 2015).

The growing impacts of soil degradation and its potential to disrupt the food system have therefore led to calls for urgent action to tackle this problem, which will require the adoption of new agricultural practises that can increase food yields whilst simultaneously reducing soil degradation (Panagos et al., 2020). Managing agricultural land in ways that boost earthworm populations has been proposed as one potential way to achieve this (Blakemore and Hochkirch, 2017; Lavelle et al., 1989; Yvan et al., 2012), given the integral role that earthworms play in promoting and maintaining soil health described previously. This is because in addition to combatting soil degradation (Blouin et al., 2013), many studies have also shown that the presence of abundant and diverse earthworm populations can be hugely beneficial for crop production too (Bertrand et al., 2015). Positive relationships between earthworms and plant growth are well documented, and a meta-analysis conducted by van Groenigen et al. (2014) found that the presence of earthworms increased crop yields by an average of 25%. The

authors also noted that the level of earthworm-related yield increases was markedly higher in infertile soils that were nitrogen deficient or had undergone disturbance, suggesting that in addition to boosting yields earthworms can also help to reduce the reliance on nitrogen fertilisers. As well as increasing plant growth directly, the activity of earthworms and resulting changes in soil microbiological activity can also suppress crop disease and increase plant resistance to herbivory, in some cases reducing incidences of disease and herbivory by up to 70% and 81% respectively (Elmer, 2009; Xiao et al., 2018). Taking all of these benefits into account suggests that earthworm-focused management could be a promising prospect to enable the sustainable intensification of agriculture going forward, as this can maintain or even increase crop production without sacrificing soil health.

1.5 Earthworms in agriculture: land use and management effects on earthworm populations

Harnessing the potential of earthworm populations and the services they provide requires managing agricultural land in ways that allow them to proliferate. However, many common intensive cropping practices have been found to be harmful for earthworm populations in a number of ways. Continuous cropping depletes levels of soil organic matter that earthworms depend on for food (Dou et al., 2016; Pervaiz et al., 2020), and the removal of crop residues for biofuels or other uses can exacerbate this further (Birkás et al., 2004; Blanco-Canqui and Lal, 2009). Regular and intensive tillage has also been shown to reduce earthworm populations (Briones and Schmidt, 2017), through a combination of direct mortality resulting from the mechanical action of the plough and indirect mortality associated with surface litter removal, burrow destruction and changes in soil physical conditions (Chan, 2001; Johnston et al., 2018). The effects of tillage can also manifest themselves in different ways for the different earthworm ecological categories, with epigeic and anecic populations particularly sensitive to tillage-related disruption (Briones and Schmidt, 2017; Crittenden et al., 2014). If cultivation and tillage are particularly intensive the negative impacts on earthworm populations can be severe. For example, Curry et al. (2002) showed that intensive tillage practises associated with potato cultivation led to earthworm populations being reduced to barely detectable levels in a single season, with few signs of recovery observed in the years following the disturbance. There is also evidence that the use of pesticides in intensive cropping systems can harm earthworms (Gunstone et al., 2021; Céline Pelosi et al., 2014; Treder et al., 2020). Gaupp-Berghausen et al. (2015) showed that glyphosate, the most widely applied herbicide globally (Maggi et al., 2019), caused a dramatic reduction in *L. terrestris* casting activity and cocoon production, whilst Capowiez et al. (2005) found significant reductions in

the weight of *Aporrectodea nocturna* and *Allolobophora icterica* when exposed to field relevant concentrations of the insecticide imidacloprid.

As a result of these practices, conventionally managed arable farms can be inhospitable environments for earthworms. However, changing how agricultural land is managed can help to reverse any damage and increase both earthworm populations and other associated soil health indicators. Examples of earthworm-friendly management include the addition of organic matter inputs and the retention of crop residues after harvesting, which have both been shown to increase earthworm activity and abundance in cropping systems (Abail and Whalen, 2018; Estevez et al., 2011; Fonte et al., 2009). Cover cropping is another technique that can effectively increase the abundance, biomass and reproductive output of earthworms, by providing them with a good supply of food and more stable environmental conditions (Euteneuer et al., 2020; Kautz et al., 2010; Roarty et al., 2017). Reducing tillage intensity and switching to minimum tillage systems can also restore earthworm populations and reverse the damage done by conventional tillage regimes (Briones and Schmidt, 2017; Brown et al., 2003). As well as boosting earthworm numbers and biomass, reduced tillage can restore functional diversity and the associated trait profiles of earthworm communities (Pelosi et al., 2014). However, although the benefits of reduced tillage for earthworms can be considerable, there are also some potential drawbacks (Armengot et al., 2015), and the ultimate success of any of these land use changes is likely to be site and context specific (Morris et al., 2010).

The increasing focus on soil health is also bringing more attention to tradition soil management techniques that used to be more widely implemented before the onset of the green revolution (Blanco and Lal, 2008). One such practice is the addition of temporary leys into crop rotations, which involves taking cropped fields out of production for a few seasons and converting them to pasture in order to rebuild fertility. Typically, the ley is planted using a combination of grasses, legumes and occasionally forbs, which are then left for up to five years before being brought back into cultivation (Martin et al., 2020). The use of temporary arable to ley conversion was once a common technique applied throughout the UK and many other temperate areas, but as affordable synthetic fertilisers and pesticides became more accessible, its use declined in favour of more intensive and specialised continuous cropping systems (Knox et al., 2011).

Recent studies looking at reintroducing temporary leys back into arable rotations have found a range of potential benefits both for agriculture and wider ecosystem services, including weed suppression, water purification and climate regulation (Martin et al., 2020). Temporary conversion to ley has also been found to positively influence a number of soil properties, such as soil organic carbon concentrations and bulk density, which can offset potential yield reductions resulting from the break

in cropping and bring longer term benefits in the form of increased soil health (Hallam et al., 2020; Persson et al., 2008; Prade et al., 2017; Rao et al., 1997). This has led to calls for temporary arable to ley conversion to be included in policy mechanisms aimed at increasing food security and soil health going forward (Knox et al., 2011; Martin et al., 2020). However, with the exception of Hallam et al. (2020) and Lamandé et al. (2003), few studies have examined the impact of ley farming on earthworm populations. If the uptake of this management practice is to be more widely encouraged, further research examining how earthworms respond to arable to ley conversion is needed.

1.6 Monitoring earthworm populations in agroecosystems

In order to effectively manage agricultural land to benefit earthworm populations and assess how they respond to different land use changes, accurate and reliable sampling techniques and information on their wider distributions is needed. As with many soil organisms, earthworms have been severely under-recorded in previous biodiversity monitoring schemes, leading to large knowledge gaps surrounding their geographic distributions and habitat associations. Recent attempts have been made to rectify gaps in earthworm distribution data at various scales, including the first attempt at mapping global earthworm communities and abundance patterns (Phillips et al., 2019), and another study that used habitat-response models to predict local diversity across Europe (Rutgers et al., 2016). In the UK, the first earthworm distribution maps were published by Carpenter et al. (2012), and attempts have been made to develop population assessment protocols for agriculture that can be used in national soil health monitoring programmes (Stroud, 2019). Despite the progress made by these studies, all have highlighted the extensive recording gaps that remain (Figure 1.3), and have emphasised the need for much greater earthworm sampling and better monitoring programmes going forward.

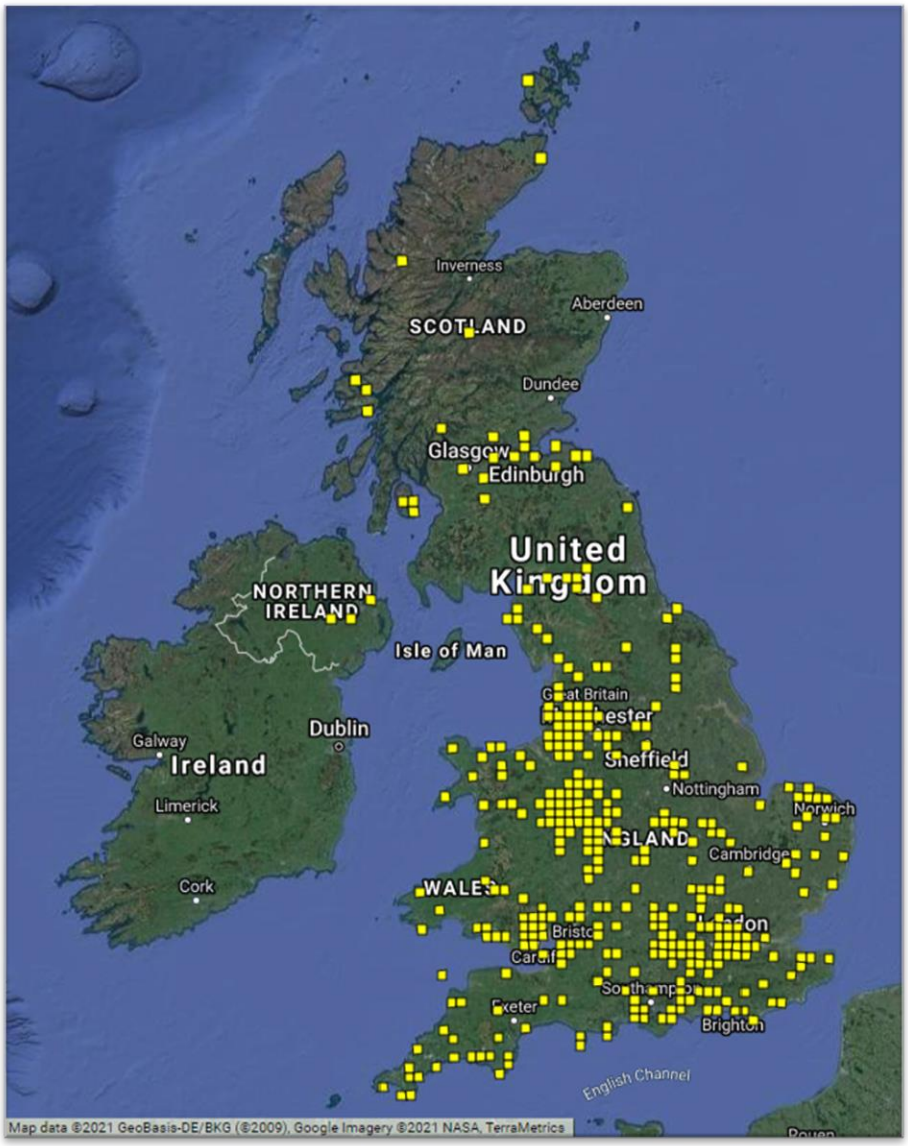

Figure 1.3. UK Distribution map of all the earthworm species records in the National Earthworm Recording Scheme database, hosted by the Earthworm Society of Britain. Each yellow square represents a 10 x 10 km grid cell that contains at least one record for any UK earthworm species. Areas that are not covered by a yellow square have no records. At the time of map creation there were 4873 verified records in the database. NBN Atlas occurrence download at https://nbnatlas.org. Accessed 3rd May 2021. (Data © Earthworm Society of Britain, 2020).

One potential explanation for the historical under-recording of earthworms could be due to limitations associated with conventional sampling techniques. Earthworm populations have traditionally been sampled using traditional hand-sorting or chemical extraction, or a combination of both (Valckx et al., 2011). The hand-sorting method is the most widely employed technique, which involves digging soil pits, sorting through the soil by hand and collecting all the earthworms that are found (Singh et al.,

2016). Hand-sorting has a number of advantages, most notably that it does not require any specialist equipment and is relatively simple to carry out. However, it also has a number of disadvantages and can be very time and labour intensive, difficult to standardise and requires a relatively large number of pits to obtain a representative sample of a given area (Bartlett et al., 2010; Čoja et al., 2008; Valckx et al., 2011). Furthermore, hand-sorting can lead to the underrepresentation of certain earthworm ecological categories, as anecic earthworms are known to flee the pit area when sensing the vibrations made during excavation. Alternatively, the chemical extraction method has been shown to be more effective at sampling anecic earthworms (Callaham and Hendrix, 1997; Chan and Munro, 2001), and does not require the energy-intensive soil excavation associated with hand-sorting. Instead, this technique involves applying a chemical vermifuge to the soil surface, such as mustard solution or allyl isothiocyanate, which irritates the earthworms and causes them to emerge at the surface (Pelosi et al., 2009). Although using a chemical expellant can be less labour intensive, it can also be prone to bias as studies have shown that endogeic earthworms are less responsive to chemical expellants (Singh et al., 2016). Combining both sampling techniques can help to reduce biases (Andriuzzi et al., 2017), but time and labour constraints remain, as highlighted by Targetti et al. (2014), who found earthworm sampling to have the highest time, labour and personnel requirements when assessing the performance of six different strategies for measuring farmland biodiversity. To further compound the issue, both hand-sorting and chemical expellant sampling rely on assessing the morphological characteristics of collected earthworms to identify them down to species level, which requires taxonomic expertise and can only be accurately carried out with mature specimens (Sherlock, 2018).

One alternative approach that could address some of the limitations of the traditional earthworm sampling techniques is environmental DNA (eDNA) sampling. This involves collecting genetic material that has been deposited by organisms as they move through their environment, which can then be extracted, amplified and sequenced using next-generation sequencing technologies. Described as a ‘revolutionary’ technology for biodiversity monitoring (Bylemans et al., 2019; Lawson Handley, 2015), eDNA sampling can be targeted to assess the distributions of a single species (‘barcoding’), groups of species or entire communities ('metabarcoding'; Lawson Handley, 2015). Despite being a relatively new technology, a plethora of studies have been published on the potential of eDNA metabarcoding for the non-invasive sampling of communities that may be difficult to observe through conventional methods, and as sequencing costs continue to decline, it is fast becoming an indispensable tool for aquatic and marine biodiversity monitoring in particular (Belle et al., 2019; Bylemans et al., 2019; West et al., 2020). Studies have also shown that it is possible to extract eDNA from soils (Oliverio et al., 2018), and the feasibility of isolating earthworm eDNA from soil samples has previously been demonstrated by Bienert et al. (2012). However, despite its potential, eDNA sampling has yet to be

used for monitoring earthworm populations in active cropping systems, and many unanswered questions remain regarding its sensitivity, standardisation potential and performance relative to traditional earthworm sampling methods. The need for more research into the performance of eDNA sampling relative to conventional techniques has been recognised by Fediajevaite et al. (2021), who issued a call to action for more quantitative comparisons between this form of sampling and traditional methods, whilst also highlighting the need for more research focused on soil eDNA in particular.

1.7 Research questions and thesis outline

As outlined in this chapter, earthworms play a fundamental role in the maintenance of agricultural soil health, and the services they provide can help to tackle some of the most pressing sustainability issues. However, in order to more effectively manage agroecosystems to boost earthworm populations, it is important that existing knowledge gaps surrounding the effects of different land use practices on earthworms are addressed, and more extensive population monitoring conducted. Improved sampling techniques could help to achieve this, whilst also proving useful for wider soil health monitoring programmes given the close links between earthworms and other soil physical, chemical and biological properties. The wider application of earthworm eDNA sampling could help to bring about the improvements in earthworm monitoring that are needed, but before this can be realised more research and development is required, including field testing and quantitative comparisons with existing sampling techniques.

This thesis aimed to address some of the knowledge gaps surrounding earthworm populations and land use, and to further develop earthworm eDNA sampling for use in agroecosystems. The subsequent chapters addressed the following overarching research questions:

1) How do different land use practices affect earthworm populations and behaviour?
    - Specifically, the effects of ley cropping and hedgerows on earthworm abundance, biomass, diversity and casting behaviour were investigated.
2) Can earthworm populations in agroecosystems be distinguished using eDNA sampling?
3) How sensitive is eDNA to changes in communities bought about by land use changes?
4) How does eDNA sampling compare with traditional earthworm sampling approaches?
5) Are changes in other soil health indicators associated with changes in earthworm populations?
    - Specifically, labile carbon concentrations were measured across different land use treatments and compared with earthworm population variables.

The research conducted to answer these questions is described in the following chapters:

Chapter 2: The effects of temporary arable to ley conversion, connection to hedgerows and weather stress on earthworm abundance, biomass, diversity and casting behaviour were investigated using a semi-controlled mesocosm experiment. The casting behaviour of earthworms in different land use and weather stress treatments was observed across a 28-day period, before destructively sampling the mesocosms and recording the abundance and biomass of the earthworms collected. To obtain diversity measures, adult earthworms were identified down to species level using morphological identification.

Chapter 3: This chapter describes the methodology development work carried out to develop an eDNA based sampling technique that can be used to monitor earthworms in agroecosystems. This included the refinement of a soil eDNA extraction protocol, *in-silico* analysis and laboratory testing of a variety of potentially suitable primer pairs identified in the literature, next-generation sequencing tests and the development of an optimised bioinformatics pipeline for the analysis of returned sequence data.

Chapter 4: The newly developed eDNA sampling technique was used to assess earthworm populations in the experimental SoilBioHedge system at the University of Leeds Field Research Unit. Soil samples were collected for eDNA analysis in conventional arable fields containing strips that had been converted to ley, and earthworm eDNA was extracted, amplified and sequenced using the methodology developed in Chapter 3. The results were compared with those acquired through the use of traditional hand-sorting that was carried out alongside eDNA sampling. The ability of eDNA sampling to detect fine-scale changes in earthworm communities bought about by land use change is discussed, alongside its potential for use as an alternative sampling approach to include earthworms in wider soil health monitoring programmes.

Chapter 5: The labile carbon concentrations of arable and ley soil samples previously used for eDNA analysis were measured to investigate the effects of temporary conversion to ley on this important soil health indicator. The relationship between labile carbon concentrations and earthworm population variables was also investigated. NB This chapter was due to include follow up experiments exploring earthworm preferences for soils containing different levels of labile carbon, but these experiments could not be conducted due to COVID-19.

Chapter 6: This chapter contains the general discussion, which explores the overall findings of each chapter and discusses how they contribute to answering the research questions listed above. Some of the limitations of the experiments are considered, and avenues for further research discussed.

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Chapter 2

The effects of arable to ley conversion, hedgerows and extreme weather on earthworm populations

2.1 Abstract

The inclusion of grass-clover leys in arable rotations has been proposed as a possible measure to tackle the growing issue of soil degradation, given its ability to restore a number of physical and chemical soil health indicators. However, there has been less consideration of the potential of leys for restoring earthworm populations, and whether their effectiveness depends on the presence of surrounding landscape features, such as hedgerows, that may act as earthworm refugia. It is also unclear how extreme weather events, due to become more frequent with climate change, may impact the potential for leys to restore earthworm communities. In this study, a novel mesocosm approach was used to investigate the effects of arable to ley conversion, connection to hedgerows and weather stress on earthworm casting activity, abundance, biomass and diversity. Intact soil blocks were excavated from conventional arable fields and leys strips that were connected or unconnected to hedgerows. The blocks were then exposed to four weeks of drought, flood or ambient weather conditions, during which earthworm casting activity was monitored by collecting and weighing any surface casts produced. On completion, earthworms within the blocks were counted, weighed and identified. Throughout the experimental period significantly higher casting rates were observed in the ley blocks when compared with the arable controls, with the leys also showing greater earthworm abundances, biomass and species richness. No clear evidence of hedgerows improving the success of ley conversion were found. Significant effects of weather stress were seen on earthworm casting activity during the experiment, but drought and flooding did not impact final abundances, biomass or diversity. The results of this study show that temporary leys can effectively restore earthworm populations in degraded arable soils in a short period of time, regardless of the presence of surrounding hedgerows. They also highlight the potential resilience of earthworm populations to weather stress, as, although significant modifications to casting behaviour were observed, no negative effects of drought and flooding were seen on overall earthworm population variables.

2.2 Introduction

Global food security remains a key issue in the 21st Century, and there is mounting pressure on agriculture to increase production in order to provide enough food for the rising human population (Foley et al., 2011; Kopittke et al., 2019). On top of this pressure, multiple issues are threatening existing crop production capacity and the long-term sustainability of agriculture, including the pervasive threats of soil degradation and climate change. Soil degradation is leading to the estimated loss of 35.9 gigatons of soil annually, the majority of which is from croplands (Borrelli et al., 2017), likely as a consequence of intensive cultivation practises such as tillage and short cropping rotations (Bennett et al., 2012; Haddaway et al., 2017; Karlen et al., 1994). Rates of soil degradation are expected to further increase if current trends continue (Borrelli et al., 2020), and in agricultural land they are already threatening the ability of large swathes of food-producing regions to continue to sustain production. For example, around 16.4 million hectares of the European Union agricultural area are experiencing prolonged high soil loss rates that are unsustainable (Borrelli et al., 2018), while in England and Wales soil degradation is estimated to be costing around £1.2 billion per year (Graves et al., 2015). Alongside soil degradation, climate change is also seen as a major threat to agriculture, with significant reductions in major crop yields expected across the globe even under modest warming scenarios (Challinor et al., 2014; Knox et al., 2012; Schmidhuber and Tubiello, 2007; Wheeler and Braun, 2013). To further confound the problems, the scale and complexities of climate change and soil degradation mean their effects can often be interlinked, resulting in positive feedback loops and amplified negative consequences for agriculture (Borrelli et al., 2020; Prăvălie et al., 2021; Tao et al., 2005).

Mitigating the effects of soil degradation and climate change on agricultural production will require multiple solutions across a broad range of disciplines, including through the shifting of agricultural management practises towards those that not only boost crop yields, but also maintain soil quality and health. Restoring agricultural earthworm populations has been proposed as one such measure that could help to achieve this (Bertrand et al., 2015; Blakemore and Hochkirch, 2017; Dewi and Senge, 2015), given the important role that these ecosystem engineers play in the promotion and maintenance of soil health in temperate areas. For example, as earthworms burrow through the soil they change its physical structure, creating macropores that facilitate soil gas exchange and increase soil water holding capacity (Chen et al., 2018; Hallam and Hodson, 2020; Schaik et al., 2014). By feeding on organic matter at the surface of the soil, breaking it down and distributing it throughout the soil profile, earthworms change the chemical composition of the soil and release key nutrients such as carbon, nitrogen and phosphorus, that can then be taken up by plants and microbes (He et al., 2018;

Le Bayon and Milleret, 2009; Zhang and Schrader, 1993). Biologically, through their interactions with other taxa earthworms can stimulate microbial activity and alter microbial diversity (Gong et al., 2019; Medina-Sauza et al., 2019), whilst also playing an important role in the soil food web (Bonkowski and Schaefer, 1997). Through these activities, earthworms in agricultural systems not only increase soil quality and functioning but have also been shown to increase crop yields, with an average yield increase of 25% when they are present (van Groenigen et al., 2014).

Despite the important services that earthworms provide for both soil sustainability and crop production, there are still knowledge gaps surrounding how best to manage agricultural soils in order to boost earthworm populations and maximise their benefits, particularly in the face of climate change. This study aimed to address some of these knowledge gaps, by investigating how different land use practises and weather stresses affected agricultural earthworm populations and their casting activity. A focus on casting activity was taken as, in addition to being a good indicator of earthworm feeding and burrowing activity (Shipitalo et al., 1988), it is an ecologically important behaviour directly linked to the formation and maintenance of healthy soils (Bonkowski et al., 1998; Bossuyt et al., 2004; Edwards, 2004), with earthworm casts also bringing several benefits for crop production. For example, earthworm casting activity has been shown to play an important role in soil aggregation and can help to reduce compaction, increase the concentration of water stable macroaggregates and reduce surface water runoff (Bertrand et al., 2015; Larink et al., 2001; Lipiec et al., 2015; Marashi and Scullion, 2003). Casts have also been shown to contain higher concentrations of important crop nutrients which could help to reduce the need for non-organic fertiliser inputs (Clause et al., 2014; Jouquet et al., 2008), on top of increasing the concentration and stabilisation of organic carbon within the soil (Bossuyt et al., 2004; Zhang and Schrader, 1993).

The land use practises investigated in this study were temporary arable to ley conversion, and the impact of connection to surrounding hedgerows was also explored. There is continued debate over which agricultural land use practises should be favoured by policy schemes in the coming decades, but both temporary ley conversion and the presence of hedgerows have been proposed as potential ways to boost soil quality and health in agroecosystems (Hamer et al., 2018; Holden et al., 2019; Knox et al., 2011; Lenka et al., 2012). Temporary arable to ley conversion involves the addition of legume pasture into the arable rotation, often using grass and clover mixes that are sown into the field and left for a few years to rest the soil and rebuild quality. Before the use of synthetic pesticides and fertilisers became widespread, farming with leys was a practise used widely across traditional mixed arable systems in the UK and elsewhere, but has since declined in favour of continuous crop production (Knox et al., 2011). However, in recent years the reintroduction of temporary leys back into arable rotations

has been gaining more attention, as concerns over soil degradation and declining soil quality grows. Previous studies have demonstrated the benefits of temporary arable to ley conversion for increasing soil carbon and subsequent crop yields (Persson et al., 2008; Prade et al., 2017), and policy mechanisms are beginning to look into encouraging their uptake (for example the UK Government 25 year environment plan, 2018). However, with the exception of Hallam and Hodson (2020), there has been little consideration of the response of earthworm populations to the reintroduction of leys, and the potential role they play in resulting improvements to soil quality.

Alongside the reintroduction of leys, the role of hedgerows in the improvement of soil health within adjacent agricultural fields has also gained increasing recognition in recent years. Studies have shown that soil quality underneath hedgerows can be significantly higher than in the surrounding arable fields (Holden et al., 2019) and connection to them can bring a number of benefits to the adjacent farmland, including reduced soil erosion and increased water storage (Adhikary et al., 2017; Ghazavi et al., 2008; Lenka et al., 2012). Given that there is often increased soil biodiversity under hedgerows it has also been hypothesised that they may act as a potential refugia for soil organisms (Montgomery et al., 2020; Spaans et al., 2019), maintaining a biodiversity reservoir which can then spread and recolonise the surrounding soils when conditions become more favourable (for instance when arable fields are converted to ley). However, while the role of hedgerows as potential refugia for the subsequent recolonisation of agricultural land by soil organisms has been demonstrated for other taxa (Alvarez et al., 2000), this has yet to be investigated for earthworms. Given the limited duration of this experiment and the active dispersal rates of earthworms described in the literature (Eijsackers, 2011), it is unlikely that a definitive conclusion on whether or not hedgerows act as refugia can be reached by this study (reasons for this are discussed further in section 2.5). However, it is nonetheless hoped that it can be a useful starting point for further investigation.

Investigating the effects of different land uses on earthworm populations to inform agricultural practises going forward also requires acknowledgement of the future climatic conditions that they will be operating in. As a result of climate change many countries, including the UK, are predicted to experience increased frequencies of more severe weather stress events, such as drought and flooding (DEFRA, 2018; Rahmstorf and Coumou, 2011). These events can severely affect agriculture through direct crop losses, increased erosion and changes to the physical and chemical profile of soils (Falloon and Betts, 2010; Lesk et al., 2016; Powell and Reinhard, 2016; Prăvălie et al., 2021; Rial-Lovera et al., 2017). However, less is known about the effects of severe weather stress on soil macrofauna, including earthworms. A recent review by Coyle et al. (2017) highlighted this, showing that whilst the number of studies on soil microbial diversity had increased exponentially in the last few decades, the number

focused on earthworms and other soil macrofauna has remained extremely low. As a consequence, the authors issued an urgent call for more research into the effects of climatic disturbances on soil macrofauna, to help ensure future soil disturbance mitigation strategies are informed and fit for purpose.

This study investigated the effects of arable to ley conversion, connection to hedgerows and weather stress events on earthworm casting behaviour, abundance, biomass and species diversity using a semi-controlled mesocosm experiment. Intact soil blocks, referred to as monoliths, were extracted from arable fields and ley strips connected or unconnected to hedgerows, and then exposed to ambient, drought or flood conditions. Casting activity within the monoliths was monitored before, during and after the weather stress events had taken place, and at the end of the experimental period the monoliths were broken up and earthworm numbers and biomass were recorded. Collected earthworms were then identified to species level and diversity patterns were investigated. It was hypothesised that casting activity and earthworm abundance, biomass and diversity would be greater in the ley monoliths compared to the arable controls and, if hedgerows are acting as refugia for earthworms, numbers in the leys connected to hedgerows would be greater still. I also hypothesised that weather stress would lead to reductions in earthworm casting activity and increase mortality, thereby reducing the abundance, biomass and diversity recorded in the droughted and flooded monoliths at the end of the experiment.

2.3 Methodology

This experiment formed part of the larger SoilBioHedge monolith study, which investigated the effects of land use and weather stress on a range of soil quality indicators. The experimental design, including the number of replicates and weather stress protocols, was inherited from the larger study. The full methodology including all the additional variables measured can be found in (Berdeni et al., 2021), but in this section only the methodology relevant to the earthworm study is described in detail.

Field site, land use factors and monolith extraction

The study site consisted of four arable fields at the University of Leeds Field Research Unit, a commercial mixed farm in West Yorkshire, United Kingdom (53° 52' 25.2 N 1° 19' 47.0" W). Three of

these fields had been conventionally managed (tilled, harrowed and cropped) since 1995 and predominantly cultivated wheat, with break crops such as oilseed rape, barley, potatoes and vining peas also included in the rotation. These were referred to as 'Big substation East', 'Big substation West' and 'Copse' (Figure S1). The remaining field, known as 'Hillside', followed the same management with the exception of a 10-year period where it was managed as pasture (1998 – 2008), before being returned to continuous annual cultivation. In April – June 2015, pairs of ley strips were established in each field by spraying the winter wheat crops with glyphosate, tilling the soil in the strips and then sowing with a grass-clover seed mixture. The strips were 3 metres wide by 70 metres long and extended out into the arable field from the bordering hedgerow and field margin (Figure 2.1). In each pair, one ley strip (referred to as the unconnected ley) had a steel mesh barrier inserted down to the bedrock between the strip and the hedgerow, preventing subsurface movement of earthworms from the hedgerow to the strip. The other ley strip (connected ley) did not have a steel barrier inserted and remained connected to the hedgerow.

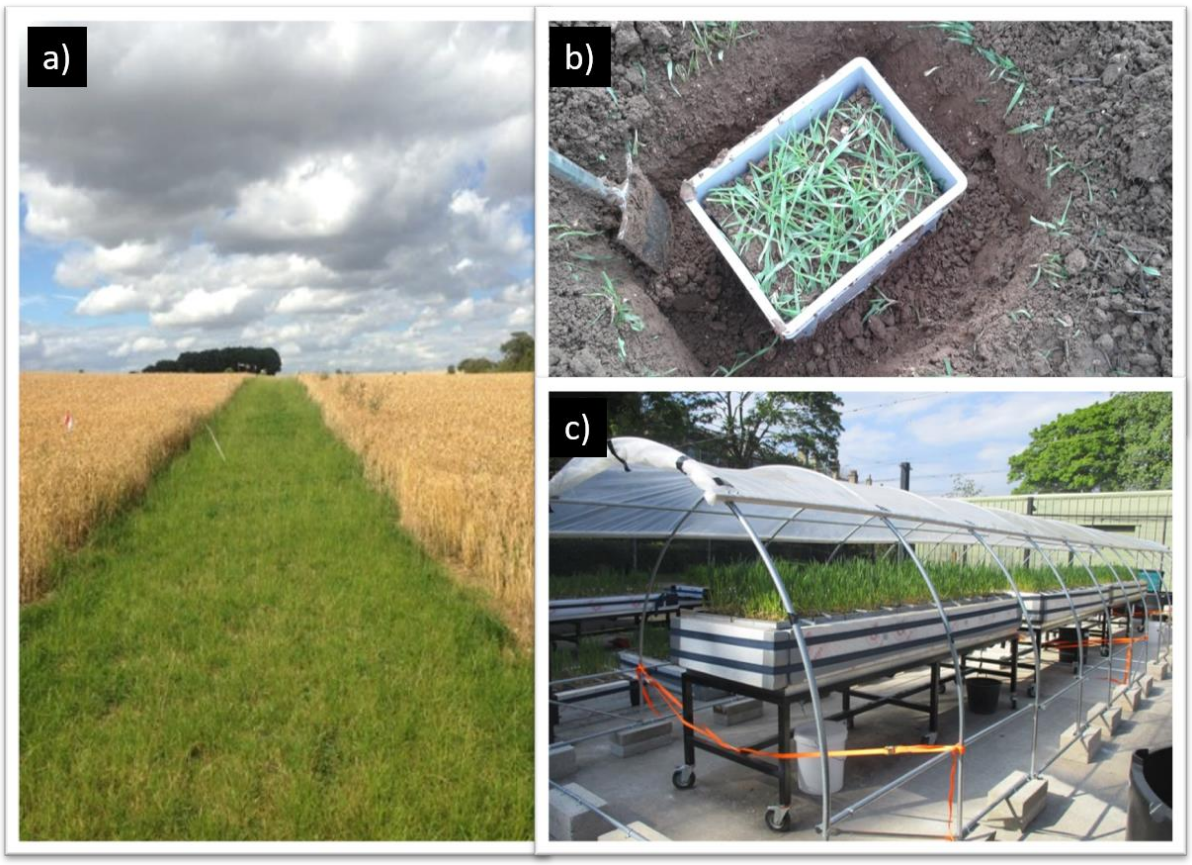

Figure 2.1. a) One of the ley strips sampled at the study site, b) the monolith extraction in progress, c) the outdoor benches where the monoliths were stored during the weather stress experiment. Photographs (a) and (b) courtesy of Despina Berdeni.

In November 2016, when the ley strips had been established for 18 months, intact soil monoliths measuring 37 cm long x 27 cm wide x 22 cm deep were excavated from the ley strips and arable fields, 68 metres from the surrounding hedgerow (Fig. 1). Three replicate monoliths were taken from each ley strip and from the arable field area between them (the control), giving a total of nine monoliths from each field and 36 in total. Twelve of these were arable field control monoliths, 12 were from the unconnected leys and 12 from connected leys. They were then placed into plastic boxes with 10 mm drainage holes in their bases. The boxes were lined with 0.5 mm pore size nylon mesh to allow drainage but prevent the loss of earthworms and soil through the holes. In mid-December 2016, 19 months after the leys had first been established, a herbicide was applied to the surface of the ley monoliths to kill the vegetation covering them. Once this had senesced, the remaining shoots were clipped to soil level and removed. The wheat seedlings that had been growing in the arable field monoliths were carefully removed by hand and did not require the use of a herbicide. In January 2017 the monoliths were taken to the Arthur Willis Environment Centre at the University of Sheffield, where they remained for the duration of the experimental period.

Monolith preparation and weather stress simulation

At the University of Sheffield, the three replicate monoliths from each sampling location were divided across three separate outdoor benches and 100 mm thick thermal insulation board was placed on the bottom and sides to simulate field conditions (Fig. 1). In an effort to prevent earthworms escaping from the monoliths or moving between boxes Velcro strips were applied to the inner rim of each, which has been reported as an effective way to stop earthworms climbing out of containers (Lubbers and van Groenigen, 2013). At the end of January 2017, 30 young wheat seedlings were inserted into each monolith in accordance with field cropping density, and in April and early May 0.48 g of ammonium nitrate fertiliser was applied to each.

Immediately prior to the onset of the weather stress experimental drought and flooding events, an open-sided transparent rain shelter was erected over the benches so that the water supply to the monoliths could be controlled (Fig 1.). On 4th May 2017 the experimental weather stress began. On one bench, referred to as the ambient treatment, the monoliths were watered three times a week with 42.6 mm of tap water (which equates to the average May rainfall for Sheffield). The second bench, referred to as the drought treatment, received no water at all for the duration of the weather stress period. The monoliths on the final bench, the flood treatment, had their drainage holes sealed with bungs before being saturated with tap water until they were completely submerged with ~3 cm of standing water. These monoliths were continuously topped up with tap water to replace the water

lost through evapotranspiration. The experimental weather stress period lasted for 28 days in total, during which daily minimum and maximum temperatures ranged between 4.1 – 26.6°C. At the end of this period, the bungs were removed from the flooded monoliths and 2 litres of water was added to the drought and ambient monoliths to bring them up to field capacity. The rain shelter was removed and all monoliths then received ambient conditions until they were dismantled in October 2017.

Earthworm casting activity measurement

In the days leading up to the 4th May 2017, just before the weather stress events began, the spatial position of every cast visible at the surface of each monolith was recorded on gridded paper. Individual casts were then carefully collected using tweezers and placed in Eppendorf tubes marked with a unique ID. The total number of casts from each monolith was recorded and all casts were taken to a nearby laboratory for storage before weighing. Within 1-2 days of collection individual casts were weighed and any that exceeded 1.5 grams were returned back to the monolith surface (marked with a pin to avoid recollection), to limit the potential of cast removal impacting other soil properties that were being measured in the wider experiment. This initial cast collection was referred to as 'collection A'. Further cast collection and mapping was carried out in the same way at two points during the weather stress events, at around two weeks ('collection B') and four weeks ('collection C') after the onset of drought and flooding. Four further cast collections were carried out immediately after the weather stress events ended to measure casting activity in the recovery period after drought and flooding. These were referred to as 'collection D' (1-2 days post-weather stress), 'collection E' (4-5 days), 'collection F' (11-13 days) and 'collection G' (21-22 days). To enable accurate comparisons to be made between the collections, daily casting rates were calculated for each monolith by dividing the number of casts by the number of days since the last collection.

Measuring earthworm abundance, biomass and diversity

In October 2017 after the wheat had been harvested, the soil of each monolith was removed from the plastic box, broken up by hand and put through a 1 cm sieve. Any earthworms found were collected and separated into adults and juveniles based on the presence of a visible clitellum. The adults and juveniles from each monolith were then weighed to obtain the fresh biomass and the total number of each was recorded, before being preserved in 90% ethanol and stored at 4°C. Species identification of the adult earthworms was then carried out by determining morphological characteristics under a

dissecting microscope, following the Sherlock (2012) key and using the Burkmar (2018) Earthworm Society of Britain online 'Identikit'.

Statistical analysis

All statistical analyses were performed in R studio using R version 3.4.3 or later (R Core Team, 2020). Model assumptions were checked and plotted using the 'autoplot' function in the ggplot2 graphics package (Wickham, 2011) and Shapiro-Wilk normality tests performed where appropriate. To investigate the effects of land use on initial monolith cast numbers before the onset of weather stress, a negative binomial generalised linear model was fitted with cast number as the response variable and field ID and land use type as the predictors. A negative binomial model was chosen to account for overdispersion in the cast number data (see Lindén and Mäntyniemi, 2011), and field ID was included to check for location-specific factors that may influence earthworm populations. Likelihood ratio testing was performed to obtain the statistical significance of each predictor variable, and post-hoc Tukey Honest Significant Difference tests were carried out using the 'Tukey HSD' function. To look at the effect of land use on cast weights before the onset of weather stress, a mixed effects model was fitted using the function 'lmer' in the package 'lme4' (Bates et al., 2014), with the log transformed cast weights as the response variable and land use and field ID as predictors. Monolith ID was also included as a random effect to account for the expectation that cast weights within the same monolith would be more similar to each other. Tukey tests were performed using the 'glht' function in the 'multcomp' package (Hothorn et al., 2008). Four further mixed effects models were fitted to test the effect of land use and weather stress on both casting rates and cast weights during and after the weather stress period. These models were structured with either log daily casting rate or log cast weight as the response variable and land use, weather stress, field ID, collection and the weather stress x collection interaction as fixed effects, and monolith ID as a random effect (to account for repeated measures). Post-hoc Tukey tests were performed to ascertain the differences between groups.

To test for possible effects of land use and weather stress on earthworm abundance in the monoliths, linear models were fitted using the function 'lm', followed by post-hoc Tukey tests. For these models either total earthworm number, juvenile number or adult number were included as response variables, with land use, weather stress and field included as predictor variables. Square root transformation was performed on the adult number data to satisfy the assumptions of ANOVA, but this was not necessary for the juvenile or total earthworm abundances, as according to diagnostic checks models using the untransformed data did not violate the assumptions. The same model structure was also used to test for possible effects of land use, weather stress and field on total

earthworm biomass. Alongside total biomass, the average individual adult and juvenile biomass was calculated for each monolith and tested in the same way, to see whether land use or weather stress had any effects on the size of individual earthworms in the monoliths. To satisfy the assumptions of the ANOVAs, square root and log transformation were performed on the mean individual adult biomass and mean individual juvenile biomass respectively. Finally, to investigate differences in earthworm diversity a linear model was fitted, which contained monolith species richness as the response variable and land use, weather stress and field ID as predictors.

2.4 Results

Earthworm casting activity

Across the duration of the experiment, 2839 casts were collected and categorised. There were 360 casts collected from the arable control monoliths, with a mean weight of 0.20g; 1404 from the connected leys, with a mean weight of 0.35g; and 1075 from the unconnected ley monoliths, with a mean weight of 0.24g. In total, 1405 casts were collected before the onset of the drought and flooding events, 335 were collected during the drought and flooding period (from the drought and control monoliths only) and 1099 were collected in the recovery period after the droughted and flooded monoliths were returned back to ambient conditions (Table 2.1).

Table 2.1. The number of casts collected in each collection period, grouped by land use and weather stress. Collection period A was prior to the onset of weather stress, collections B-C were during the weather stress events and collections D-G were after the weather stress had ended.

	Land use				**Weather stress**		
Collection period	Arable Control	Connected ley	Unconnected ley	***Total***	Ambient	Drought	Flood
A	129	730	546	***1405***	596	488	321
B	21	109	77	***207***	123	84	-
C	8	66	54	***128***	92	36	-
D	32	80	60	***172***	25	65	82
E	18	60	49	***127***	18	30	79
F	96	221	187	***504***	150	154	200
G	56	138	102	***296***	94	136	66
Total	***360***	***1404***	***1075***	***2839***	***1098***	***993***	***748***

Casting activity before drought and flooding events

There were 1405 surface casts collected from the monoliths immediately prior to the onset of the drought and flooding events. Of these, 129 were collected from the arable control monoliths, 730 from the connected leys and 546 from the unconnected leys (Table 2.1). Initial cast number was significantly affected by land use (LRT: $X^2_1 = 40.94$, $p < 0.001$), with both the connected and unconnected ley monoliths yielding significantly more casts than the arable field controls (Tukey multiple comparison tests: $p < 0.001$ and $p = 0.001$ respectively; Figure 2.2a). The number of casts collected from the connected and unconnected leys were not significantly different from each other ($p = 0.20$). The field that the monoliths originated from was also found to significantly affect cast numbers ($X^2_1 = 20.83$, $p < 0.001$), with post-hoc tests indicating significantly higher numbers in the 'Big substation West' and 'Hillside' fields when compared with the 'Big substation East' field ($p = 0.03$ and $p = 0.02$ respectively; Figure S2a).

For cast weight, casts collected before the weather stress events began were significantly affected by land use (LRT: $X^2_1 = 10.69$, $p = 0.004$; Figure 2.2b) and field ($X^2_1 = 8.81$, $p = 0.032$; Figure S2b). With a mean weight of 0.54 g, casts in the connected ley monoliths were significantly heavier than those in the unconnected leys ($p = 0.009$) and arable controls ($p = 0.003$). The casts weights of the unconnected leys and arable controls were not significantly different from each other ($p = 0.376$). For field, casts collected from 'Hillside' field were found to be significantly heavier than those from 'Big substation West' ($p = 0.029$) and 'Copse' ($p = 0.039$).

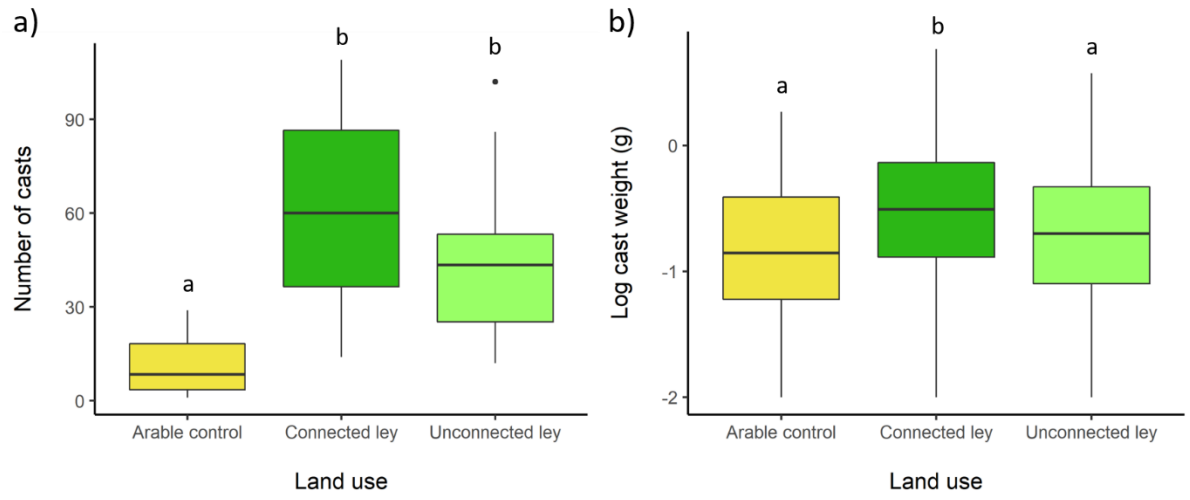

Figure 2.2. a) The total number of casts per monolith and b) cast weight by land use type before the onset of the weather stress events (collection A). Plots show the median, interquartile range and min/max values for each group. Different letters indicate the groups were significantly different from each other in post-hoc Tukey tests ($p < 0.05$).

Casting activity during drought and flooding events

During the period that the monoliths were exposed to weather stress, 335 casts were collected: 215 from the ambient and 120 from the droughted monoliths. Of the casts collected, 29 came from the arable controls, 175 from the connected leys and 131 from the unconnected leys. Daily casting rate was found to be significantly affected by land use (ANOVA: $F = 7.52$, d.f. = 2, $p = 0.004$; Figure 2.3a), with higher casting rates in the connected and unconnected ley monoliths compared to the arable controls (Tukey multiple comparison tests: $p < 0.001$ and $p = 0.005$ respectively). The casting rates in the two ley treatments were not significantly different ($p = 0.53$). No significant effect of weather stress on casting rates in the ambient and droughted monoliths was found ($F = 3.82$, d.f. = 1, $p = 0.06$; Figure 2.3b), and field did not significantly affect casting rate ($F = 2.35$, d.f. = 3, $p = 0.11$; Figure S3).

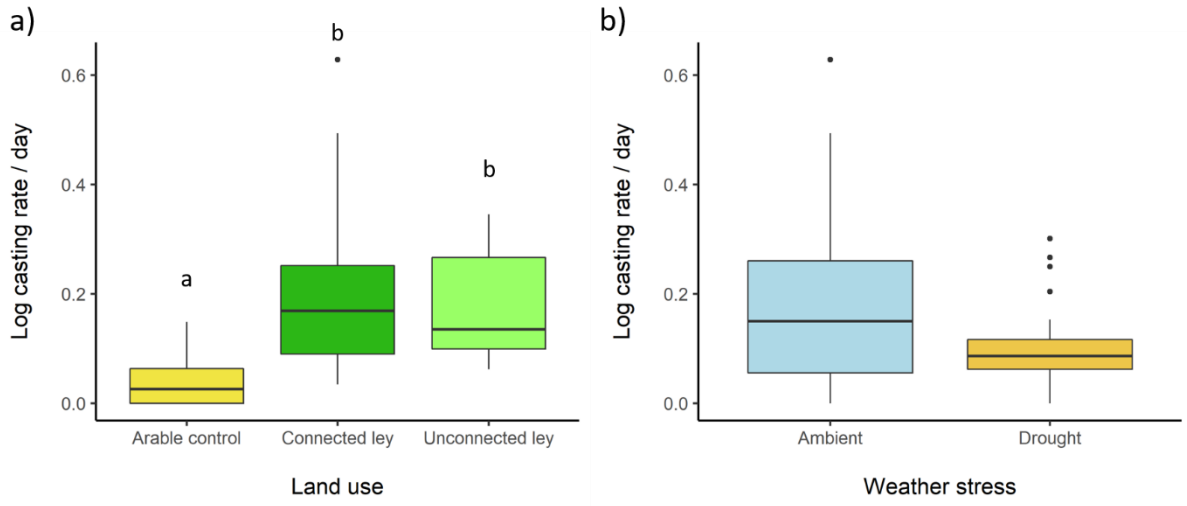

Figure 2.3. Daily casting rates by a) land use type and b) weather stress treatment, during the drought and flooding period (collection B-C). Plots show the median, interquartile range and min/max values for each group. Different letters indicate the groups were significantly different from each other in post-hoc Tukey tests ($p < 0.01$).

There was no overall effect of land use (ANOVA: $F = 1.84$, d.f. = 2, $p = 0.19$), drought ($F = 0.002$, d.f. = 1, $p = 0.96$), or field ($F = 0.09$, d.f. = 3, $p = 0.96$) on the weight of casts collected during the weather stress period (Figure 2.4). However, a significant effect of collection period was observed, with collection B casts - taken around two weeks after the onset of weather stress - weighing significantly more than collection C casts, which were taken around 4 weeks after onset ($F = 9.51$, d.f. = 1, $p = 0.002$; Figure 2.5). A significant interaction between weather stress and collection also suggests that cast

weights in the ambient and drought monoliths were affected differently by the collection period ($F = 22.98$, d.f. = 1, $p < 0.001$), with Figure 2.5 indicating declines in cast weights in droughted monoliths between collection B and C, in contrast with the ambient monoliths where cast weights remained stable throughout.

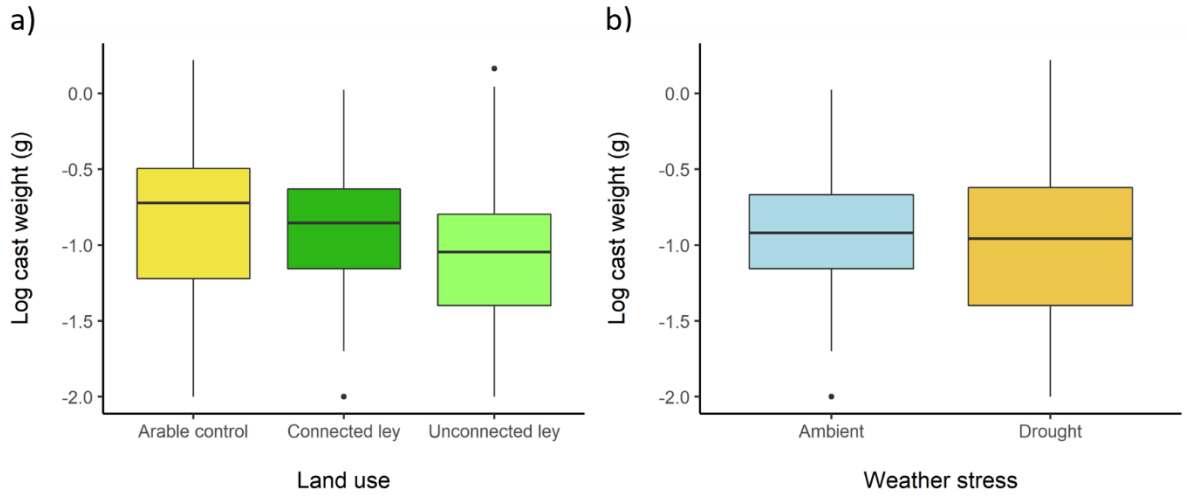

Figure 2.4. Cast weight by a) land use type and b) weather stress treatment during the drought and flooding period (collections B-C). Plots show the median, interquartile range and min/max values for each group. No significant effects of land use or weather stress on cast weight were detected at this stage of the experiment.

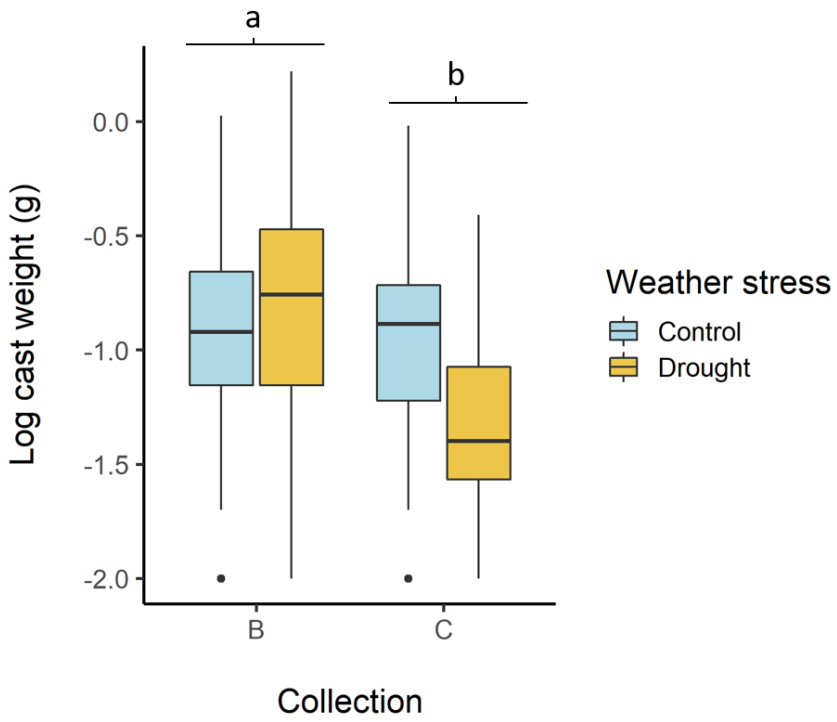

Figure 2.5. The weight of casts taken at collection point B (two weeks after the onset of weather stress) and C (four weeks after the onset), grouped by weather stress treatment. Plots show the median, interquartile range

and min/max values for each group. Lower case letters indicate the significant difference in log cast weights between collection points B and C ($p = 0.002$).

Casting activity after drought and flooding events

In the collection periods after the weather stress events had ended, a total of 1099 casts were collected from the monolith surfaces. There were 202 casts collected from the arable control monoliths, 499 from the connected leys and 398 from the unconnected leys (Table 2.1). Land use was again found to significantly affect casting rate ($F = 9.14$, d.f. = 2, $p < 0.001$; Figure 2.6a), with the connected and unconnected leys showing higher rates than the arable controls ($p < 0.001$ and $p = 0.002$, respectively), and no significant difference between each other ($p = 0.44$). In contrast with casting rates during the weather stress events, after the events had ended a significant effect of weather stress on casting rates was observed ($F = 5.26$, d.f. = 2, $p = 0.01$; Figure 2.6b), with higher rates in the monoliths that had undergone both drought and flooding compared with the ambient control (both $p < 0.001$). The overall casting rates in the droughted and flooded monoliths were not significantly different after the weather stress events had ended ($p = 0.27$). Collection period was also found to affect casting rates post-weather stress ($F = 17.16$, d.f. = 3, $p < 0.001$) and a significant interaction between weather stress treatment and collection was observed ($F = 8.53$, d.f. = 6, $p < 0.001$), indicating the effects of previous weather stress on casting rates were expressed differently across the collection periods. This is highlighted by Figure 2.7, which shows casting rates in post-droughted and flooded monoliths were markedly higher than the ambient controls at collection point D (1-2 days after weather stress ended), before gradually declining and converging with the ambient controls in later collections.

As in the previous experimental stages, the weight of casts collected after the drought and flooding periods was not affected by land use ($F = 1.03$, d.f. = 2, $p = 0.37$) or field ($F = 0.35$, d.f. = 3, $p = 0.79$). However, a significant effect of weather stress treatment was seen ($F = 3.82$, d.f. = 2, $p = 0.039$; Figure 2.8a), with heavier casts in the monoliths that had previously experienced drought conditions rather than ambient ($p = 0.002$) or flood conditions ($p < 0.001$). The latter two did not differ significantly from each other ($p = 0.21$). Collection period also affected cast weight ($F = 16.78$, d.f. = 3, $p < 0.001$), and a significant interaction between weather stress and collection period was again observed ($F = 3.87$, d.f. = 6, $p < 0.001$), indicating that the way cast weights changed across the D-G collection periods depended on which weather stress treatment they had been exposed to (Figure 2.8b).

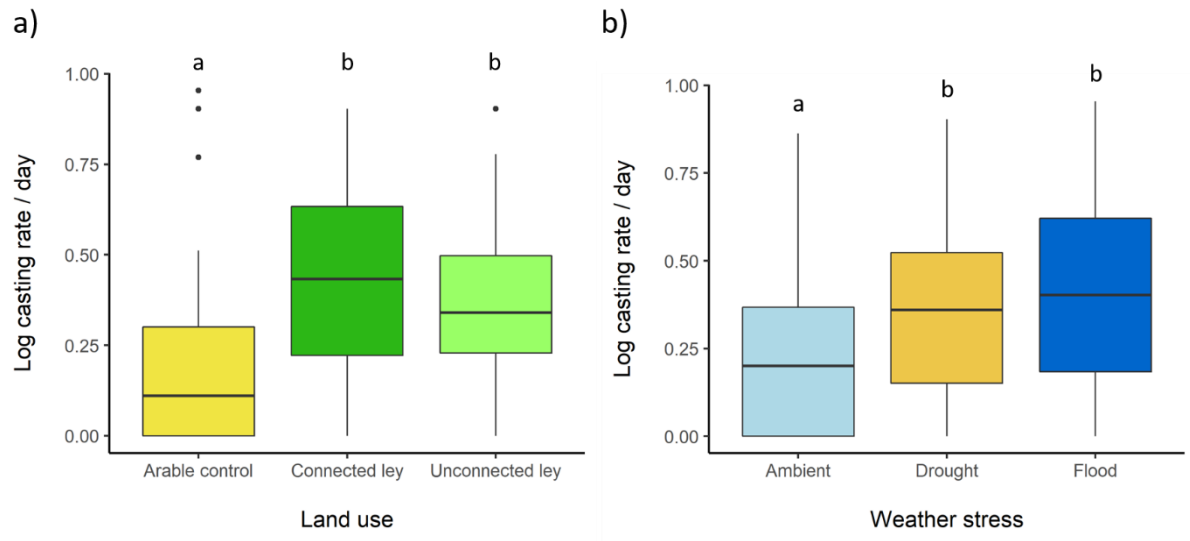

Figure 2.6. Daily casting rates by a) land use type and b) weather stress treatment, after the drought and flooding period had ended (collections D-G). Plots show the median, interquartile range and min/max values for each group. Different letters indicate the groups were significantly different from each other in post-hoc Tukey tests ($p < 0.01$).

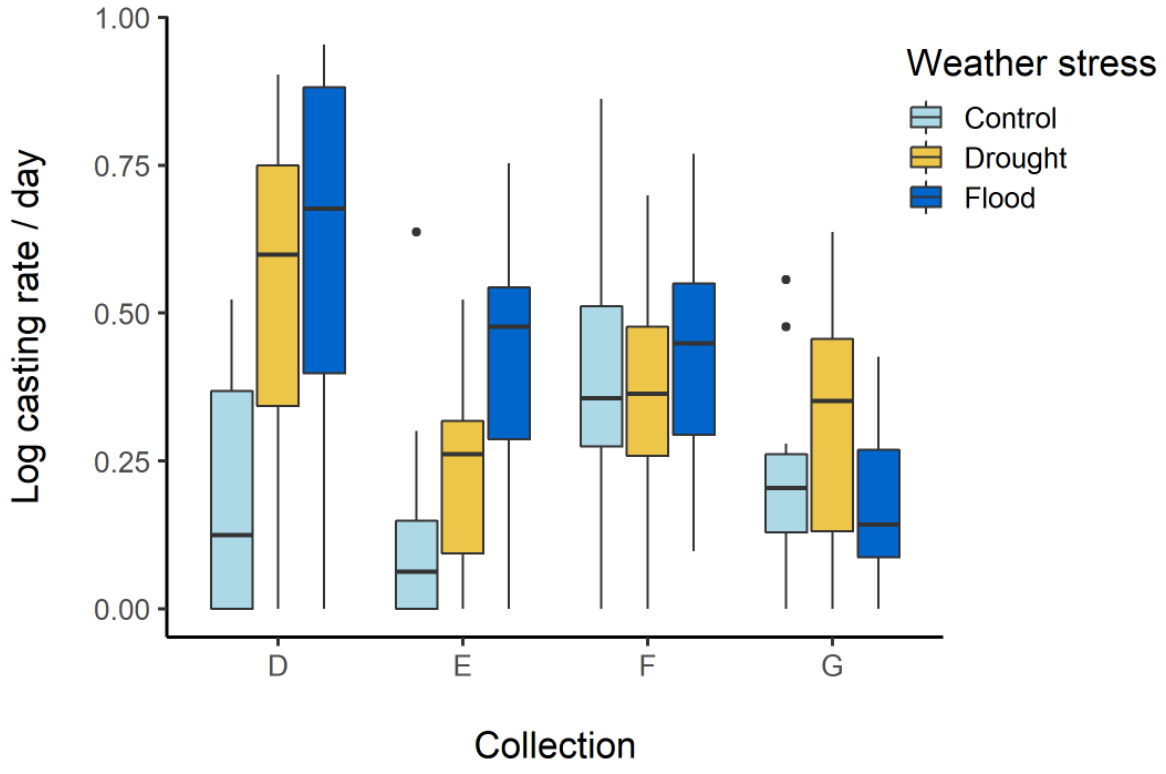

Figure 2.7. Daily casting rates in the recovery period after the weather stress events had ended. Collection D was 1-2 days after the weather stress period ended, E was 4-5 days, F was 11-13 days and G was 21-22 days. Plots show the median, interquartile range and min/max values for each group.

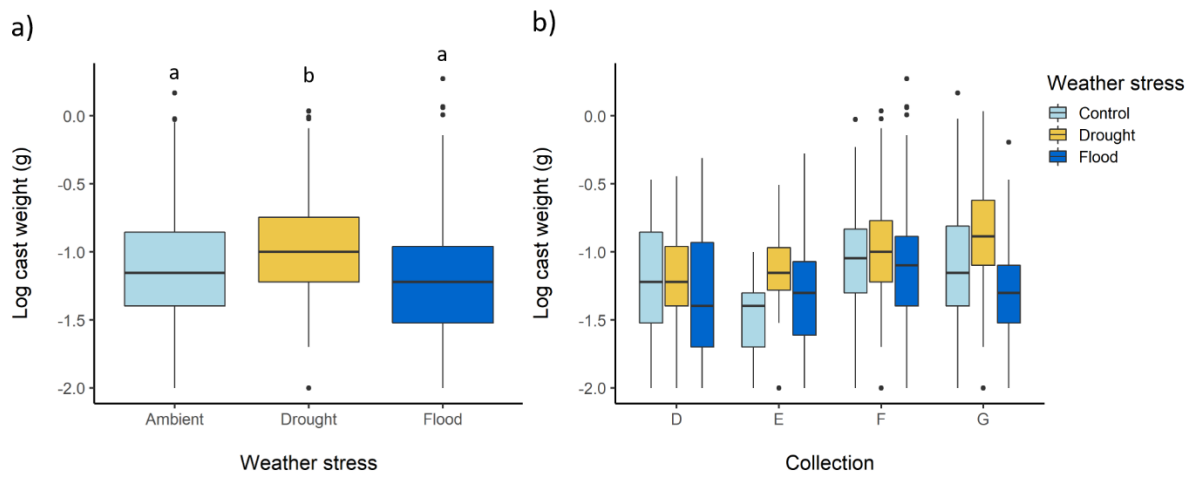

Figure 2.8. a) Overall cast weights in the ambient, drought and flood monoliths in the recovery period after the weather stress events had ended (collections D-G). Different letters indicate the groups are significantly different from each other in post-hoc Tukey tests ($p < 0.01$). b) The cast weights for each collection within the recovery period, grouped by weather stress treatment. Collection D was 1-2 days after the weather stress period ended, E was 4-5 days, F was 11-13 days and G was 21-22. Both plots show the median, interquartile range and min/max values for each group.

Earthworm abundance and biomass

At the end of the experiment 830 earthworms were collected from the monoliths, consisting of 304 adults (36.6%) and 526 juveniles (63.4%). There were 132 earthworms collected from the arable controls, 323 from the connected leys and 375 from the unconnected leys. The total number of earthworms was found to be significantly affected by land use ($F = 29.64$, d.f. = 2, $p < 0.001$), with the connected and unconnected leys containing significantly more earthworms than the arable control monoliths (both $p < 0.001$; Figure 2.9a). Within these both the juvenile and adult abundances followed the same patterns (for juveniles $F = 13.68$, d.f. = 2, $p < 0.001$; for adults $F = 13.21$, d.f. = 2, $p < 0.001$), showing significantly higher numbers in the connected ($p < 0.001$ and $p = 0.003$ respectively) and unconnected (both $p < 0.001$) leys compared to the arable controls. For both juveniles and adults there was no significant difference in abundance between the two ley types ($p = 0.77$ and $p = 0.14$). No significant effects of weather stress ($F = 2.12$, d.f. = 2, $p = 0.14$) or field ($F = 1.11$, d.f. = 3, $p = 0.36$) were seen on the final earthworm totals, with the same outcome when analysing juvenile and adult abundances separately.

As expected, given the higher numbers in the ley monoliths, total earthworm biomass was significantly affected by land use ($F = 8.79$, d.f. = 2, $p = 0.001$), with greater earthworm biomass in the connected

(p = 0.02) and unconnected (p = 0.002) leys when compared with the arable controls (Figure 2.9b). No significant difference between the two ley types was observed (p = 1), and neither were significant effects found for weather stress (F = 0.58, d.f. = 2, p = 0.57) or field (F = 2.68, d.f. = 3, p = 0.07). When looking at the mean individual biomass of adults, the effect of land use was no longer significant (F = 1.28, d.f. = 2, p = 0.29) and no effect of weather stress (F = 1.28, d.f. = 2, p = 0.29) or field (F = 0.57, d.f. = 3, p = 0.64) was observed. Similar results were observed for mean individual juvenile biomass, except field ID was found to have a significant effect (F = 5.11, d.f. = 3, p = 0.006), with a higher mean biomass in 'Copse' and 'Hillside' fields compared with 'Big substation West' (p = 0.04 and p = 0.005, respectively; Figure S6).

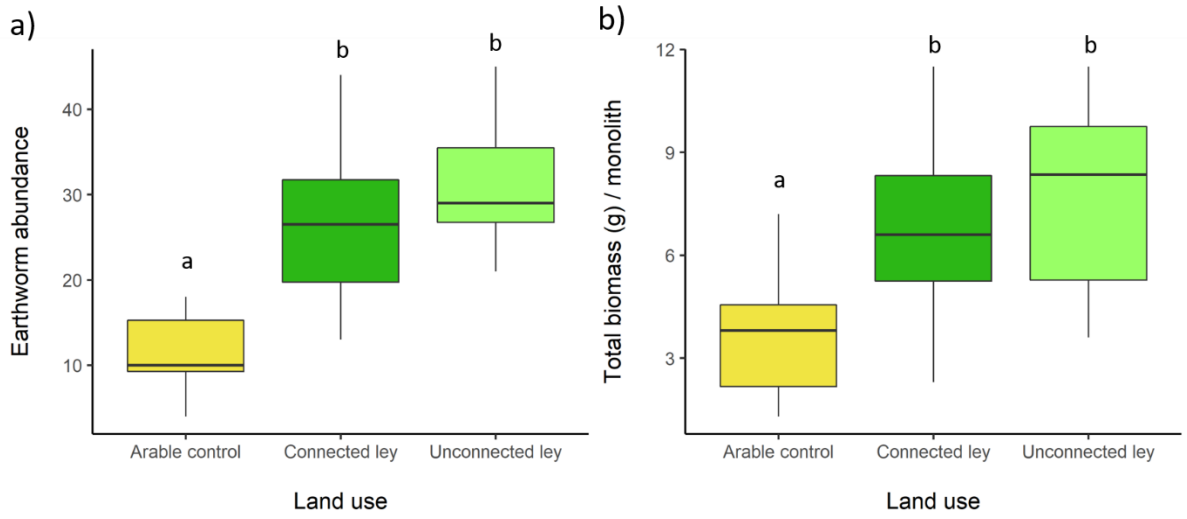

Figure 2.9. a) Total number of adult and juvenile earthworms per monolith and b) Total biomass per monolith grouped by land use type. Different letters indicate the groups were significantly different from each other in post-hoc Tukey tests (for a) p < 0.001; for b) p < 0.05). Plots show the median, interquartile range and min/max values for each group.

Earthworm diversity

Of the 304 adult earthworms collected after the experimental period, 298 could be identified to species level. Species richness in the monoliths was found to be significantly affected by land use (F = 6.10, d.f. = 2, p = 0.006), with higher mean species richness in the unconnected ley monoliths than in the arable controls (p = 0.004, Figure 2.10). The connected leys also showed a higher mean species richness than the arable controls, but this was not significantly different from either the arable control

or unconnected ley monoliths (both p = 0.21). Weather stress had no significant effect on species richness (F = 2.03, d.f. = 2, p = 0.15), but field ID did (F = 3.37, d.f. = 3, p = 0.03; Figure S7).

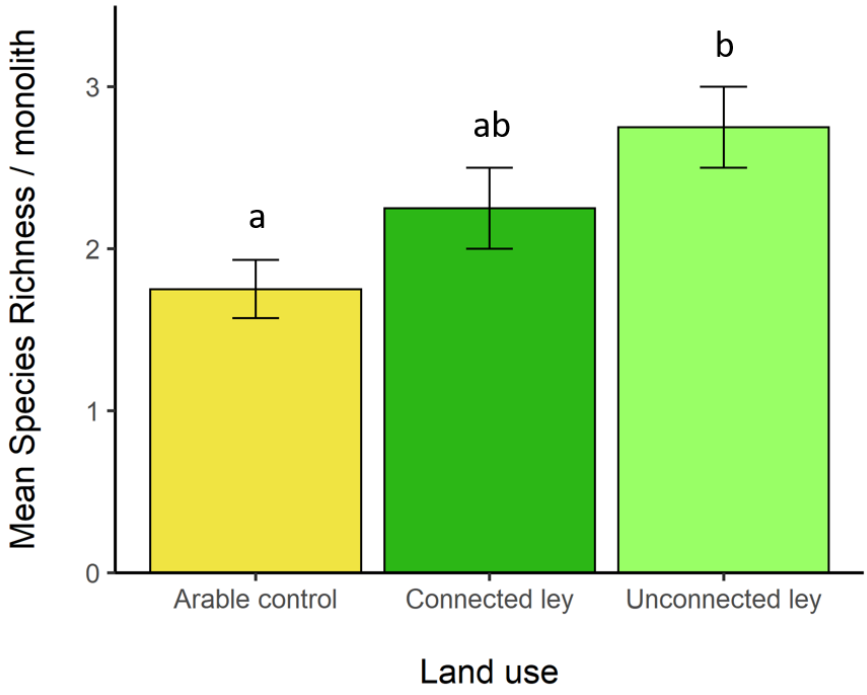

Figure 2.10. Mean species richness per monolith (+/- standard error) according to land use type. Different letters indicate the groups are significantly different from each other in post-hoc Tukey tests (p < 0.01).

Across all monoliths a total of five earthworm species were found, by far the most common of which was *Allolobophora chlorotica* (212 individuals), followed by *Aporrectodea rosea* (51) and *Aporrectodea longa* (17). Observations of the abundances of each species and the number of monoliths they occupied across the different land use categories (Table 2.2) supported the earlier overall finding of greater abundances in the ley monoliths compared to the arable controls. This was particularly pronounced for *A. rosea*, which were over five times more abundant in the connected and unconnected ley monoliths than in the arable controls (Table 2.2). When looking at particular species abundances and monolith occupancy as a result of the different weather stress categories, no clear species-specific responses were observed, with the possible exception of *A. chlorotica,* which was 1.5 times more abundant in the flooded monoliths (Table 2.3).

Table 2.2. The total number of each earthworm species collected across the different land use types. Numbers in parentheses represent the total number of monoliths that a particular species occupied, out of 36 in total and 12 for each treatment group.

	Number of earthworms (no. monoliths occupied)			
Species	Total	Arable Control	Connected ley	Unconnected ley
A. chlorotica	212 (33)	43 (9)	78 (12)	91 (12)
A. rosea	51 (23)	4 (3)	22 (9)	25 (11)
A. longa	25 (17)	7 (6)	5 (4)	13 (7)
A. caliginosa	8 (7)	2 (2)	3 (2)	3 (3)
L. castaneus	2 (1)	2 (1)	0 (0)	0 (0)

Table 2.3. The total number of each earthworm species collected across the different weather stress treatments. Numbers in parentheses represent the total number of monoliths that a particular species occupied, out of 36 in total and 12 for each treatment group.

	Number of earthworms (no. monoliths occupied)			
Species	Total	Ambient	Drought	Flood
A. chlorotica	212 (33)	62 (10)	61 (11)	89 (12)
A. rosea	51 (23)	18 (7)	22 (10)	11 (6)
A. longa	25 (17)	6 (6)	9 (5)	10 (6)
A. caliginosa	8 (7)	3 (2)	5 (5)	0 (0)
L. castaneus	2 (1)	0 (0)	0 (0)	2 (1)

2.5 Discussion

The results of this study demonstrate the potential of temporary arable to ley conversion for restoring earthworm populations in agricultural soils, and show that these populations can be resilient to future climate change induced weather stress. Land use had a clear effect throughout the experiment, with higher initial cast numbers in the ley monoliths, higher casting rates throughout the experiment and greater earthworm abundance, biomass and diversity overall. No clear effects of connection to hedgerows on earthworm populations and their casting activity were seen, though casts were slightly heavier in the connected leys before the experiment began. Surprisingly, during the weather stress

period casting rates were not significantly affected by drought, although casts in the droughted treatment did seem to become lighter as the drought progressed. The end of the weather stress events led to a significant increase in casting rates and cast weights in the monoliths that had been exposed, indicating a boost in earthworm activity in response to the harsh conditions ending. No effects of weather stress were seen on the final overall earthworm abundances, biomass and species richness within the monoliths, but potential positive effects of flooding on the abundance of the endogeic *A. chlorotica* were observed.

Firstly, this study showed that conversion to ley can boost earthworm numbers, biomass and diversity, and have beneficial effects for earthworm casting activity that is important for the maintenance of healthy agricultural soils. This indicates that as well as bringing other benefits such as increased soil carbon, water holding capacity and reduced bulk density (Berdeni et al., 2021; Persson et al., 2008; Prade et al., 2017), temporary conversion to ley can also have multiple beneficial effects for agricultural earthworm populations, supporting the findings of Spurgeon et al. (2013). Given that the monoliths had been taken from the leys only 19 months after they had been established, our results show that arable fields need only be taken out of production for a few seasons to see significant increases in earthworm numbers, diversity and activity. The improvements seen in earthworm populations and other soil quality indicators mean that potential losses in income associated with converting arable fields into temporary grass-clover leys are likely to be at least partially offset by increased yields when crops are subsequently grown in the improved ley soils. This effect is demonstrated by the results of Berdeni et al. (2021), who report significantly greater grain, chaff and shoot biomass in wheat harvested from the ley monoliths when compared with the arable controls.

Higher earthworm species richness in the ley monoliths was another benefit of arable to ley conversion observed in this study. Several studies have demonstrated that earthworms can have different effects on soil quality restoration depending on their species-specific behaviours and functional ecotype. For example, Haynes et al. (2003) showed that despite feeding on the same substrate, the epi-anecic earthworm species *L. rubellus* and endogeic *A. caliginosa* produced casts with distinctly different physical properties, levels of microbial activity and nutrient concentrations. Furthermore, Hallam and Hodson (2020) and Ernst et al. (2009) demonstrated varying influences of earthworm species and ecotypes on soil water holding capacity, infiltration rates and other hydrological properties. Higher species richness could therefore increase the variety of behaviours and functions performed by earthworms in the temporary leys, potentially boosting the rate and extent of soil quality improvements further.

Although most species of earthworms identified in this study were found to be more abundant in the ley monoliths, it is worth noting that *A. rosea* in particular showed markedly higher numbers in the leys compared with the arable controls. This is perhaps surprising given that the endogeic *A. rosea* has been described as 'disturbance tolerant' (Ivask et al., 2007; Sherlock, 2012), but in this case the numbers of this species are much lower in the more disturbed arable soils. One potential explanation for this may be the association of *A. rosea* with higher moisture content soils, as reported in previous studies (for example Falco et al., 2015). Under normal conditions the ley monoliths in this study were found to have significantly higher soil moisture contents than the arable controls (Berdeni et al., 2021), which may therefore explain why more *A. rosea* were present in these monoliths. Whatever the cause, the large increase in numbers of *A. rosea* is another positive reason for the uptake of temporary arable to ley conversion, as the presence of this particular species has been shown to bring both yield increases and disease suppression benefits when cultivating common crops like wheat and barley (Doube et al., 1997; Stephens et al., 1994a, 1994b).

In contrast to the improvements bought about by arable to ley conversion, we did not find any evidence to support the hypothesis that connection to hedgerows increases in-field earthworm populations. This was surprising given the results described for other soil macrofauna (Alvarez et al., 2000), but supports the findings of previous studies that found no clear evidence of earthworm populations in field margins dispersing into and colonising arable fields (Lagerlöf et al., 2002; Roarty and Schmidt, 2013). However, it is possible that limitations with the monolith study approach (discussed further in Chapter Six) meant that possible effects of hedgerow connection on the earthworm populations in the leys were missed. For example, the monoliths were removed from the ley strips 68 metres away from the field margin, so, given the limited active dispersal rates reported for common earthworm species (Butt et al., 2004; Eijsackers, 2011), it is likely that colonising earthworms would not have been able to reach this far into the leys in the 19 months leading up to monolith removal. Investigation into the ley strip earthworm populations closer to the hedgerows would allow for more insight into the role that these features play in earthworm colonisation, in a similar way to Hoeffner et al. (2021) who showed earthworm species richness was higher in newly-established grassland soils that were directly adjacent to hedgerows as opposed to ditches. Whilst our study indicates that the recovery of earthworm populations seen in the ley monoliths is more likely to be seeded from earthworms that have managed to persist in the arable fields throughout the previous cultivation period, further research is needed to explore the potential for hedgerows to act as refugia. The deployment of capture mark recapture techniques and laboratory habitat preference testing may help to shed further light on this (Butt and Lowe, 2007; Mathieu et al., 2018).

Focusing on the effects of weather stress, by the time the monoliths were dismantled there were no clear effects of drought on overall earthworm abundance, biomass or diversity, indicating that UK populations may be fairly resilient to one-off drought events. However, there was evidence that earthworms in the monoliths had modified their behaviour in response to the drought, both during the weather stress and in the recovery period after it ended. Previous studies have demonstrated the ability of many earthworm species to survive drought stress by moving to deeper soil layers and aestivating in chambers (Garnsey, 1994; Gerard, 1967; McDaniel et al., 2013), during which time their metabolism and activity are severely reduced (Bayley et al., 2010). A reduction in casting rates would be expected if more earthworms were reducing their activity and aestivating in response to drought but, while reduced casting rates were seen in the droughted monoliths, these were not quite significantly lower than those experiencing ambient conditions ($p = 0.06$). The lack of a significant difference in casting rates between the drought and ambient treatments may be explained by the unseasonably dry conditions at the start of the weather stress period, which meant the ambient monoliths actually experienced a moderate drought. This likely reduced the casting rates in this group, thereby resulting in a non-significant outcome. Despite this, the reduced weight of surface casts collected during the second half of the weather stress period does suggest that as the drought progressed, there were reductions in earthworm feeding and/or burrowing activity in the monoliths experiencing drought. The clearest indication of behavioural changes in response to drought came in the recovery period when the drought monoliths were rewet, with significantly higher casting rates observed in these monoliths compared with the ambient. This change in casting rate suggests that the earthworms that experienced severe drought were boosting their activity above normal levels in response to the end of the weather stress. The higher cast weights in the post-drought monoliths also supports the idea that these earthworms may have boosted their feeding activity to compensate for the time they spent inactive, as these cast weight increases were not observed in the ambient or flooded monoliths where earthworms were able to feed throughout.

Like the drought conditions, flooding appeared to have no overall effects on earthworm abundance, biomass or diversity, but behavioural modifications were again seen in the form of higher casting rates at the end of the experimental period when compared with the ambient monoliths. Previous studies have shown that the response of earthworm populations to flooding can often be complex and context-specific, with a range of positive and negative responses observed depending on factors such as the severity of flooding, the composition of the earthworm community or the presence of interacting land use factors (Kiss et al., 2021a, 2021b; Singh et al., 2019; Zorn et al., 2005). The creation of hypoxic conditions in inundated soils is one of the key ways flooding is thought to negatively impact earthworm abundance and biomass in the field (Coyle et al., 2017), but due to the nature of the

mesocosm experiment it is unlikely that these conditions were adequately replicated here. This is because the flooded monoliths had to be continuously topped up with fresh water in order to keep them saturated, which is likely to have prevented the oxygen levels from dropping too low. Earthworms can survive long durations fully submerged in water provided it is sufficiently oxygenated (Roots, 1956), which could explain why no negative effects of flooding were observed in this study. This is supported by the higher casting rates observed in the flooded monoliths at the end of the experiment, indicating that earthworm activity levels were not negatively affected by the inundation. On the contrary, it is thought that the increased moisture levels combined with sufficient oxygenation actually allowed more feeding activity, a phenomenon previously demonstrated by Perreault and Whalen (2006). Unlike the drought treatment, these high casting rates were not considered to be a response to the flood ending, but were more likely to be a continuation of high casting rates that would have been observed if it had been possible to measure them during the flood event. Finally, whilst no effects of flooding on overall abundance and diversity were seen, it is interesting to note that numbers of the endogeic *A. chlorotica* were found to be 1.5 times greater in the flooded monoliths. This reflects similar findings of Schütz et al. (2008) who previously showed that *A. chlorotica* abundance and biomass increased after short flooding periods followed by long recoveries, and therefore provides more evidence that under certain conditions some earthworm species can benefit from flood related disturbance.

2.6 Conclusions

The results of this study show that changes to agricultural land use can bring substantial improvements in earthworm populations in a relatively short space of time, providing more evidence that temporary arable to ley conversion is an effective way to boost soil health and prevent further degradation of arable soils. Given the short 19-month time frame in which large increases in earthworm activity, abundance, biomass and diversity were observed, reintroducing leys into arable rotations offers a promising solution for improving soil health without major impacts on crop yield, and should therefore be considered for inclusion in future agricultural policy schemes. No evidence was found for hedgerows providing additional benefits on top of those brought about through ley conversion, although this may be due to the possibility that earthworms from the hedgerows did not have sufficient time to reach the area that the monoliths were extracted from, due to their limited dispersal rates. Further investigations are needed to determine whether hedgerows act as refugia, but these

results do indicate that the increases in earthworm abundance seen in the ley strips was likely seeded by surviving earthworms already present in the arable fields. Although no lasting effects on earthworm population variables were seen as a result of extreme weather stress, the results indicate that earthworm behaviour was modified in response to one-off drought and flooding events. More research is needed into the long-term implications of weather stress on earthworm populations and behaviour, particularly with regards to the effect of repeated drought and flooding events, which are likely to become more regular occurrences in the coming decades.

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2.8 Supplementary materials

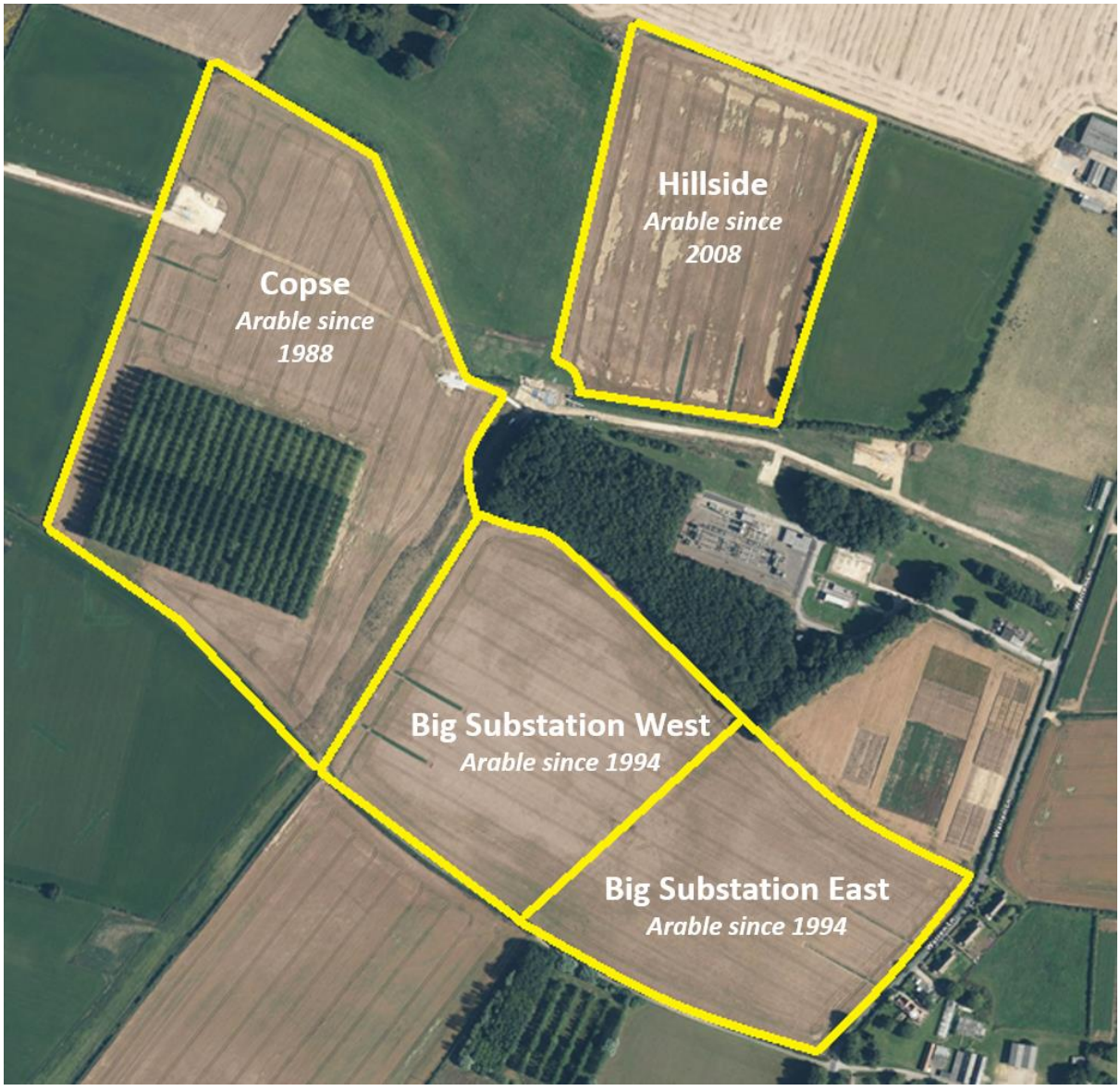

Figure S1. An aerial view of the four fields at the University of Leeds Field Research Unit from which the monoliths were extracted. The ley strips are visible, extending inwards from the edge of the fields. Google Earth (2017), earth.google.com/web/.

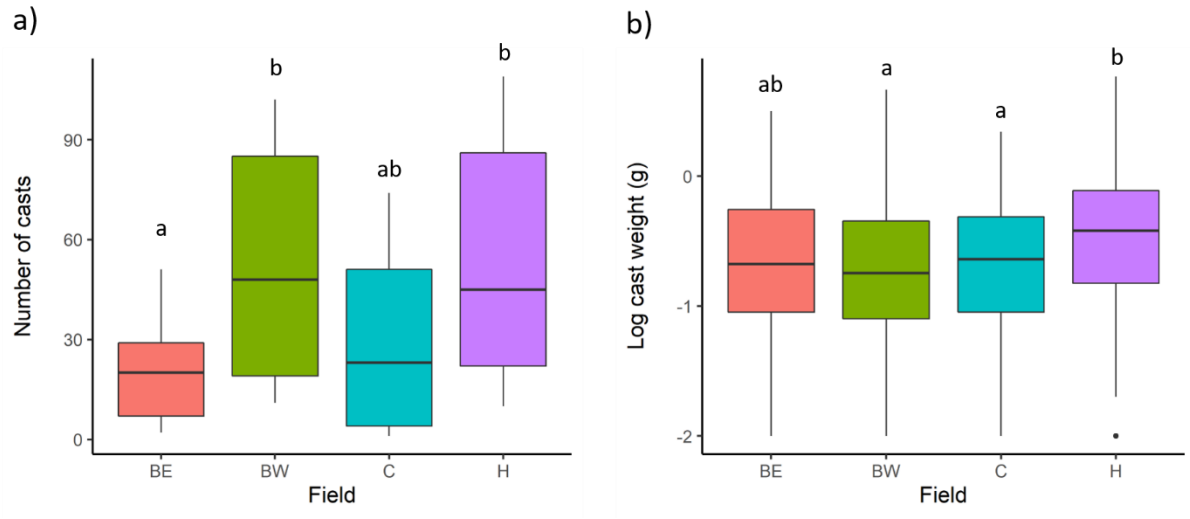

Figure S2. a) The total number of casts per monolith and b) cast weight by field, of casts taken before the onset of the weather stress events (collection A). Different letters indicate the groups are significantly different from each other in post-hoc Tukey tests ($p < 0.05$). For field, 'BE' = Big Substation East, 'BW' = Big Substation West, 'C' = Copse and 'H' = Hillside.

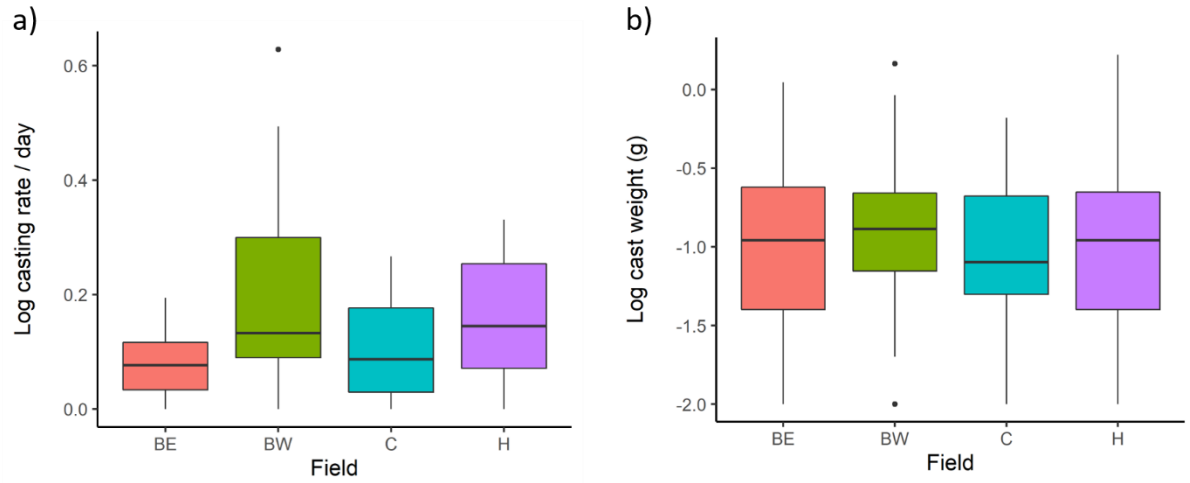

Figure S3. A) Casting rate and b) cast weight by field, during the drought and flooding period (collections B-C). No significant effects of field on log casting rates or weights were found during this stage of the experiment ($p > 0.05$). For field, 'BE' = Big Substation East, 'BW' = Big Substation West, 'C' = Copse and 'H' = Hillside.

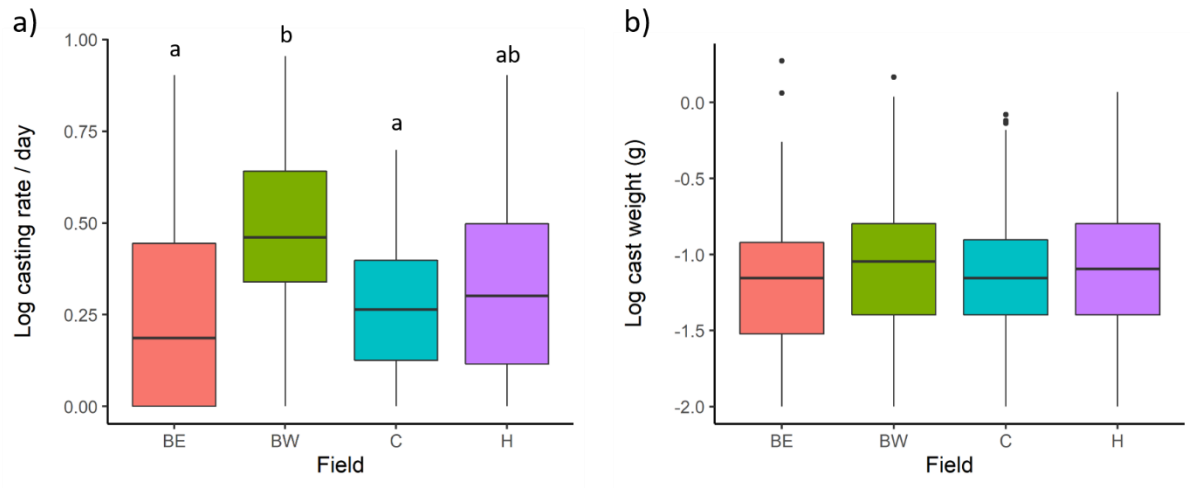

Figure S4. A) Casting rate and b) cast weight by field, after the weather stress events had ended (collections D-G). For casting rate, different letters indicate the groups are significantly different from each other in post-hoc Tukey tests ($p < 0.05$). No significant effects of field on cast weights were found during this stage of the experiment ($p > 0.05$). For field, 'BE' = Big Substation East, 'BW' = Big Substation West, 'C' = Copse and 'H' = Hillside.

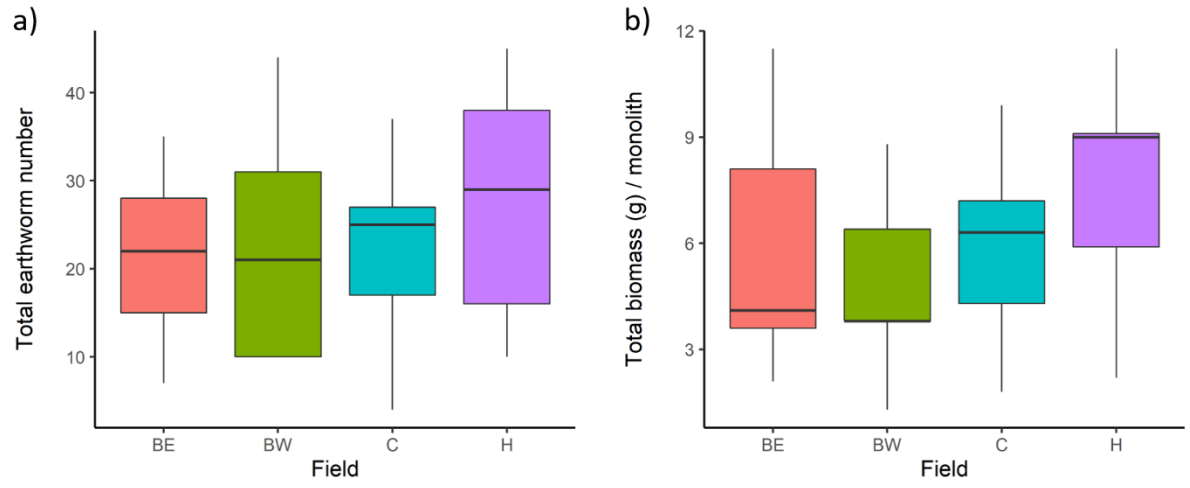

Figure S5. a) Total number of adult and juvenile earthworms per monolith and b) Total biomass per monolith grouped by field. No significant effects of field on total earthworm abundance or biomass were found ($p > 0.05$). For field, 'BE' = Big Substation East, 'BW' = Big Substation West, 'C' = Copse and 'H' = Hillside.

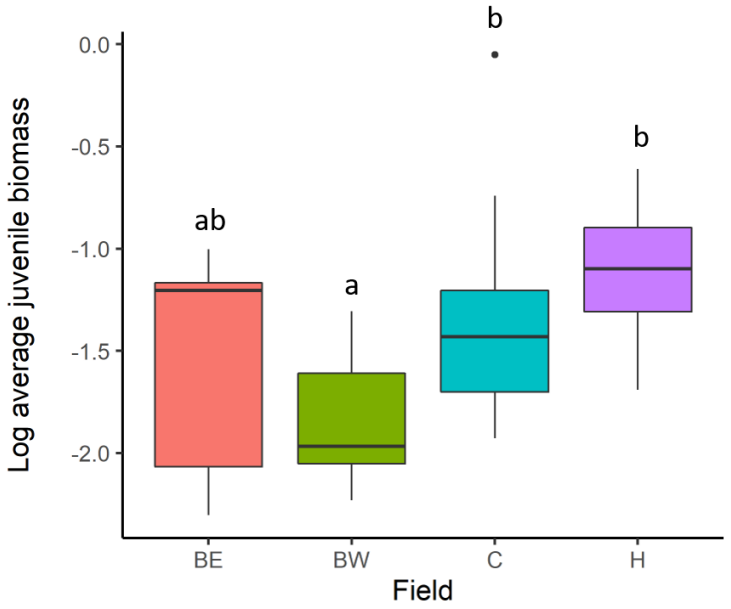

Figure S6. Mean individual juvenile biomass per monolith grouped by field. Different letters indicate the groups are significantly different from each other in post-hoc Tukey tests ($p < 0.05$). For field, 'BE' = Big Substation East, 'BW' = Big Substation West, 'C' = Copse and 'H' = Hillside.

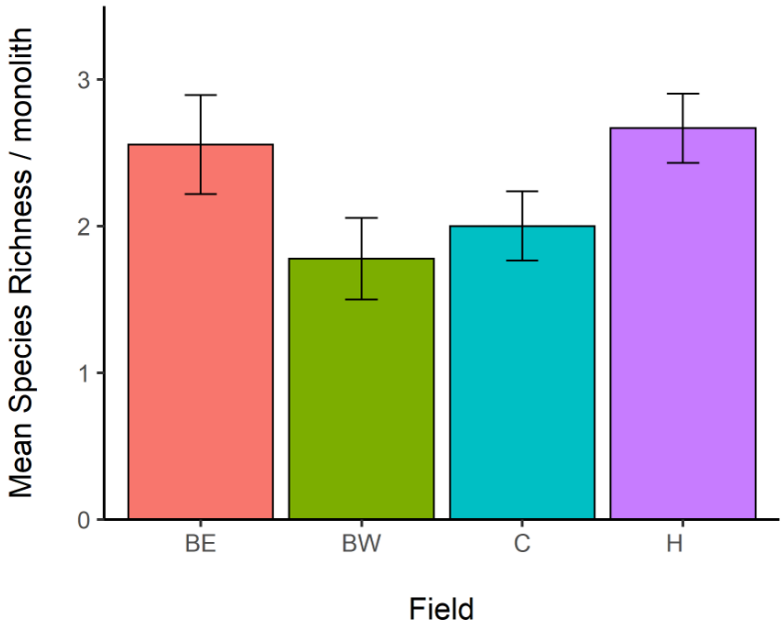

Figure S7. Mean species richness per monolith grouped by field. Field ID was found to have a significant overall effect on species richness ($F = 3.37$, d.f. = 3, $p = 0.03$), but in post-hoc Tukey tests only marginal significance was observed between 'Big Substation West' and 'Hillside' ($p = 0.054$). For field, 'BE' = Big Substation East, 'BW' = Big Substation West, 'C' = Copse and 'H' = Hillside.

Chapter 3

Developing an eDNA-based methodology to assess earthworm populations in agricultural systems

3.1 Abstract

The rapid development of environmental DNA (eDNA) metabarcoding technologies has revolutionised biodiversity monitoring in recent years, allowing the non-invasive sampling of a range of different taxa in terrestrial, aquatic and marine environments. The application of eDNA metabarcoding could be particularly useful for sampling earthworm populations in agricultural soils, as they play a vital role in promoting soil health but remain largely under-recorded. In this study, laboratory testing and *in-silico* analyses were performed to develop and refine a methodology for extracting, amplifying and sequencing earthworm eDNA from agricultural systems. An extraction protocol, originally described by Taberlet et. al (2018) for extracting extracellular DNA from soil, was tested using a subset of soil samples collected for the main eDNA study in Chapter 4. The performance of promising primer pairs identified from the literature were assessed using *in-silico* alignment tests, and PCR conditions were manipulated to find the most effective amplification conditions. Sanger sequencing and a pilot MiSeq Nanorun were carried out, to aid the development of a bioinformatics pipeline and determine which primer combinations were most suitable for amplifying earthworm eDNA. The methodology development tests showed that earthworm eDNA could be detected in agricultural soils under active management, and allowed the development of an optimised workflow for use in Chapter 4. The pilot MiSeq Nanorun also revealed that a set of 16S primer pairs, originally developed by Bienert et al. (2012), proved most effective in amplifying products that could be sequenced to a high quality. Alongside the shorter ewD/ewE pair, the previously untested ewB/ewE 16S primer combination performed particularly well and detected the most species. Finally, the results showed that issues with negative contamination could be sufficiently addressed by taking several measures, including using single-use reagents and primer stocks, performing reactions in PCR tubes instead of plates and including multiple negative controls throughout the sequencing preparation process.

3.2 Introduction

As organisms move through their environment they leave behind DNA in many forms, including through the shedding of skin cells and hair, the deposition of mucus or the excretion of faeces. Known as environmental DNA (eDNA), it can be extracted from environmental samples, and then subsequently amplified and sequenced to provide insights into the organisms that are present. eDNA sampling can be targeted to look for specific taxa or to analyse whole community patterns (Biggs et al., 2015; West et al., 2020). Over the last few decades, the rapid development of eDNA metabarcoding technologies has revolutionised biodiversity monitoring and allowed new ecological insights to be made (Lawson Handley, 2015). The uptake of eDNA sampling has been particularly prominent in surveys of aquatic and marine communities (Belle et al., 2019), where it is now an important tool regularly applied in fisheries management, endangered or invasive species monitoring and ecosystem assessment (Pfleger et al., 2016; Seymour et al., 2020; Thomas et al., 2020; Wang et al., 2021).

In addition to aquatic and marine ecosystems, eDNA sampling can also be applied to terrestrial systems, and a number of studies have shown that extracting and sequencing eDNA from soils can provide insights into both above and belowground communities (Andersen et al., 2012; Oliverio et al., 2018; Yoccoz et al., 2012). The application of eDNA sampling to soils can be viewed as an exciting development that could illuminate habitats that have typically been difficult to sample, and in the process help to shed light on soil communities that have previously been underrepresented in conventional biodiversity surveys (Parker, 2010; Phillips et al., 2017). A prime example of this is earthworms, which despite being key ecosystem engineers and important bioindicators (Le Bayon et al., 2017; Pérès et al., 2011; Ritz et al., 2009), have remained under-recorded in biodiversity databases and mapping projects (Carpenter et al., 2012). The feasibility of sampling earthworm populations using soil eDNA was demonstrated by Bienert et al. (2012), who developed a set of metabarcoding primers to amplify short regions of 16S mitochondrial DNA that can be extracted from soil (Figure 3.1). Short barcodes like these are particularly useful for sampling soil eDNA, as it is typically highly degraded and fragmented as a result of the metabolic activity of soil microbes (Nielsen et al., 2007). Building on the work of Bienert et al. (2012), Taberlet et al. (2012) further improved the eDNA extraction protocol by developing a phosphate buffer extraction method that can be carried out using larger amounts of soil than the traditional commercial DNA kits previously employed (which are optimised for microbial analysis). Pansu et al. (2015) then utilised this extraction method and one of the 16S primer pairs to show that eDNA sampling can be used to demonstrate differences in earthworm communities at the landscape level.

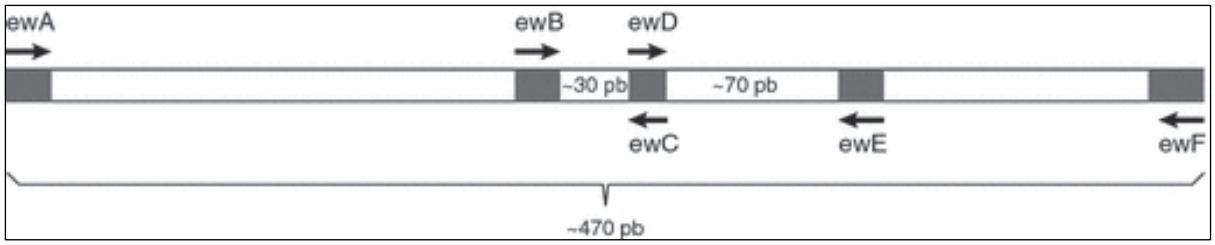

Figure 3.1. Visual representation of the 16S DNA region in earthworms, showing the positions of the primers in grey and the variable regions between them in white (taken from Bienert et. al, 2012).

These pioneering studies on earthworm eDNA sampling are particularly timely, given the growing concerns surrounding soil degradation and its impact on food security (Jie et al., 2002; Tamene et al., 2019). An estimated 35.9 Petagrams of soil are lost globally each year and degradation is disproportionately affecting croplands (Borrelli et al., 2017), which is leading to growing recognition amongst policymakers that more must be done to monitor and restore soil health (Panagos et al., 2020; UK Government, 2018). Given the benefits that earthworms bring to crop production (van Groenigen et al., 2014), their suitability as bioindicators (Pérès et al., 2011) and the central role that they play in maintaining the health of agricultural soils, including but not limited to the creation of macropores, the mixing of organic matter throughout the soil profile and the stimulation of microbial activity (Binet et al., 1998; Blouin et al., 2013), they have been proposed as promising candidates for use in wider soil monitoring schemes (Fründ et al., 2011; Stroud, 2019). However, progress towards developing wider-scale earthworm population surveys and increasing their use for monitoring soil health on farms has so far been limited. This is perhaps due to the high labour and time costs associated with traditional earthworm sampling methods (Targetti et al., 2014), which typically involve digging soil pits and manually sifting through the soil to extract earthworms by hand. As well as being time and energy intensive, traditional earthworm sampling can also be difficult to standardise, prone to biases and requires taxonomic expertise to identify adult worms to species level (Bartlett et al., 2010; Jiménez et al., 2006; Valckx et al., 2011).

The application of soil eDNA sampling could therefore represent an alternative to traditional sampling techniques that addresses some of these limitations, enabling less invasive and more standardised measurement of earthworm populations in agroecosystems. In turn, this would facilitate the development of wider earthworm biodiversity surveys and the inclusion of earthworms in soil health monitoring schemes. However, to our knowledge the extraction, amplification and sequencing of earthworm eDNA has yet to be performed using soil that is being actively worked for agriculture. A study examining the feasibility of using eDNA sampling to measure earthworm populations in

agricultural fields was therefore performed, which included testing the ability of eDNA to detect fine-scale differences in earthworm communities bought about by changes in agricultural management, and comparing its performance to that of the traditional hand-sorting method (Chapter 4). Before this could be carried out however, it was necessary to develop and test the laboratory protocols and practises that would be used, to ensure an optimised and smooth workflow.

This chapter summarises the methodology development and laboratory testing that was carried out prior to the main eDNA extractions, amplifications and sequencing in Chapter Three. First, various modifications to the eDNA extraction protocols described in Taberlet et al. (2012) and Taberlet et al. (2018) were carried out and assessed to find the most suitable extraction procedure. Next, the 16S primers developed by Bienert et al. (2012) and a pair of 12S primers described in Harper et al. (2005) were analysed *in-silico*, to determine their suitability for use in amplifying DNA from UK earthworm species and highlight promising primer combinations for further PCR testing. The optimum PCR conditions for each primer combination were then explored by making adjustments to the annealing temperatures, dilution factors and prior DNA purification process, before assessing the results via gel electrophoresis. To further examine the performance of the different primer pairs and check they were amplifying the target organisms, Sanger sequencing was conducted and the returned sequences compared to those listed in the NCBI BLAST database. In addition to Sanger sequencing a pilot Illumina MiSeq Nanorun was performed, the results of which were used to develop an optimised bioinformatics pipeline for the returned sequence data and determine the most effective primer contributions to use in Chapter 4. Throughout the methodology development process various contamination control measures were put in place and assessed, in order to develop effective protocols that minimised the potential for contamination between samples. The findings of all of these investigations are discussed with regards to their implications for the research described in Chapter 4, and ways to further optimise laboratory eDNA protocols are explored.

3.3 Methodology

Earthworm and soil sampling in the field

This section describes the main field sampling that was performed for Chapter three, which was carried out before the laboratory methodology development tests began. Fieldwork was carried out at Leeds University Farm in the 'SoilBioHedge' experimental fields, in March and April 2018. Earthworm and soil sampling were performed in broadly similar weather conditions (overcast days

around 7-12°C). The sampling sites were conventional arable fields surrounded by hedgerows that each had two 70m grass-clover ley strips sown into them, which had then been allowed to establish for three years (Figure 3.2a). In each field, one of the ley strips had a steel mesh barrier inserted down to the bedrock between the strip and the hedgerow, to prevent the movement of earthworms below the surface level. The other ley strip did not have a steel mesh barrier and remained connected to the hedgerow. Previous investigations found no clear effects of the steel mesh barrier on earthworm populations in the ley strips (see Chapter 2), and so all of the ley strips were subsequently treated as one treatment group.

Traditional hand-sorting for earthworms and eDNA soil sampling was done concurrently, with a total of 8 earthworm pits and 24 eDNA soil samples taken per field (Fig. 2b). Four pits were dug in the ley strips and four in the arable field surrounding them, at distances of 32 and 64 m from the hedge. Pits were dug by excavating an 18 x 18 x 15 cm block of soil and immediately placing it into a bucket with a sealable lid. Any earthworms visible at the base or sides of the excavated pit were also added to the bucket. Using a pallet knife and small trowel, smaller 5 x 5 x 15 cm soil cores were also extracted from the centre of the removed soil blocks, as well as from 2 m either side of the excavated pits (Fig. 2b) for eDNA sampling. Extracted soil cores used for eDNA sampling weighed ~175g on average. To prevent contamination from reusing equipment, individual tools were cleaned with 10% Bleach and left to soak for at least 15 minutes before being used again. Disposable gloves were worn and changed between taking each eDNA sample. Each eDNA soil core was transferred to a sealable plastic sample bag and carefully broken up so that any stones, earthworms or cocoons could be removed. After the samples had been taken from a given field, the soil blocks from the pits were hand-sorted and any earthworms collected were categorised into adults or juveniles based on the presence/absence of a clitellum, before being preserved in 90% ethanol. Earthworms and soil samples were then taken back to the University of Sheffield. The preserved earthworms were kept refrigerated at 4°C and the soil samples were frozen at -20°C.

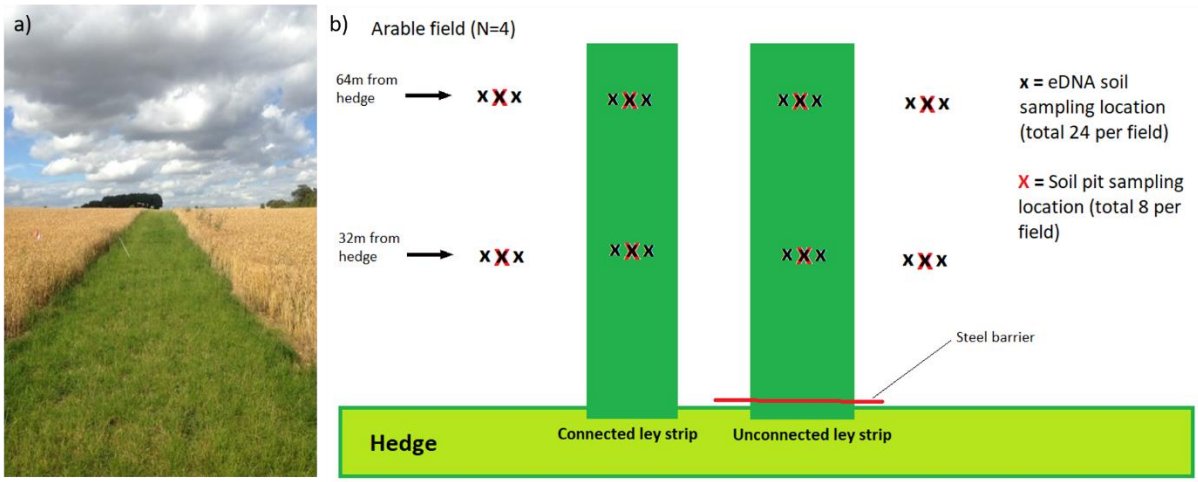

Figure 3.2. The study site and sampling protocol. A) One of the ley strips in an arable wheat field, b) The positions and number of samples taken per field, for both hand-sorting and eDNA sampling (see Chapter 4). A total of 32 pits for hand-sorting and 96 soil samples for eDNA analysis.

eDNA extraction

Three soil samples, one from an arable field and two from the ley strips, were selected for the preliminary methodology development and protocol testing. The arable field sample was expected to have a low diversity and abundance of earthworms based on the traditional hand-sorting results, whilst the ley samples were expected to have higher diversities and abundances. The samples came from two different fields and contained above average amounts of soil, which minimised the risk of running out of a soil sample before the main study conducted in Chapter 4. Modified versions of the phosphate buffer extracellular DNA extraction method described in Taberlet et al. (2018) were tested, using equipment and reagents from the Macherey-Nagel NucleoSpin® Soil kits. The amount of starting material, time spent mixing the buffer and soil sample and the amount of supernatant loaded into the NucleoSpin® soil columns were all varied to see how these factors affected DNA amplification.

DNA extractions were all performed in a pre-PCR room inside lamina-flow hoods, which were sterilised before and after use by wiping down with 10% bleach and leaving UV cabinet lights on overnight. Disposable plastic spoons and weigh boats were used during the weighing steps, and gloves were changed and equipment wiped with 10% bleach between the handling of each sample. Soil samples were defrosted and thoroughly homogenised whilst still in their sealed sample bags, after which either 15 g or 25 g of soil was weighed out and placed into a 50ml Falcon Tube to assess the effectiveness of different amounts of starting material. Phosphate buffer was then added to the falcon tubes at a 1:1 volume (i.e. 15ml of buffer added to 15g of soil) and a negative control consisting of a falcon tube with

just buffer was included for each round of extractions. After adding phosphate buffer, the tubes were mixed by rotating in a falcon rotator for either 15 or 25 minutes to investigate whether longer mixing times aided desorption of the extracellular DNA. The sample mixes were then centrifuged, and the resulting supernatant loaded into NucleoSpin soil columns on a vacuum manifold. Again, varying amounts of supernatant were used to determine whether DNA yield could be maximised by loading larger volumes. The final steps of the procedure were then completed using buffers from the Macherey-Nagel NucleoSpin Soil kit, and the resulting eluted eDNA samples were diluted to varying concentrations to discover the ideal dilutions for downstream use (described below). All samples were subsequently stored at -20°C when not in use.

Primer selection

Several possible primer pairs were identified from the literature that were deemed to be potentially suitable for measuring earthworm diversity from soil eDNA. The sets of primers used for further analysis were originally described in Bienert et al. (2012) and Harper et al. (2005), and amplified the 16S and 12S mitochondrial DNA regions respectively (Table 3.1). *In silico* analyses were performed for all primers using Mega7 (Kumar et al., 2016) and R version 3.4.3 (R Core Team, 2020). Sequences for known UK earthworm species listed on BLAST were collected and alignments with the primers were performed to check for good binding and sufficient variation in the target region. Based on these analyses, primer combinations deemed suitable were tested using PCRs with extracted eDNA and positive controls (DNA extracted from earthworm tissue), and amplification success was judged via gel electrophoresis. The PCR products from successful primer pairs then underwent Sanger sequencing (described below) to investigate whether the expected species were being amplified correctly, before proceeding with the pilot Illumina MiSeq Nanorun.

Optimising PCR conditions

All PCR preparation was carried out in a room where post-PCR products were not present. Plates, tubes and reagents were prepared in lamina flow cabinets sterilised with 10% bleach and UV-C light, and plates and tubes were sealed before removal from the cabinets. Initial PCR cycling conditions were based on those listed in Bienert et al. (2012) and Harper et al. (2005) and varied according to the primers being used. Varying the annealing temperature from between 55-57°C was tested for the 16S primer pairs and 52-57°C for the 12S pairs. Diluting the eDNA extracts to different concentrations (undiluted, 1:5 or 1:10) was assessed to gauge the effect on amplification success. The effectiveness

of purifying the PCR products using AMPure XP Beads before sequencing preparation was also examined. The relative effectiveness of the different conditions was assessed using gel electrophoresis of the PCR products and subsequent UV imaging. The final optimised PCR conditions are listed in Table 3.1.

Table 3.1. The primer combinations that were trialled, including the size of the region they amplified, their optimised PCR conditions and their original source. The pairs in bold indicate those that were included in the pilot MiSeq Nanorun.

Primer pair	Sequence (forward / reverse)	Product size	PCR conditions	Source
ewA/ewF	CGACTGTTTAACAAAAACAT/ CGCGGTCTGAACTCAGCTCATG	~ 428 bp	95°C for 15 mins; 49 cycles 94°C for 30 secs, 55°C for 90 secs and 72°C for 90 secs; 72°C for 10 mins.	Bienert et al. (2012)
ewA/ewC	CGACTGTTTAACAAAAACAT/ GGTCGCCCCAACCGAAT	~ 250 bp	As above	Bienert et al. (2012)
ewB/ewC	**CAAGAAGACCCTATAGAGCTT/ GGTCGCCCCAACCGAAT**	**~ 30 bp**	**As above**	**Bienert et al. (2012)**
ewB/ewE	**CAAGAAGACCCTATAGAGCTT/ CTGTTATCCCTAAGGTAGCTT**	**~ 120 bp**	**As above**	**Bienert et al. (2012)**
ewD/ewE	**ATTCGGTTGGGGCGACC/ CTGTTATCCCTAAGGTAGCTT**	**~ 70 bp**	**As above**	**Bienert et al. (2012)**
185F/14233R	TGTGTACTGCCGTCGTAAGCA/ AAGAGCGACGGGCGATGTGT	~ 225 bp	95°C for 15 mins; 40 cycles 94°C for 30 secs, 57°C for 90 secs and 72°C for 90 secs; 72°C for 10 mins.*	Harper et al. (2005)

* For the 12S 185F/14233R primers it was not possible to optimise the PCR conditions to successfully amplify soil eDNA, so the conditions listed are those that worked well in amplifying DNA extracted from earthworm tissue.

Sanger Sequencing

For some of the potential primer pairs, Sanger sequencing was performed to assess whether they were amplifying the desired target organisms, before deciding which primer pairs to include in the pilot MiSeq Nanorun. Although Sanger sequencing is typically unsuitable for samples with multiple species and individuals, in this case it was deemed useful to check the identity of the most dominant sequences occurring in the samples. For Sanger sequencing, 2µl of EXO-sap was added to 6 µl of PCR products and incubated for 15 minutes at 37°C, followed by 15 minutes at 80°C to purify them. A sequence PCR was then performed that included positive and negative controls, and subsequent ethanol precipitation and formamide addition steps were carried out to prepare samples for sequencing. Samples were run on an ABI3730 sequencer and the results were analysed using BioEdit software (Hall, 1999). Derived dominant sequences were aligned to the BLAST database to obtain species level identifications for each sample.

Pilot MiSeq Nanorun and bioinformatics pipeline development

After narrowing down a final subset of promising primer pairs, a 2 x 120 bp pilot Miseq Nanorun was performed to assess the success of the developed protocol. Thirteen DNA samples and negative extraction controls that had been initially obtained from the three soil samples described earlier (with varying extraction methods used on each subsample) were diluted to 1:5 concentration with lowTE. Initial PCRs (PCR1) were performed using region-specific sequences with an overhang for Illumina sequencing adaptors and with the cycling conditions listed in Table 3.1; the three most promising primer pairs based on earlier investigations were selected for this sequencing test (ewB/ewC, ewD/ewE & ewB/ewE in order of shortest to longest amplified product). Successful amplification was checked using gel electrophoresis. Bead cleans with AMPure XP Beads were then performed at concentrations of 0.5:1 and 1:1 bead to sample ratios to remove unwanted large and small fragments respectively. After bead cleaning a second PCR (PCR2) was performed to add unique index sequences and Illumina sequencing adaptors to the amplicons. The conditions for PCR2 consisted of an initial incubation step of 95°C for 15 minutes, followed by 12 cycles of 98°C for 10 seconds, 65°C for 30 seconds and 72°C for 30 seconds, with a single final step at 72°C for 5 minutes. Size analysis using a TapeStation was then performed on one sample from each primer pair to check that the Illumina tags had been successfully added and to see whether any primer dimer was present. A second bead clean was performed to remove any primers or tags that had not successfully bound to the amplicons. The concentration of DNA in each subsample was then assessed using a fluorometer, and the results were used to pool each sample by equimolar amounts. This resulted in three pools (or 'libraries'), one for each primer pair. Serial dilutions of the libraries were created and a qPCR was performed to accurately quantify the amplicons in each. The results of the qPCR were used to pool the libraries in equimolar amounts, aiming for a final concentration of 4 nM. The concentration of the final pool was then checked using a Qubit and adjusted accordingly by adding more lowTE or pooled product. The final pool was then sent to Sheffield Children's Hospital for running on the Illumina MiSeq.

In addition to assessing the success of the extraction protocol, the pilot Miseq Nanorun was also used to develop a streamlined bioinformatics pipeline for each primer pair. The original pipeline structure was based on one developed by the NERC Biomolecular Analysis Facility at the University of Sheffield, for metabarcoding analyses with multiple samples. A number of different parameters were adjusted to explore how they affected the quality, quantity and taxonomic assignment of the final sequences returned. Adjustments were made to the quality filtering scores and minimum sequence lengths in the trimming steps, the percentage similarity values when clustering similar sequences into molecular operational taxonomic units (mOTUs) and the minimum sequence identity percentages when running BLAST searches.

Reducing contamination

Throughout the method testing and optimisation period, contamination of negative controls was recognised as an issue, with unacceptably high contamination rates (evident from gel electrophoreses) occurring at several stages during the development process. A number of tests were conducted to isolate the causes of contamination combat the problem. These measures included using different equipment for PCR reactions (plates, tubes and different types of lids); replacing reagents, primers and buffers and developing a 'single-use' reagent policy; changing gloves more frequently and using filter tips; including both extraction and PCR negatives to more accurately assess when the contamination occurs; using lamina-flow hoods with or without blowers; changing laboratories where PCR preparation was carried out; and performing sanger sequencing on contaminated negatives to determine what DNA was entering the controls.

3.4 Results

eDNA extraction

Varying the amount of soil that was mixed with buffer from 15 to 25 g had no observable effects on the quality, concentration or diversity resolution as measured by gel electrophoreses, fluorometer and MiSeq Nanorun respectively. Mixing the soil and buffer for 25 min instead of 15 min caused the resulting supernatant that was loaded into the columns to be heavily coloured, and this colouration persisted through to the final elution. Given that this may potentially hamper downstream uses (quantification with Qubit etc.) and indicate higher concentrations of PCR inhibitors, it was concluded a mixing time of 15 min was preferable. The addition of more than 400 ul of supernatant to the Nucleospin columns caused the filters within them to become blocked, which increased the time it took to complete the process and left the final extract highly coloured. This blockage issue was particularly a problem with the ley soils, which tended to block the filters more quickly than the arable samples. The optimal amount of supernatant for the Chapter 4 extractions was therefore deemed to be 400ul. The final complete extraction protocol is listed in the Supplementary Materials.

Primer Selection

In-silico MEGA7 and BLAST analysis was performed to check the alignment of the eight primers with the target regions, and to determine whether there was sufficient sequence variation in the regions between the proposed primer pairs to allow each species to be distinguished (16S: ewB/ewC, ewD/ewE, ewB/ewE, ewA/ewF; 12S: 185F/14233R). For the 16S primer pairs, 35 sequences describing the target regions were found in GenBank and used in the alignment tests, representing 24 different UK earthworm species and several of their haplotypes. For the 12S primer pair, 16 sequences were included, which represented 16 species and no haplotypes.

For 16S, four primers (ewB, ewC, ewD and ewE) showed good binding to the target sites for all the UK earthworm species and haplotypes predicted to occur at the site. Primer ewE aligned perfectly with all 24 species and 35 sequences, whereas primers ewB, ewC and ewD aligned perfectly with 23/24 species. EwB contained one base difference from *Aporrectodea limicola* (C instead of T) and both ewC and ewD contained one base difference from *A. rosea* (G instead of A). The ewA and ewF primers did not successfully align, but this may have been because the partial BLAST sequences only included the region between these two primers, and not the region they bind to. Alignment of the different sequences showed that within the ewB/ewC region there was sufficient variation in the sequences to distinguish all of the species included in the analysis and a number of their haplotypes. The size of the differences between distinct species ranged from 2 base pairs (*Lumbricus terrestris* and *L. castaneus*) to 26 base pairs (*Dendrobaena veneta* and *D. attemsi*), with an mean difference of 12.6 base pairs between sequences. For the longer ewD/ewE region, there was not only enough variation to distinguish between all the species tested but also to technically discern almost all of the haplotypes examined (except for *A. caliginosa*). The size of the differences between distinct species ranged from 8 base pairs (*L. terrestris* and *L. friendi*) to 34 base pairs (*A. cupulifera* and *Microscolex phosphoreus*), with a mean difference of 20.9 base pairs. The primer combination ewB/ewE was a combination of the ewB/ewC and ewD/ewE amplified regions and therefore was not analysed separately.

For the 12S primers, *in-silico* analysis indicated good binding to the target sequences. Both the forward 185F and reverse 14233R primers aligned perfectly with 15/16 species that had sequences available. 185F contained one base difference from *D. rubidus* (G instead of A) and 14233R contained one base less than *A. rosea* (which contained an inserted G). Alignment of the primers with the retrieved GenBank sequences indicated an amplified area of ~190 base pairs, with sufficient variation between species to distinguish all those included in the analysis. The size of the differences between distinct species ranged from 14 base pairs (*Octolasion lacteum* and *O. cyaneum*) to 56 base pairs (*Eisenia fetida* and *D. attemsi*), with a mean difference of 40.6 base pairs between sequences.

Optimising PCR Conditions

For the majority of the 16S primer pairs (ewB/ewC, ewD/ewE, ewB/ewE and ewA/ewC), lowering the annealing temperature from 57°C to 55°C gave clearer bands when run on a gel – this was the recommended temperature from Taberlet *et al.* (2018). Diluting the DNA extracts also improved PCR performance, giving clearer bands at both 1:5 and 1:10 dilution levels, with 1:5 judged to be best as it gave the brightest bands (Figure 3.3). Although visualisation on a gel was inconclusive when judging the effectiveness of bead cleaning, subsequent Tapestation analyses on PCR products pre- and post-bead cleans showed that bead cleaning was effective in reducing the concentration of PCR artefacts and primer dimer in the samples, and was therefore included in the final protocol. The ewA/ewF primer pair was the only 16S combination that did not successfully amplify DNA despite repeated attempts using varying PCR conditions, and so it was excluded from testing at this stage and not taken forward for the sequencing trials.

PCRs using the 12S primer pair were problematic from the start, producing inconsistent and low-quality amplifications that were not improved by adjusting the PCR conditions (Fig. 3). As a result, this primer pair was not included in any subsequent tests. The final PCR primer pairs taken through to the pilot Miseq Nanorun, including their optimised conditions, are listed in Table 3.1.

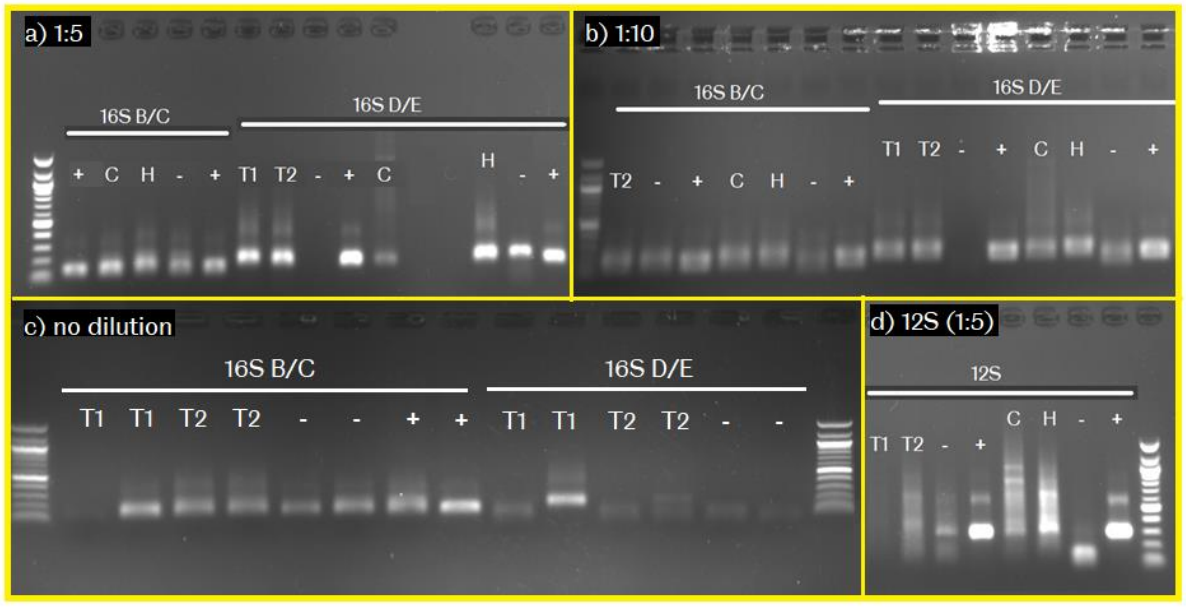

Figure 3.3. Electrophoresis gels photographed under UV light, showing differences between the success of the PCR reaction when using eDNA extracts diluted to a) 1:5 concentration b) 1:10 concentration and c) no dilution. Clear, bright and distinct bands indicate more successful amplification. Panel d) shows a typical result of soil eDNA extracts amplified using the 12S primers, showing poor quality streaky bands compared with the positive

controls (labelled as '+'). Negative controls are indicated by '-' and eDNA samples are shown by letters and numbers (i.e. 'C', 'H', 'T1', 'T2').

Sanger sequencing

To check that the four primer pairs carried through from previous tests were amplifying the desired taxa, Sanger sequencing was performed on the pilot DNA extracts alongside positive tissue controls. The positive tissue controls were correctly sequenced to a high level of accuracy, identifying the correct earthworm species (*A. chlorotica* and *A. longa*). As expected, the eDNA extracted from the pilot soil samples did not sequence as cleanly, given that there were multiple sequences present in any given sample. This led to multiple overlapping peaks indicating several competing signals at the same location (Figure 3.4). However, in some cases for the ewD/ewE, ewB/ewE and ewA/ewC pairs a 'dominant' sequence was able to be distinguished (made up of the highest peaks at each position) and a species ID generated with reasonable confidence (i.e. an alignment score above 80 and a low e-value). The Sanger results for the ewB/ewC pair did suggest amplification was occurring, but there were too many overlapping peaks to isolate clear dominant sequences that could be confidently identified using BLAST.

Surprisingly, the dominant sequences that arose most frequently were not from earthworms but members of the closely related Enchytraeidae family (including *Enchytraeus christenseni*, *Frederica christeri* and *Enchytronia parva*). Only the ewA/ewC primer pair had some samples with dominant sequences corresponding to earthworms; *L. terrestris* was identified in one of the arable samples, *A. longa* was identified from the first ley and *Allolobophora chlorotica* from the second ley sample. However, sequencing with ewA/ewC yielded small peaks with very few overlapping sequences compared with other pairings (Fig. 4), which suggested that only a small proportion of the potential eDNA signatures present in the samples were being picked up by this primer pair. Therefore, the ewA/ewC primer pair was dropped from consideration and the remaining ewB/ewC, ewD/ewE and ewB/ewE pairs were included in the MiSeq sequencing pilot experiment.

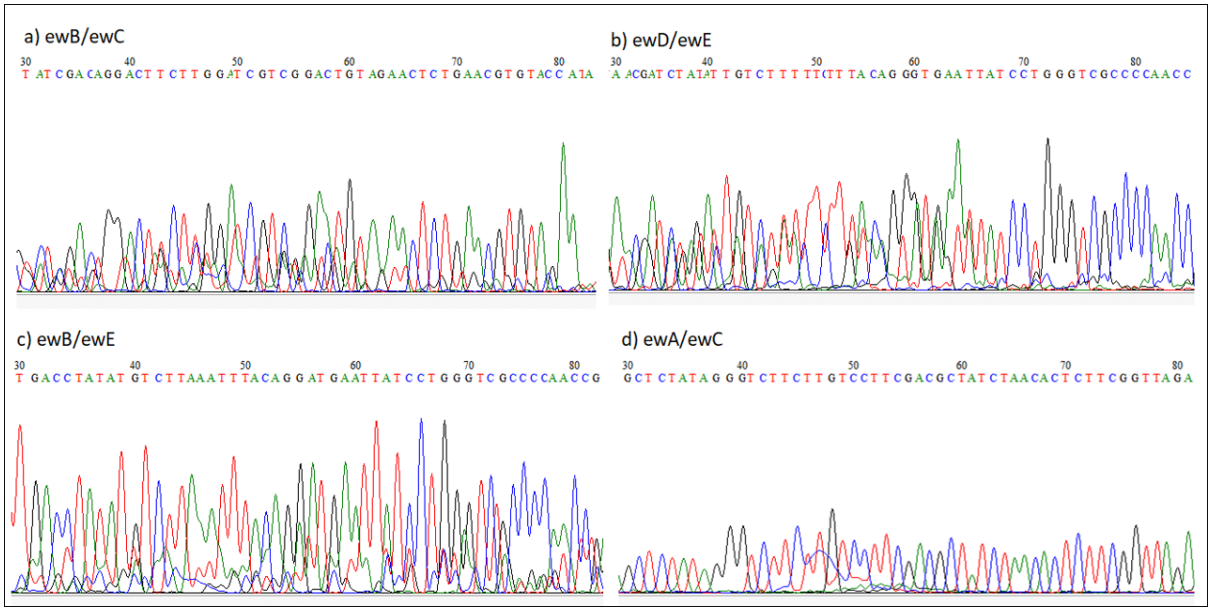

Figure 3.4. Examples of the results from Sanger sequencing soil eDNA samples. Each panel shows the returned sequences from a single sample using a) ewB/ewC, b) ewD/ewE, c) ewB/ewE and d) ewA/ewC primer pairs. Taller peaks indicate stronger sequence signal (good quality reads). Multiple overlapping peaks indicate the presence of several different sequences in the sample (as expected for environmental samples).

Bioinformatics pipeline development and pilot Miseq Nanorun

Before assessing the success of the different primer pairs and the performance of the protocol, the sequence data obtained from the pilot MiSeq Nanorun was first used to optimise the bioinformatics pipeline. During the quality filtering and trimming steps, the criteria that yielded the highest possible quality data without discarding too many sequences was deemed to be a minimum Phred quality score of 30 over a 4 base pair sliding window. This meant that each sequence read was inspected 4 base pairs at a time, and if the mean Phred score dropped below 30 the sequence was trimmed. The most suitable minimum sequence lengths were 20-bp for ewB/ewC, 50-bp for ewD/ewE and 100-bp for ewB/ewE, and sequences shorter than this were discarded. When clustering sequences into mOTUS, grouping reads that were found to be at least 97% similar worked well for both ewD/ewE and ewB/ewE. For these two primer pairs the final sequence numbers yielded by the MiSeq Nanorun did not seem to be greatly affected by variations in the minimum sequence identity value when conducting a BLAST search to assign taxonomy, so it was initially decided that the cut-off would be set to 95% minimum sequence identity and a maximum e-value of 0.00001. However, after these criteria were applied to the main MiSeq results in Chapter 4, it became evident that 95% sequence identity was not sufficiently high enough to maximise species-level assignment in ewD/ewE (Table 3.2). In this case a significant number of the ewD/ewE sequences were assigned to the *Apporectodea* genus level,

which was not an issue with the longer ewB/ewE primer pair. This was difference was likely due to the lowest common ancestor taxonomic assignment algorithm applied by the Megan software, that was used for visualising the BLAST results. Subsequently, it was decided that the minimum percentage identity should be increased to 97% for the ewD/ewE pair, which solved the issue and allocated the majority of genus-level sequences to *A. longa*.

Table 3.2. The sequences numbers returned for the ewD/ewE primer pair from the full MiSeq run in Chapter 4, showing the variations brought about by different mOTU clustering and minimum sequence identity BLAST values. Numbers in bold indicate the 'best' outcome (i.e., highest sequence numbers, except for *Aporrectodea sp.,* where the lowest sequence numbers indicate a better outcome).

Species	97% Cluster / 95% Blast	98% Cluster / 95% Blast	98% Cluster / 97% Blast	97% Cluster / 97% Blast
A. chlorotica	**385,282**	385,278	385,233	385,233
Aporrectodea sp.	60,464	60,118	**2**	**2**
A. caliginosa	35,045	35,045	35,045	35,045
A. longa	3120	3466	**63,582**	**63,582**
A. rosea	**115,582**	115,563	115,563	**115,582**
L. castaneus	49,864	49,864	49,864	49,864
L. terrestris	18,820	18,820	18,820	18,820
O. cyaneum	**14,225**	**14,225**	14,223	**14,225**
S. mammalis	15,609	15,609	15,609	15,609

After the bioinformatics pipelines had been optimised, the Nanorun yielded a total of 638,841 sequences across the three primer pairs used (ewB/ewC, ewD/ewE and ewB/ewE). The ewD/ewE and ewB/ewE primer pairs, which amplify longer DNA fragments than ewB/ewC, both showed similar performance, with the former yielding an initial total of 232,820 sequence reads and the latter 208,407 reads. After quality filtering and trimming these totals were reduced to 122,188 for ewD/ewE and 113, 041 for ewB/ewE. Sequence clustering resulted in 75 and 61 mOTUs respectively. After the final BLAST steps, 5076 of the returned ewD/ewE sequences were found to be from earthworms, compared with 2466 for ewB/ewE. Both of these primer pairs detected the same four earthworm species, but ewD/ewE also identified additional *L. castaneus* sequences that were not picked up by ewB/ewE (Table 3.3). As predicted in Bienert et al. (2012) these primer pairs also amplified sequences from the closely related Enchytraeidae family, with ewD/ewE yielding 16,439 enchytraeid sequences representing nine species compared to 39,294 sequences across 11 species for ewB/ewE. The Nanorun results also supported the decision to use a maximum of 400 ul of supernatant in the eDNA

extraction phase, as samples which exceeded this returned lower sequence numbers on average in the ewD/ewE primer pair (mean of 1170 compared with 3743.5 in the 400 ul samples).

MiSeq sequencing of the shorter ewB/ewC primer pair proved more problematic. Eventually an optimised bioinformatics pipeline was achieved, most notably by increasing the sequence similarity to 99% when clustering and using a 97% minimum sequence identity cut off for the BLAST search. The initial number of sequences returned was 197,614, which was reduced down to 145,661 after quality filtering and trimming. There were 66 mOTUs generated from the clustering step. After all the bioinformatics steps had been completed, a total of 4707 earthworm sequences belonging to four different species were detected. EwB/ewC did not pick up the sequences of *O. cyaneum* that were found by both the longer primer pairs, or the *L. castaneus* sequences that were identified in the ewD/ewE sequences. Given that ewB/ewC seemed to show less sensitivity when compared with the other two primer pairs, and also because the size of the ewB/ewC products may exacerbate contamination issues and cause further losses of sensitivity when sequencing the other primers (see discussion), it was decided that it would not be used for the main eDNA experiment in Chapter 4.

Table 3.3. The final sequence numbers reported from the pilot MiSeq Nanorun. Species in bold are the earthworms, with the rest belonging to the family Enchytraeidae.

Species	ewB/ewC	ewD/ewE	ewB/ewE
Allolobophora chlorotica	**3197**	**1184**	**608**
Aporrectodea longa	**71**	**2131**	**1488**
Aporrectodea rosea	**945**	**541**	**41**
Lumbricus terrestris	**494**	**914**	**69**
Lumbricus castaneus	**0**	**50**	**0**
Octolasion cyaneum	**0**	**256**	**260**
Enchytraeus sp.	0	0	4361
Buchholzia fallax	0	0	580
Enchytraeus buchholzi	0	0	13
Enchytraeus bulbosus	0	7632	1126
Enchytraeus christenseni	0	1909	0
Enchytraeus coronatus	0	0	6264
Fridericia sp.	0	0	2474
Fridericia christeri	0	66	8337
Fridericia galba	0	176	5226
Fridericia heliota	0	0	756
Fridericia isseli	0	3057	9171
Fridericia paroniana	0	34	2
Fridericia tuberosa	0	3482	792
Henlea perpusilla	0	3	0
Marionina communis	0	80	192

Reducing contamination

During the PCR optimisation period it become apparent that levels of contamination in both extraction and PCR negatives (which substituted sterilised molecular grade water for DNA) was unacceptably high, despite attempts to reduce the risk of contamination during DNA extraction and PCR prep (see above). Slight reductions in contamination were achieved by using Microseal silica lids to seal the plates instead of adhesive ones, but it was still prevalent. Switching from performing reactions in PCR plates to individual 0.2 ml PCR tubes substantially reduced contamination levels. However, occasional contamination was still evident, so Sanger sequencing of contaminated negatives from earlier plate and tube tests was performed to elucidate the origin of contamination.

Of eight negatives that showed possible contamination (indicated by the presence of a band on a gel), six gave a positive match to a sequence listed on BLAST. Five proved to be contamination resulting from enchytraeid DNA, originating from three different species (*Friderica galba, Enchytraeus christenseni* and *Chamaedrilus varisetosus*), suggesting multiple sources of contamination. One negative had contamination that matched DNA originating from a moth species, *Herminia grisealis*, which indicated that the lamina-flow cabinets and/or a shared buffer stock could have been the source, as these had been used previously for another experiment involving this species. Subsequently, buffer stocks were replaced, cabinets thoroughly cleaned, and some of the samples were prepared for PCR in another clean laboratory on a different site. Contamination of primer stocks was also possible, so new protocols to reduce contamination of primer stocks and other reagents were developed and implemented. As a result, primer stocks were aliquoted out and stored in single use tubes, and no primer aliquots were used for more than one set of reactions. Implementing these measures greatly reduced contamination, as indicated by presence of bands on a gel. If contamination of PCR negatives still occurred after taking these measures, the compromised reactions were repeated in a laboratory off-site using the same procedure. No PCR negatives showed any visual contamination after being repeated off-site.

3.5 Discussion

The results of the methodology development tests enabled the most effective eDNA extraction technique to be determined, and identified the primer combinations ewD/ewE and ewB/ewE as the most promising candidates for use in Chapter 4. Despite showing promising results in the original Bienert et al. (2012) study, the shortest primer pair ewB/ewC was found to be less sensitive than the

longer 16S primer pairs and required challenging sequencing conditions, so was dropped ahead of the main study. Both ewD/ewE and ewB/ewE detected more sequences from the closely related Enchytraeidae family in this pilot study, matching the predictions made by Bienert et al. (2012). The results of the investigations into negative control contamination and the subsequent implementation of effective control measures also highlighted the importance of having dedicated protocols, control measures and workspace in place when working with short, degraded eDNA sequences.

Firstly, the extraction and PCR optimisation tests indicated that diluting eDNA extracts by a factor of 5 gave better amplification results than using undiluted extracts. The difference in results was likely due to the presence of PCR inhibitors in the soil samples that were extracted alongside the eDNA, which may have disproportionately inhibited the PCR reactions and led to sensitivity losses when included in high enough concentrations (Wilson, 1997). An example of PCR inhibitors commonly found in soils are humic substances, such as humic and fulvic acids, which are polymers with complex structures that have been shown to disrupt the activity of the *Taq* DNA polymerase enzyme during PCR, leading to a decrease in the quality and quantity of resulting DNA extracts (Braid et al., 2003; Wnuk et al., 2020). The presence of these inhibitors in the soil eDNA extracts is also supported by the observation that some of the undiluted extracts showed some yellow discolouring, particularly in those that had originated from columns that were loaded with more than 400 ul of supernatant, which is often associated with humic acid inhibitors (Kemp et al., 2006). Dilution of the extract reduces the inhibitors, but this can be a trade-off, as it also lowers the concentration of template DNA in the final sample, causing a loss in sensitivity and making it more likely that eDNA present in lower quantities (i.e., rare species) will be missed (King et al., 2009). In this study, dilution to a factor of five was chosen as it seemed to give better amplification and resolve any potential effects of inhibitors, whilst still yielding sufficient sequence numbers downstream. Although there were no noticeable effects on the final sequence results of different humic substance levels between the sample groups (arable or ley) either here or in Chapter 4, alternative solutions to deal with potential inhibitors may be required (for example Kermekchiev et al. 2009, Braid et al. 2003 or Kemp et al. 2006) when comparing between soil samples with significantly different organic matter amounts, as the concentration of inhibitors present may be substantially different (Wnuk et al., 2020).

Assessing the performance of the different primer combinations throughout the methodology development process reduced the original six possible primer pairs down to two. Initially the *in-silico* analyses showed promising results for five of the six possible combinations. ewA/ewF were unable to be assessed as the sequences in the NCBI database were not present for this region. Three of the 16S primers contained a single base mismatch with either *A. limicola* or *A. rosea*, which were all located towards the middle of the primers. However, these were not considered problematic as a single

mismatch should not have a big effect on binding, provided it is not at the 3' end of the primer (Christopherson et al., 1997; Ficetola et al., 2015). One advantage of using the ewD/ewE and ewB/ewE primer pairs, as revealed by the *in-silico* analysis, was the ability of these combinations to not only distinguish between all the different earthworm species, but also between some of the sequenced haplotypes too. Although here we chose to focus on diversity at the species level, distinguishing between haplotypes could be important if investigating factors such as intraspecific genetic diversity or local adaptation (Epp et al., 2018; Sigsgaard et al., 2020).

Although initially yielding acceptable results in the *in-silico* analysis, the 12S primer pair developed by Harper et al. (2005) did not perform well during the eDNA extract PCR testing (Fig. 3) and was ruled out at this stage. Unlike the 16S primers which were specifically designed for soil eDNA analysis, the 12S pair had originally been employed for use in analysing the gut contents of invertebrate predators. Of all the primers included in the methodology tests they amplified the longest region - around 230 base pairs including primers (Harper et al., 2005). They were found to work well in the original dietary study, where the template DNA was likely to be fresher and in both intracellular and extracellular states. However, the extracellular eDNA in soils extracted here was likely to be heavily degraded by microbial activity and exposure to the soil environment (Nielsen et al., 2007; Sirois and Buckley, 2019). This would explain why the 12S primers were effective at amplifying the positive control DNA extracted from earthworm tissue but not the soil eDNA extracts in any of the PCR conditions tested, as the eDNA is heavily fragmented and requires primers that amplify shorter regions (like those developed by Bienert et al., 2012).

In contrast to the 12S primers, the ewB/ewC primer combination amplified the shortest region tested and seemed to perform well up to the Miseq Nanorun. However, the extremely short nature of the region amplified (~30bp), even in comparison to the other short 16S ewD/ewE and ewB/ewE combinations that amplified regions of ~70bp and ~120bp respectively, caused difficulties at a number of stages during the development process. The amplified PCR products were difficult to distinguish from primer dimer when visualising the results of gel electrophoresis (Fig. 3), and there was also concern that the short PCR fragments may become potent sources of contamination that could evade control measures if released into the wider laboratory environment (Champlot et al., 2010). A key difficulty also arose during the running of the MiSeq Nanorun, as Illumina technical support staff advised the inclusion of the very short ewB/ewC fragments increased the chance that the sequencing run would fail. To reduce the probability of the run failing spiking in a large volume of PhiX sequences was recommended, which are sequences originating from the bacteriophage PhiX and commonly used as a control in Illumina MiSeq runs or put in to increase sequence diversity in low complexity libraries (Mukherjee et al., 2015). The sequencing run therefore went ahead with 50% PhiX spiked in, which

successfully prevented failure but also meant that a large proportion of the returned sequences were taken up by PhiX. We were advised that dropping the ewB/ewC pair and only running ewD/ewE and ewB/ewE would allow for a more balanced run that would require a much lower percentage of PhiX to be spiked in (10-15%), which would improve the sequencing coverage and increase the numbers of useful reads returned (Jeon et al., 2015). Taking these factors into consideration, combined with the fact that the final sequence results showed ewB/ewC to be less sensitive than the two longer 16S primer pairs, it was decided to drop this combination for the Chapter 4 study and use ewB/ewE and ewD/ewE only.

Another interesting finding from the methodology development was the promising performance of the ewB/ewE primer pair, which although mentioned as a possible combination in Bienert et al. (2012) had not been tested until now. As predicted this pairing did amplify enchytraeid sequences alongside those of earthworms, and like ewD/ewE returned more enchytraeid sequences than earthworms. This could have occurred because although we were able to remove earthworms from the soil samples prior to eDNA extraction, it was not possible to remove enchytraeids due to their small size. Therefore, less degraded DNA from enchytraeid tissue may have been included in the extractions and then preferentially amplified. However, despite the sequencing of the Enchytraeidae these primer pairs still picked up more earthworm species than the smaller ewB/ewC pair, and with the added sensitivity due to be gained by reducing the PhiX volume spiked into the main run it was deemed not to be an issue. It is also worth noting that enchytraeids also play important roles in soils and have been proposed as potential indicators (Jänsch et al., 2005; Koutika et al., 2001), so their inclusion in eDNA surveys using these primers could be viewed as a benefit and not a hindrance.

The contamination of negative controls seen at various points through the methodology development tests highlighted the importance of having dedicated contamination prevention procedures in place when working with short, degraded eDNA fragments. Although measures to reduce contamination were taken from the start, the conventional controls used for other types of DNA analysis performed in the laboratory were not sufficient to prevent repeated contamination events. The use of lidded PCR tubes instead of plates, storing reagents separately and employing a single-use policy for primer aliquots and other PCR ingredients markedly reduced contamination, and are therefore recommended actions for future studies using soil eDNA for earthworm diversity analyses. It is worth noting that the only way to completely eliminate all noticeable negative contamination was to combine the measures above with running the PCR reactions in another clean laboratory facility off-site. This demonstrates the importance of having laboratory facilities that are physically separated from those that are not working with eDNA, which can be treated separately and have specialist equipment installed to reduce contamination (Goldberg et al., 2016). Since undertaking this work, the

facility has invested in a dedicated eDNA lab, which is anticipated to greatly reduce the occurrence of contamination in eDNA projects going forward. Finally, this investigation also shows the importance of including negative controls in as many stages of the protocol as possible, so that when contamination events do occur they can be investigated further and dealt with effectively (Sepulveda et al., 2020).

3.6 Conclusions

As well as determining the workflow to be employed in Chapter 4, the methodology development tests described here strengthened the main study by allowing the exploration of novel techniques and solutions to improve the extraction, amplification and sequencing of earthworm eDNA. For example, these preliminary analyses enabled the investigation of the novel ewB/ewE primer combination that had not been tested in previous studies, but proved to be a promising option for earthworm diversity analysis which was subsequently utilised in the main eDNA experiment in Chapter 4. It is hoped that these results will benefit future projects working on earthworm eDNA collected from soils, through the protocols that have been developed and the subsequent recommendations made for dealing with issues like PCR inhibition and negative contamination. Most importantly, these findings indicate that it is feasible to collect and sequence soil eDNA from agricultural soils under active management, paving the way for further investigation in Chapter 4.

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3.8 Supplementary Materials

Protocol for the extraction of eDNA from soil samples using a phosphate buffer

This protocol has been adapted from a protocol outlined in Taberlet *et al.*, 2018: Environmental DNA for Biodiversity Research and Monitoring (page 37).

I chose to run through the below protocol using three soil samples, taking two replicates of each (i.e. six 15 g soil samples) at a time, plus a negative control. This was due to the falcon rotator being used only having six slots, but also as taking on more samples at a time would have led to more difficulties during the time-sensitive steps. I usually ran through the protocol twice a day, getting through six soil samples (12 replicates) out of 96 total soil samples – plus two negative controls – per day. However, this can be increased or decreased as needed. All of the steps were performed in a lamina-flow hood, unless stated. The protocol uses equipment and chemicals from the Macherey-Nagel NucleoSpin® Soil Kit.

1. Ensure all equipment has been properly cleaned and sterilised before use. Cleaning with 10% bleach after each use and placing in the UV lamina-flow hood overnight is best.
2. Remove the soil samples (in sealed bags) from the -20 °C freezer and defrost in the store room overnight, for use the next day.
3. Prepare 500 ml of saturated phosphate buffer solution by adding 0.985 g NaH_2PO_4 and 7.35 g Na_2HPO_4 to 500 ml ddH_2O. Mix thoroughly until phosphate is dissolved, then autoclave. This buffer cannot be kept for longer than 24 hours after autoclaving, so new buffer will need to be made fresh regularly.
4. Label 50 ml falcon tubes with unique codes corresponding to the soil sample they will correspond to, or with a negative control number.
5. Homogenise the collected soil in the sealed bags, by working the soil with your fingers – taking care that stones do not pierce the bag itself. Often, particularly with the arable soils, this will lead to the soil coalescing as one large clump. When this happens, continue to work the clump like a ball of clay or dough, to ensure proper homogenisation of the soil.
6. Weigh out 15 g of soil from each bag using a disposable plastic spoon and wooden toothpick. Try to sample different parts of the soil in the bag to make up the 15 g (i.e. not just one single large

lump), and if the soil is already in a single clump, take pieces from various parts of it. Add this soil to the relevant 50 ml falcon tube and seal.

7. To get two replicates from each bag (i.e. two 15 g samples), repeat step 6. Then dispose of the used plastic spoon, toothpick and weighing boat between each sample bag.
8. Clean and sterilise the weighing scales and surrounding areas by wiping down with 10% bleach between each soil sample bag, and change your gloves. I found that laying a paper towel down next to the scales and where homogenisation took place was useful, to catch any dropped soil and make disposing/subsequent cleaning easier.
9. Add 15 ml of the autoclaved phosphate buffer to each of the falcon tubes containing soil. Include a negative extraction control (a falcon tube that contains only phosphate buffer). If the phosphate buffer has come fresh from the autoclave and is still hot, run the exterior of the bottle under the cold tap until it cools.
10. Use the falcon rotator to rotate the falcon tubes for 15 minutes (out of the lamina-flow hood).
11. While the tubes are rotating, distribute 250 ul of SB buffer into Eppendorf tubes, one tube for each extraction.
12. While the tubes are rotating, put the spin columns on the vacuum manifold ('hedgehog') connectors, close the tops and label.
13. After 15 minutes, take the falcon tubes out of the rotator and take them down to the 50 ml tube centrifuge. Centrifuge the tubes for 5 minutes at 4700 rpm.
14. While the tubes are in the centrifuge, put the elution buffer SE in the oven and set to 80 °C.
15. Remove the tubes from the centrifuge and wipe them with 10% bleach, before taking them back up to the lamina-flow hood. Be careful to walk slowly and try not to mix the supernatant with the floating debris in the tube!
16. Remove 400 ul of the supernatant from the 50 ml falcon tube and transfer it into the Eppendorf tube containing 250 ul SB buffer. Take the supernatant from around 10 mm above the sediment and try to avoid transferring bits of floating debris with the supernatant.
17. Thoroughly mix the supernatant with the buffer using the same filter tip and transfer the 650 ul mix to the relevant spin column.
18. Put the vacuum on (i.e. open the tap on the connector) and the liquid will pass through the column.
19. After all columns have been loaded and all liquid has passed through, break the vacuum for each column.
20. Load 500 ul of SB buffer to each column and put the vacuum on again.

21. Once all liquid has passed through, break the vacuum and load 550 ul of SW1 buffer. Open the taps.
22. Once all the liquid has gone through, break the vacuum and load 750 ul of the SW2 buffer to the columns. Remember to break the vacuum before loading this buffer, so that it can clean the very top parts of the columns.
23. Put the vacuum on again until all the liquid has passed through.
24. Close the columns and transfer each of them to a 2 ml collection tube without cap, and centrifuge for 2 minutes at 11,000 x g to dry the silica membrane
25. If necessary, remove the columns and tap the collection tubes on a dry paper towel to remove the liquid residue inside, ensuring no contamination between tubes. Return each column to its original collection tube after dabbing.
26. Add 680 ul of SW2 buffer, close the columns and then vortex for 2 seconds.
27. Centrifuge the columns for 30 seconds at 11,000 x g
28. Pour out the liquid from each collection tube into a sink or container, and tab the tube on a dry paper towel to remove excess liquid. Ensure no contamination between each tube occurs, and dilute the solution running down the drain by running the taps for a short time.
29. Return the columns to their collection tubes and centrifuge for 2 minutes at 11,000 x g for drying the silica membrane.
30. Put each column on a labelled collection tube with cap and discard the previous collection tube.
31. Collect the elution buffer SE from the oven, which should now be heated to 80°C.
32. Take this back up to the lamina-flow hood (quickly!) and add 100 ul of elution buffer SE to each column.
33. Wait 1 minute at room temperature, then centrifuge for 30 seconds at 11,000 x g.
34. Remove the columns from the collection tubes and store the DNA extract collected in the tubes in the -20 °C freezer. This extract will likely need to be diluted – x5 is best - before use to limit the influence of PCR inhibitors that are coextracted with the DNA.
35. Make sure all equipment to be reused is cleaned with 10x bleach and UV'ed in the lamina-flow hood before next use. To clean the hedgehog, 10% bleach was allowed to soak in the connectors for 15 minutes before opening the taps. The bung was then taken out and the contents emptied into a sink and rinsed with plenty of water.

Chapter 4

Using soil eDNA to measure earthworm diversity and assess the impact of land management in agricultural systems

4.1 Abstract

Restoring and maintaining soil biodiversity is important for the sustainability of our food systems, particularly as agricultural soils continue to degrade at alarming rates. Earthworms are key components of soil biodiversity and provide many benefits to soil functioning, but effective and standardised approaches to measure their populations are lacking. In this study, the feasibility of using soil environmental DNA (eDNA) metabarcoding for surveying earthworm populations in agroecosystems was explored. Soil samples were collected from arable fields with temporary grass-clover ley strips sown into them, and extracellular earthworm eDNA was extracted, amplified and sequenced using next-generation sequencing. Alongside eDNA sampling, earthworm populations were also sampled using a traditional hand-sorting approach, which allowed quantitative comparisons between the different methods. The eDNA method was found to detect significantly higher local species richness when compared to the traditional hand-sorting approach, with the site occupancy proportions indicating that it was markedly better at detecting anecic earthworms. The results also showed that eDNA sampling was sensitive enough to detect differences in the earthworm communities bought about by different land use treatments in the same field, with significantly higher earthworm diversity detected in the 33-month old ley strips when compared to the arable soils. With regards to relative abundance, there was considerable within-site variation in the relative abundances depending on which methodology was being used to measure it, but overall site occupancy proportions calculated by the eDNA and traditional hand-sorting methods were found to be more consistent. It was concluded that eDNA sampling could become a useful tool for monitoring agricultural earthworm diversity and biological soil health, and could be utilised for use on farms across the UK to promote better management and soil care.

4.2 Introduction

Producing enough food for the worlds growing population remains a key challenge, and has been prioritised in the United Nations Sustainable Development Goals (UN, 2019a). Given that humanity derives 98.8% of our food from soils (Kopittke et al., 2019), protecting and enhancing this resource will be essential for feeding the predicted 9.7 billion people on the planet in 2050 (UN, 2019b). The ongoing degradation of soils across the globe, attributed to multiple factors, including intensive agriculture, climate change and land use change (Lal, 2013; Muñoz-Rojas et al., 2017; Zhao et al., 2005), is therefore causing widespread concerns for the ability of humankind to meet the rising food security challenges (Jie et al., 2002; Tamene et al., 2019). Recent estimates suggest that around 25% of the global land surface can be classed as 'highly degraded or experiencing high degradation', 8% as 'moderately degraded or experiencing moderate degradation' and a further 36% as stable but 'slightly or moderately degraded' (FAO, 2011). Borrelli et al. (2017) estimates that 35.9 Petagrams of soil are lost globally per year, with 50.5% of total soil erosion occurring in cropland in 2012, despite it only accounting for 11.2% of the land area studied. For England and Wales, the economic cost of soil degradation has been calculated at £1.2 billion per year (Graves et al., 2015).

In light of the importance of soils for food production and the need to address soil degradation, there has been an increasing focus on the concept of 'soil health' in recent years (Karlen et al., 2019; Liu et al., 2020). Soil health is often defined as "the continued capacity of soil to function as a vital living system, within ecosystem and land-use boundaries, to sustain biological productivity, promote the quality of air and water environments, and maintain plant, animal, and human health" (Doran, 1996). The term is often used interchangeably with 'soil quality', although this practise is debated (for example see Lal, 2016). Both soil health and soil quality are affected by complex physical, chemical and biological properties and interactions that govern the workings of a soil. Some of the key physical soil properties that contribute to soil quality and health include bulk density, porosity, compaction and aggregate stability; whilst some key chemical properties include organic matter content, aeration status, salinity and availability of plant nutrients (Nortcliff, 2002).

The biological properties of soils – and soil biodiversity in particular - is another key aspect determining soil health and quality in agriculture (Brussaard et al., 2007; Lehman et al., 2015; Pulleman et al., 2012). In recent decades soil biodiversity has received increasing attention in the research literature, but this attention has mainly been focused on soil microbial diversity, with the number of studies looking at soil meso and megafauna lagging far behind (Coyle et al., 2017). Despite receiving less attention, earthworms play a particularly important role in maintaining and promoting the health of temperate soils, and are recognised as 'ecosystem engineers' for the disproportionate impact they have on the

soil environment and pedogenesis (Cunha et al., 2016; Lavelle et al., 1997). Through their burrowing and feeding behaviour they influence the physical, chemical and biological properties of the soil. Physical effects include the creation of macropores and the formation/restructuring of macroaggregates as a result of burrowing and casting, which can increase soil water holding capacity and gas exchange (Blanchart et al., 1999; Hallam and Hodson, 2020; Schaik et al., 2014; Sheehy et al., 2019; Zhang and Schrader, 1993). The activity of earthworms and resulting bioturbation affect soil chemistry in numerous ways, in particular through the breakdown of organic matter, distribution of it throughout the soil profile and by increasing the availability of other nutrients that are important for the development of plants and microbes (Chaoui et al., 2003; Lavelle et al., 1998; Le Bayon and Milleret, 2009). The biological properties of soils are also greatly affected by earthworms, as a result of their direct or indirect interactions with other organisms and their ability to stimulate microbial activity (Bart et al., 2019; Binet et al., 1998; Monroy et al., 2011; Wang et al., 2020).

Given the major role that earthworms play in temperate soils, it is no surprise that they also bring great benefits to agriculture too (Bertrand et al., 2015). As well as maintaining and promoting agricultural soil health, a meta-analysis by van Groenigen et al. (2014) found that the presence of earthworms increased crop yields by an average of 25%, with higher increases seen in nitrogen deficient soils or those that had previously been disturbed and lost their structure. Despite their importance, earthworms remain understudied and their species diversity largely unmapped. Efforts have been made to rectify this both at international and local levels, including research that has mapped global distribution patterns of earthworm biodiversity (Phillips et al., 2019) and the more local #60minworms UK study that tasked farmers with sampling and recording earthworm numbers in their fields (Stroud, 2019). However, progress towards increasing earthworm diversity sampling and population monitoring may be hampered by the limitations of traditional earthworm sampling techniques, which can be time and energy intensive, difficult to standardise, prone to biases and require taxonomic expertise (Andriuzzi et al., 2017; Bartlett et al., 2010; Čoja et al., 2008; Jiménez et al., 2006). Addressing these limitations and developing more standardised methods for sampling earthworm populations is therefore seen as important for monitoring their distributions and realising their potential as both indicators and promoters of soil health.

Environmental DNA (eDNA) sampling is one possible alternative method for sampling earthworm populations that could address some of the limitations of traditional methods. eDNA sampling involves collecting, amplifying and sequencing genetic material that has been left behind by organisms in their environment, which may originate from deposits such as hair, skin cells, mucus or faeces. As sequencing technologies have evolved studies investigating macroorganism eDNA have increased, but the majority have tended to focus on eDNA sampling in aquatic environments (Belle et al., 2019),

perhaps because eDNA can be readily amplified and sequenced from water samples (Rees et al., 2014). However, studies have also demonstrated the feasibility of applying eDNA sampling techniques to terrestrial environments, including through the sampling of ice cores, sediments, plant material and scat (for example Bohmann et al., 2011; Hofreiter et al., 2003; Thomsen and Sigsgaard, 2019; Willerslev et al., 2007). Soil is also a potential reservoir of eDNA that can be sampled in order to build a picture of both above and belowground biodiversity. For example, Yoccoz et al. (2012) showed that eDNA sampled from boreal, temperate and tropical soils was consistent with the aboveground plant diversity seen in the corresponding sites, and Andersen et al. (2012) sampled top soil from safari parks, ostrich farms and zoological gardens with known species compositions to demonstrate the viability of soil eDNA for vertebrate biodiversity sampling. Furthermore, Bienert et al. (2012) also demonstrated that eDNA can be used for sampling earthworm communities in undisturbed woodland and meadow soils. This research was later built on by Pansu et al. (2015), who showed differences in earthworm diversity at the landscape level in the Northern French Alps. However, to our knowledge earthworm eDNA sampling has yet to be applied to active agricultural soils, where it has the potential to be used for closer monitoring of field earthworm populations and associated soil health.

As well as its potential use for monitoring soil health, eDNA sampling could be useful in agricultural systems for testing the effectiveness of management techniques that have been suggested for restoring earthworm populations. One relevant example is the inclusion of grass-clover leys into arable rotations, where cropped fields are sown with grass and clover and left for around 2-3 years to rebuild soil quality. This was a common practise in the UK and elsewhere before affordable pesticides and fertilisers became widely available, but has since declined with the onset of increasing intensification and the loss of livestock from mixed arable systems (Knox et al., 2011). However, as more focus is put on soil health and evidence grows of continued declines in soil carbon and other soil quality indicators in the UK (Squire et al., 2015), the idea of reintroducing grass-clover leys to aid soil restoration has gained more attention in recent years. This led to the inclusion of grass leys as a suggestion for maintaining soil organic matter in the Cross Compliance Soil Protection Review (DEFRA, 2006), which farmers receiving payments were required to complete as part of the EU Good Agricultural Environmental Conditions standards (European Commission, 2013). The reintroduction of leys is also mentioned in the UK Government's 25 year environment plan (UK Government, 2018), and there have been calls for any post-Brexit agricultural payment schemes to further promote the reintroduction of leys into crop rotations (for example Hamer et al., 2018). However, there are still unanswered questions surrounding the speed and extent to which leys are effective at boosting arable soil health, which includes gaps in our understanding of how soil fauna such as earthworms respond to their use.

In this study, we aim to demonstrate the application of earthworm eDNA sampling to active agricultural systems, for use as both a possible tool to measure biological soil health and to assess the effectiveness of soil management interventions (in this case temporary grass-clover leys). To do this, we collected soil samples and performed traditional earthworm hand-sorting in arable fields that had grass-clover ley strips sown into them. To sample and isolate earthworm eDNA from the soil, we used an adapted form of the protocols listed in Taberlet et al. (2012) and Taberlet et al. (2018), using earthworm specific primers pairs developed by Bienert et al. (2012). We performed next-generation MiSeq sequencing to obtain earthworm sequence numbers and species diversity, and compared these with the results from the traditional hand-sorting method and across different soil management treatments. We explored the diversity patterns generated by the different methodologies using NMDS ordinations and PERMANOVAs, assessed the required sampling intensity using species accumulation curves and investigated some potential proxy measures of abundance (relative abundance and site occupancy proportions). Finally, we discuss the potential effectiveness of earthworm eDNA sampling in agricultural systems for assessing soil health and monitoring the progress of soil management interventions, and identify areas for further research and development.

4.3 Methodology

Study site

Traditional pit sampling for earthworms and soil sampling for environmental DNA was performed at the University of Leeds Field Research Unit, a commercial mixed farm in West Yorkshire, United Kingdom (53° 52' 25.2 N 1° 19' 47.0" W). Sampling was done at the end of March/early April 2018 in broadly similar weather conditions (overcast with some sunny spells, around 7-12°C). Four arable fields were sampled, with each field containing two 70-metre-long x 3-metre-wide strips that had been temporarily converted to ley (Figure 4.1). These ley strips were planted as part of the SoilBioHedge project to examine the impact of ley conversion and hedgerows on arable soil quality, and had been established for around 33 months at the time of our sampling. The ley strips were seeded with grass and clover seed mixtures, whilst the surrounding arable fields were in a conventional arable rotation dominated by wheat. More details of the SoilBioHedge experimental setup can be found in Chapter 2.

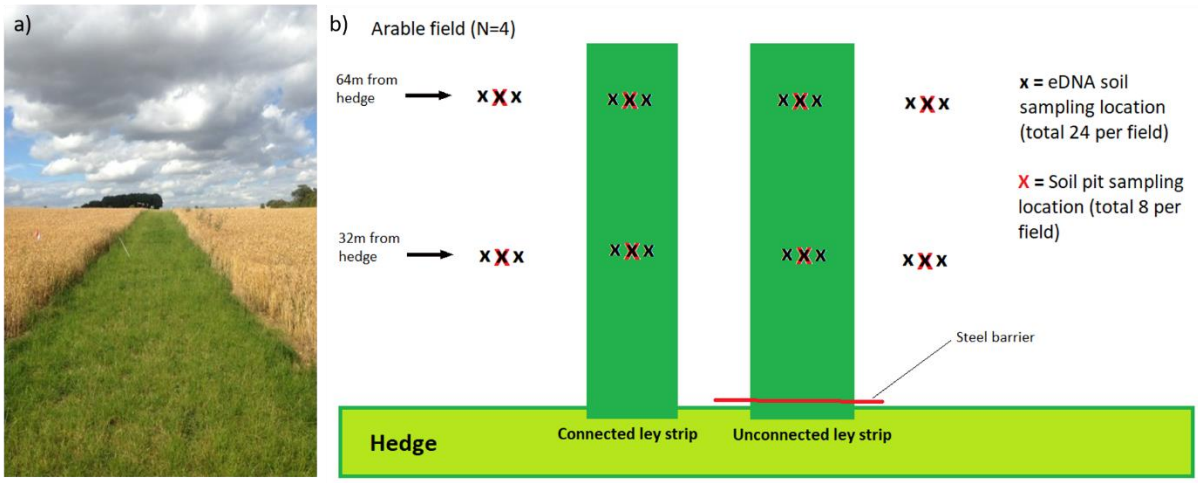

Figure 4.1. The study site and sampling protocol. A) one of the ley strips in an arable wheat field, b) the positions and numbers of samples taken per field, for both hand-sorting and eDNA sampling. This equated to a total of 32 pit samples for hand-sorting and 96 soil samples for eDNA analysis.

Traditional soil pit sampling

Pit excavation for earthworms and eDNA soil sampling was done concurrently, with a total of eight soil pits excavated per field (24 pits in total, Fig. 1). Four pits were dug in the ley strips and four in the arable field surrounding them, at distances of 32 and 64 metres from the field margin. Earthworm pits were dug by excavating an 18 x 18 x 15 cm block of soil and immediately placing it into a bucket with a sealable lid. Any earthworms visible at the base or sides of the excavated pit were also added to the bucket. After all earthworm pits and soil samples for eDNA analysis had been taken from a given field, the sealed buckets were transferred to a sorting site (either beside the field or in a nearby building) to be processed. The soil blocks were hand-sorted and all collected earthworms categorised into adults or juveniles before being preserved in 90% ethanol. Collected earthworms were then taken back to the University of Sheffield, where the preserved earthworms were kept refrigerated at 4°C prior to species identification. The numbers of preserved adults and juveniles per pit were counted and individual earthworm weights recorded. Adults were then identified to species level under a dissecting microscope using morphological characteristics (Sherlock, 2018).

Soil environmental DNA sampling

Alongside the pit excavation (and prior to the handling of earthworms) smaller soil cores were extracted for soil eDNA analysis. These cores measured approximately 5 x 5 x 15 cm and were excavated using a pallet knife and a small trowel. Two were taken from 2 m either side of the larger soil pit and one was extracted from its centre, resulting in three soil samples for eDNA analysis per one traditional earthworm sampling pit (totalling 24 eDNA soil samples per field, Fig. 1). The soil cores were given unique coded labels that identified which earthworm sampling pits they were associated with, so that the eDNA and traditional hand-sorting results could be directly compared later on. Each eDNA soil core was transferred to a transparent, sealable plastic sample bag and carefully broken up within it so that any stones, large pieces of plant matter, earthworms or cocoons could be removed. This process resulted in an average of ~175g of soil per eDNA sample. To prevent possible contamination, between each soil core extraction individual tools were cleaned and soaked in 10% bleach for at least 15 minutes before being used again. Disposable gloves were worn and changed between every core extraction. At the end of each day, all equipment was cleaned again and hand tools were soaked in 10% bleach overnight. Excavated eDNA soil samples were taken back to the University of Sheffield and stored at -20°C prior to eDNA extraction.

Earthworm environmental DNA extraction

After all field work, methodology testing and development had been completed (Chapter 3), eDNA extractions were performed using a modified protocol that had been adapted from Taberlet et al. (2012) and Taberlet et al. (2018). All molecular biology work was completed at the NERC Biomolecular Analysis Facility at the University of Sheffield. Soil samples were defrosted and thoroughly homogenised whilst still in sealed sample bags, by massaging the outside by hand. Using disposable plastic spoons and weigh boats to prevent cross contamination, 15 g of the homogenised soil was taken from the bag and transferred to a 50 ml falcon tube. This step was repeated for each soil sample, so that two extraction replicates were taken for every soil sample. Freshly prepared and autoclaved saturated phosphate buffer (Na_2HPO_4, pH 8) was added to the falcon tubes containing soil in a 1:1 ratio (i.e. 15ml of buffer added) using a bulb pipette. For every three soil samples (totalling six extraction replicates), a negative extraction control, which consisted of a falcon tube containing only phosphate buffer, was included. These negative extraction controls were subsequently treated in the same way as the falcon tubes containing soil and buffer. The falcon tubes were sealed, transferred to a tube rotator, and rotated for 15 minutes. After this, the tubes were centrifuged for 5 minutes at 4700 rpm and 400 µl of the resulting supernatant was pipetted off and mixed with buffer solution

from the Macherey-Nagel Nucleospin® Soil kits. Reagents and filter columns from the Nucleospin® Soil kits were used to complete the remaining extraction steps on a vacuum manifold, as fully outlined in Chapter 3. The resulting DNA extracts were diluted to 1:5 levels with lowTE buffer and stored in sealed tubes at -20°C prior to amplification. To avoid possible contamination, extractions were all performed in a pre-PCR room inside lamina flow cabinets, which were thoroughly cleaned before and after use with 10% bleach and exposed overnight to UV-C light. Disposable gloves were worn and changed between the handling of each sample and equipment such as weighing scales, tube racks, pipettes and rotator were wiped clean with 10% bleach and exposed to UV-C light overnight. Centrifuges were wiped clean with 10% bleach before and after each use.

Environmental DNA amplification, purification and sequencing

After preliminary experiments (see Chapter 3) two primer pairs were selected for use in this study, using primers developed and described in Bienert et al. (2012) that amplify short sequences of mitochondrial 16S rDNA. The two primer pairs consisted of primers 'ewD' paired with 'ewE', which are 17 bp and 21 bp in length respectively and amplify a region of ~70 bp; and primers 'ewB' paired with 'ewE', which are both 21 bp in length and amplify a region of ~120 bp. *In-silico* analysis of the primers was performed prior to selection, to check for appropriate primer binding and sufficient variation between earthworm species within the target region. This was performed using MEGA7 and R version 3.5.0 or later (Kumar et al., 2016; R Core Team, 2020), using sequences publicly available on GenBank® (Sayers et al., 2019) covering 24/29 of the native earthworms species. The remaining five UK earthworm species that did not have sequence data available and were therefore not included in the *in-silico* analysis were considered either rare or very rare and not likely to be found in agricultural soil habitats (Sherlock, 2012).

For the initial amplification stage (PCR1), 2 µl of the diluted DNA extracts were mixed with 1 µl each of forward and reverse primers, 10 µl of Qiagen Multiplex PCR Master Mix and 6 µl of molecular biology grade sterile water, leaving a reaction volume of 20 µl. All forward and reverse primers were tailed with Illumina sequencing primer sites (F 5'-3' TCTACACGTTCAGAGTTCTACAGTCCGACGATC; R 5'-3' GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT). The PCR1 mixtures were prepared inside lamina flow cabinets in a pre-PCR room and the reactions performed in individual 0.2 ml PCR tubes with sealable lids. In addition to the extraction negatives described earlier, two PCR negatives containing sterile water instead of extracted DNA were included for every batch of reactions. For each batch, 20% of the extracted DNA samples were replicated (i.e. two PCR replicates produced for 20% of the samples – a random number generator was used to determine which samples would be replicated) to

enable reliability and sensitivity checks. The PCR1 conditions consisted of an initial denaturation step at 95°C for 15 minutes, followed by 49 cycles of 94°C for 30 seconds, 57°C for 90 seconds and 72°C for 90 seconds, before ending with final step at 72°C for 10 minutes. After PCR1, 4 µl of each PCR product was run on a 1% agarose gel for 45 minutes at 110 volts and imaged under UV light to ensure amplification had occurred, and to check for any negative contamination. All PCR1 products were then frozen at -20°C before proceeding to the next stages.

After PCR1 had been completed for all samples, PCR extracts were purified and excess reagents removed by performing 0.5:1 and 1:1 magnetic bead cleans using AMPure XP beads. A second PCR step was then performed (PCR2), in order to add Illumina adapters and unique dual-indexed sequences, to enable samples to be identified after pooling and sequencing. PCR2 preparations were done in semi-skirted PCR plates on ice and consisted of making up a total volume of 20 µl with 1 µl each of Fi5 and Ri7 primers (at 2 µM), 8 µl of PCR1 product and 10 µl of Qiagen Multiplex PCR Master Mix. The samples were then incubated at 95°C for 15 minutes, followed by 12 cycles of 98°C for 10 seconds, 65°C for 30 seconds and 72°C for 30 seconds, with a single final step at 72°C for 5 minutes. A subset of samples was then selected and run on the TapeStation, with an observed increase in amplicon size between the pre- and post-PCR2 samples indicating successful addition of the identifying sequences.

The concentration of amplicons in each sample was then quantified using a fluorometer and the results used to pool equimolar amounts to a concentration of 40 ng/µl. Twenty-four pools (or 'libraries') were created for each primer pair, after which each library underwent a further 1:1 bead clean. Serial dilutions of the libraries were made prior to quantification with qPCR. On ice, 2 µl of the diluted sample pools were then mixed with 6 µl of KAPA SYBR® FAST master mix (including primers) and 2 µl of molecular biology grade sterile water, leaving a reaction volume of 10 µl. Negative controls and standards of known concentrations were included in each qPCR run. The qPCR conditions consisted of 5 minutes at 95°C followed by 35 cycles of 30 seconds at 95°C and 45 seconds at 60°C. After plotting the standard curve to check precision the qPCR results were used to calculate the concentrations of the libraries (in nM). Further equimolar pooling was then carried out of the libraries (initially to around 150 nM) resulting in one library pool for each primer pair. Vacuum concentration and resuspension in ddH_2O were used to get the final volume of these pools down to 20 µl, before both library pools were then gradually diluted with ddH_2O and bought down to a final concentration of 4 nM (measured using a Qubit fluorometer). The two library pools were then run on the TapeStation which revealed a small peak that indicated the presence of some lingering primer dimers, so size selection using the BluePippin system was performed according to the manufacturer's instructions. After a further 1:1 bead clean to remove unwanted reagents a repeated inspection of product size

peaks on the TapeStation showed the primer dimers had been successfully removed. The qPCR and dilution down to 4 nM steps were then repeated and 10 µl of each library pool was combined and taken for sequencing. Next generation Illumina MiSeq sequencing (using a MiSeq v2 2x 150bp run) was then carried out at Sheffield Children's Hospital.

Bioinformatics and statistical analyses

Returned sequences were put through a bioinformatics pipeline using the Sheffield Advanced Research Computer (ShARC) high performance computing system at the University of Sheffield. First, the quality of returned sequences was assessed and summary plots produced using the FastQC and MultiQC tools (Andrews, 2010; Ewels et al., 2016). Next, sequences underwent trimming using the Trimmomatic tool (Bolger et al., 2014) to remove the Illumina adapter sequences from the data and trim lower quality sequences. Reads were trimmed when the average Phred score dropped below 30 over a 4-base sliding window and reads below a minimum length threshold of 50 bp were discarded. The MultiQC plots were then generated again on the trimmed sequences to check that only high-quality data remained. The paired reads were aligned and converted to FASTA file format using the FLASH alignment tool (Magoč and Salzberg, 2011), with maximum overlap and proportion of mismatches set to 150 bp and 0.1 respectively. The primer sequences were trimmed from the remaining sequences using the 'trim.seqs' command in the mothur software (Schloss et al., 2009), which was also used to label the sequences with their corresponding primer pair groups. After demultiplexing the sequences and producing separate FASTA files for each primer pair, USEARCH v9.2 (Edgar, 2010) was used to dereplicate the sequences (by condensing identical sequences down to one sequence with a count of how many were present), remove chimeric sequences and cluster highly similar sequences with 97% identity or greater. Unique mOTUs were generated for each primer pair and blasted against the NCBI nucleotide database, using a quality filter to only take forward hits with a maximum e-value of 0.00001 and 95% percentage identity (for the ewD/ewE primer pair this was increased to 97% percentage identity). The results were visualised using MEGAN 6 (Huson et al., 2016) and tables containing sequence ID and assigned taxon name were produced. Presence/absence and sequence number by sample matrices were then produced for each primer pair to be used for further statistical analyses.

All statistical analyses were performed in R Studio using R version 4.0.1 (R Core Team, 2020). To investigate the effect of the different sampling methodologies and agricultural treatments on earthworm species richness, a linear model was fitted using the function 'lm' with sampling method, agricultural treatment and field ID as predictor variables and species richness as the response variable.

Model assumptions were checked and plotted using the 'autoplot' function in the ggplot2 graphics package (Wickham, 2011) and post-hoc Tukey Honest Significant Difference tests carried out using the 'TukeyHSD' function. Bar charts were also plotted using ggplot2 (Wickham, 2011). To further examine possible community composition differences across the different agricultural treatments and fields (and whether or not sampling methodology affected the picture observed), non-metric multidimensional scaling (NMDS) was performed on the relative abundances from each sampling method using the 'metaMDS' function in the vegan package (Oksanen et al., 2013). The Bray-Curtis dissimilarity index was applied, and the ordinations were plotted with the environmental data overlayed using the 'ordihull' and 'orditorp' vegan functions. Shephard plots and stress by dimensions plots were also produced to check the suitability of the ordinations. One outlier site was removed from the ewB/ewE analysis as it skewed the NMDS2 axis and was deemed unrepresentative, likely as a result of low sequence numbers. PERMANOVA tests were used to calculate the statistical significance of any potential differences in community dissimilarity brought about by the treatment groups, using the function 'adonis2' in vegan. To evaluate the sampling intensities of each method and to check that the diversity present had been appropriately captured, species accumulation curves were plotted for each method, with one curve for each treatment type, using the 'specaccum' function in vegan. To investigate how closely the relative abundances of species in a sample compared across the different methodologies, relative abundance scores per sample were plotted in stacked bar charts, overlayed with sequence numbers and abundances from traditional hand-sorting. Site occupancy proportions for each method were also calculated as potential proxies for overall species abundances.

4.4 Results

Traditional hand-sorting of earthworms from pits

A total of 718 earthworms were collected using the traditional hand-sorting method, 594 of which were juveniles (82.8% of the total numbers) and 124 were adults (17.2%). Morphological ID of the adults enabled 119 earthworms to be identified down to species level. Five earthworms could not be confidently identified due to damage inflicted during the sampling process. A total of eight species were identified, with *Allolobophora chlorotica* the most common (65 individuals), followed by *Aporrectodea rosea* (15) and *Lumbricus castaneus* (11; Table 4.1). Of the 119 adult earthworms morphologically identified to species level, 88 were found in the ley strips compared to 31 from the arable field samples.

Environmental DNA sampling

MiSeq sequencing yielded a total of 9,464,039 paired reads from the eDNA samples (including sequences from both primer pairs but not negatives and repeats). After the initial trimming based on sequence length and quality, 4,693,446 paired reads remained, with an additional 1,607,739 forward only and 837,293 reverse only reads surviving. The average percentage of reads dropped across all samples was 24.87%. The sequences then underwent further quality filtering and selection, including through the removal of chimeras and stringent BLAST criteria which reduced the sequence numbers further.

After all bioinformatics clean up and filtering steps had been completed, the total number of sequences classified to species level was 794,350 for ewB/ewE and 707,750 for ewD/ewE (not including the sequences obtained from negatives and repeats). For ewB/ewE, 464,287 were earthworm sequences and 330,063 were enchytraeid, compared with 542,974 earthworm and 164,776 enchytraeid sequences for ewD/ewE. Both eDNA primer pairs found a total of eight earthworm species, with ewB/ewE identifying an additional nine enchytraeid species compared with eight for ewD/ewE. Of the eight earthworm species identified by both methods, the species with the highest sequence numbers overall was *A. chlorotica* (with 303,375 and 292,637 sequences found by ewB/ewE and ewD/ewE, respectively), followed by *A. rosea* (57,521 and 96,958) and *Aporrectodea longa* (35,524 and 50,800; Table 4.1).

Sampling methods, agricultural treatment and earthworm diversity

Both eDNA primer pairs and the traditional hand-sorting method identified eight earthworm species overall, with no single method picking up species that were not also found in the others. However, the mean earthworm species richness per pit sample was significantly affected by the sampling method (ANOVA: $F = 12.79$, $df = 2, 87$, $p < 0.001$), with traditional hand-sorting yielding lower earthworm species richness than both the ewB/ewE (Tukey multiple comparison test, $p = 0.006$) and ewD/ewE ($p < 0.001$) eDNA methods (Figure 4.2). There was no significant difference in mean earthworm species richness between the two eDNA methods ($p = 0.17$). Across all sampling methods, the land use treatment was also found to significantly affect species richness (ANOVA: $F = 57.78$, $df = 1, 87$, $p < 0.001$), with higher mean species richness per pit seen in the ley treatments compared with the arable fields (Tukey multiple comparison test, for hand-sorting $p = 0.008$, for both eDNA methods $p < 0.001$; Figure 4.2). Location specific effects were also observed as field ID was found to affect earthworm species richness (ANOVA: $F = 5.65$, $df = 3, 87$, $p = 0.001$), which was driven by significantly higher mean

species richness in the 'Hillside' field compared with 'Big Sub-Station West' field (Tukey multiple comparison test, $p < 0.001$). There were no further significant differences in mean earthworm species richness between the remaining fields.

Table 4.1. The total numbers of individuals and sequences found for each identified earthworm species, and the proportion of pit samples that they were present in (i.e. site occupancy), using the different methodologies (HS = Hand-sorting).

Species	**No. hand-sorted individuals**	**Total no. sequences (ewB/ewE)**	**Total no. sequences (ewD/ewE)**	**% of pits present (HS)**	**% of pits present (ewB/ewE)**	**% of pits present (ewD/ewE)**
Allolobophora chlorotica	65	303,375	292,637	74.29	96.88	93.75
Aporrectodea rosea	15	57,521	96,958	34.38	37.50	50.00
Lumbricus castaneus	11	25,415	37,120	28.13	31.25	37.5
Aporrectodea caliginosa	9	14,789	28,665	9.38	21.88	18.75
Satchellius mammalis	9	7610	10,228	18.75	6.25	18.75
Aporrectodea longa	7	35,524	50,800	18.75	46.88	43.75
Lumbricus terrestris	2	11,962	16,423	6.25	37.50	53.13
Octolasion cyaneum	1	8090	10,137	3.13	6.25	12.50

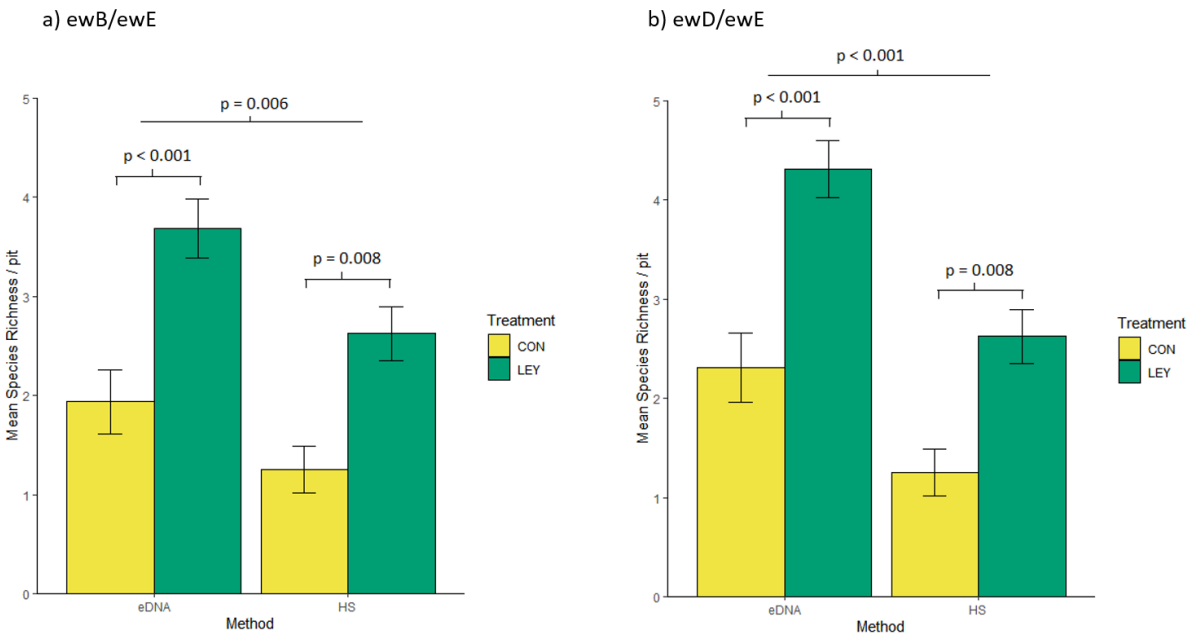

Figure 4.2. Mean earthworm species richness per pit (+/- standard error) between both eDNA primer pairs (a) ewB/ewE and b) ewD/ewE) and traditional hand-sorting (HS). Treatment refers to land use treatment ('con' = arable field control, 'ley' = ley strips) and p-values indicate significant differences between the methods and land use treatments.

The species accumulation curves shown in Figure 4.3 show that for all three methodologies a suitable number of samples were taken to capture the earthworm diversity present, and the levelling out of all the curves indicates that additional sampling was not likely to yield more species. For traditional hand-sorting, the arable control treatment curve levelled at four species, indicating that this sampling method did not find *O. cyaneum*, *S. mammalis*, *A. caliginosa* or *L. terrestris*. Similarly, the arable curve levelled out at six species for the ewB/ewE eDNA method as *O. cyaneum* and *S. mammalis* were not detected. In contrast, all eight species of earthworm are recorded in the arable control treatments using the ewD/ewE method, although it took more samples for the arable species accumulation curve to level out when compared with the ley treatment curve. This reflects the species richness results described earlier, as lower species richness in the arable control treatment means greater sampling effort is needed to find the same number of species.

Using Bray-Curtis dissimilarity calculations, NMDS ordinations successfully reached convergent solutions after 40 iterations for all three methods. With two-dimensional scaling, the reported stress levels were 0.130 for hand-sorting, 0.156 for ewB/ewE and 0.154 for ewD/ewE. Visual inspection of the NMDS ordinations suggest considerable overlap between communities belonging to both the arable control and ley treatments (Figure 4.4), indicating that communities in the ley treatment were

not clearly dissimilar from arable communities at this stage. This was supported by subsequent PERMANOVA tests, which did not find significant differentiation between the earthworm communities according to land use treatment (for hand-sorting pseudo-F = 1.37, p = 0.25; ewB/ewE pseudo-F = 1.33, p = 0.23; ewD/ewE pseudo-F = 2.21, p = 0.07). The land use treatment groups were also found to have homogenous dispersions that did not differ significantly (betadisper tests, for hand-sorting p = 0.59, ewB/ewE p = 0.29, ewD/ewE p = 0.33). There was some distinction between communities as a result of field ID observed in the hand-sorting method (PERMANOVA; pseudo-F = 2.35, p = 0.02), although this was not observed by the two eDNA methods (ewB/ewE pseudo-F = 1.32, p = 0.21; ewD/ewE pseudo-F = 1.32, p = 0.19; see Figures S1-S3 for NMDS plots overlayed with field ID groups).

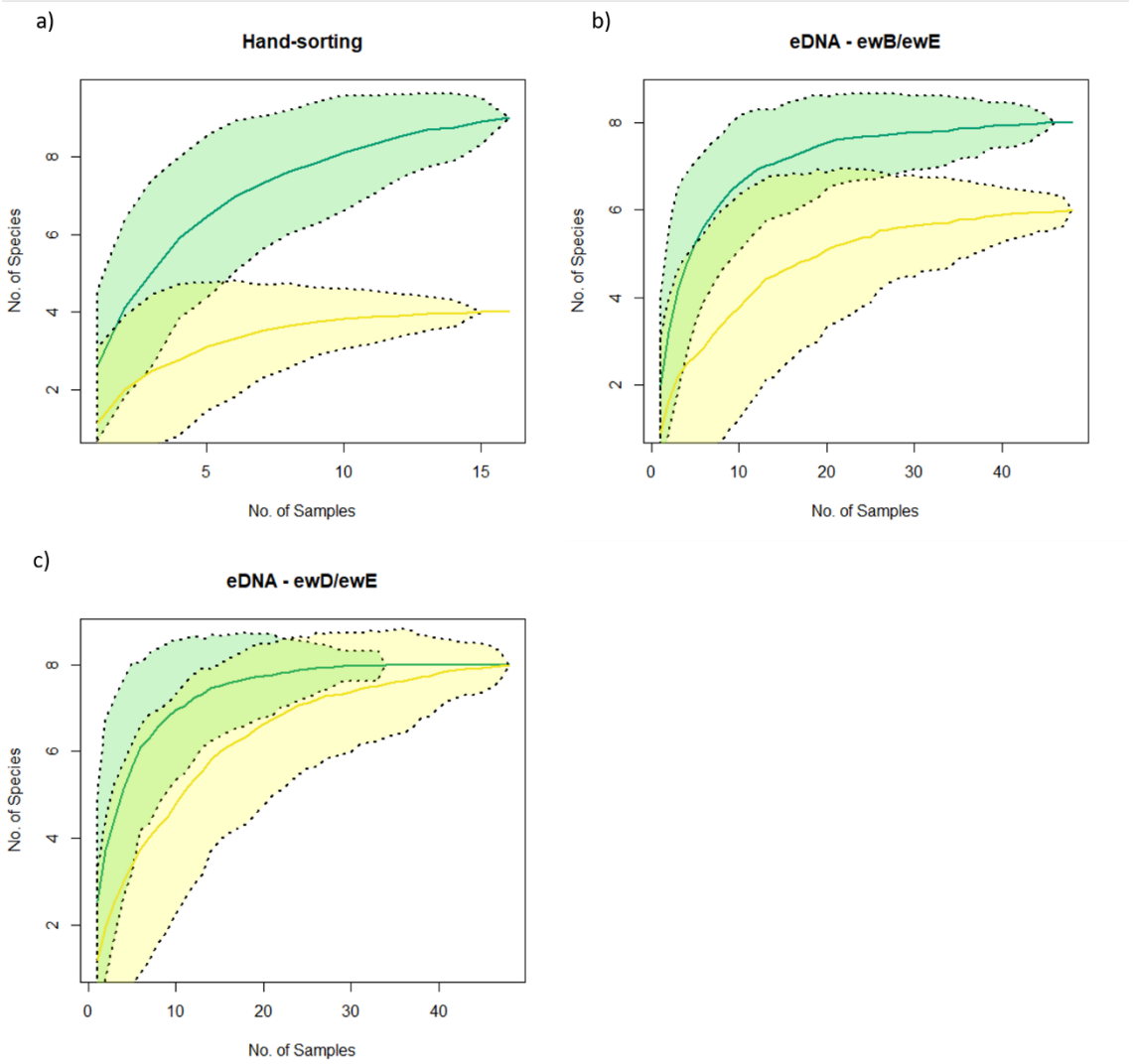

Figure 4.3. Species accumulation curves for a) hand-sorting, b) eDNA sampling using ewB/ewE primers c) eDNA sampling using ewD/ewE primers. Green lines represent curves for the ley treatment and yellow lines are for the arable treatment. Coloured areas around the lines with the dotted line borders indicate confidence intervals.

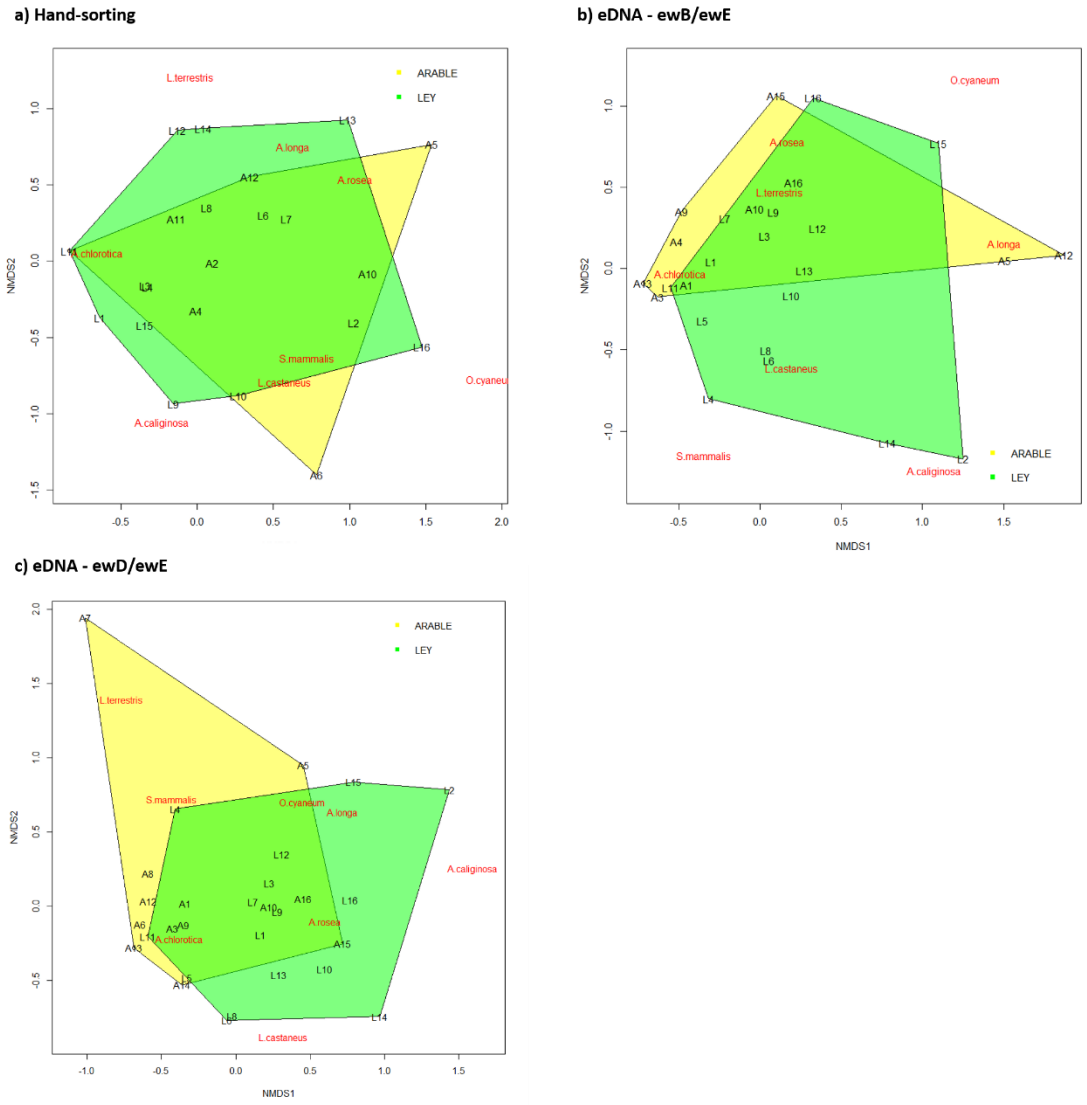

Figure 4.4. NMDS ordinations for a) hand-sorting, b) eDNA sampling using ewB/ewE primers c) eDNA sampling using ewD/ewE primers. The site scores in ordination space are represented by the site labels (arable sites = 'A1', 'A2' etc., ley sites = 'L1', 'L2' etc.), and the species labels are positioned at the weighted average of the site scores. The polygons connect the vertices of points made by the communities in the ley (green polygons) and arable (yellow) treatments.

Comparison of earthworm relative abundances and site occupancy proportions

Calculating the relative abundances of species per sample revealed substantial within-sample variation when using the different methodologies. This can be seen in Figure 4.5, which compares the relative abundances generated by the ewB/ewE eDNA method with those from hand-sorting. ewD/ewE comparisons with hand-sorting gave the same outcome. Plotting the relative abundances in this way does show the wider diversity patterns between the arable and ley treatments described

earlier, but the variations in reported relative abundances at the sample level show that specific abundance estimations should not be inferred. At the broader scale, looking at overall site occupancy (the proportion of sites in which a given species has been found) showed slightly better consistency between the three methodologies (Table 4.1), but even at this broader scale variation can be seen.

Figure 4.5. Stacked bar charts comparing the relative abundance of species within samples, as measured by the ewB/ewE eDNA method and hand-sorting for the a) arable and b) ley samples. Species relative abundances are marked by the different coloured bars and labelled with the actual sequence numbers and hand-sorting abundances.

4.5 Discussion

The results of this study show that environmental DNA sampling can be an effective tool for monitoring earthworm populations in agricultural ecosystems. For measuring earthworm species richness, both eDNA primer pairs performed favourably when compared with the traditional hand-sorting method and identified more species per pit sample on average. They revealed the same pattern of increased species richness in soils under ley management as was observed using the traditional hand-sorting method, thus highlighting the beneficial effects of conversion to ley with regards to increasing earthworm diversity. The same eight earthworm species were found across all the methodologies, indicating consistent results and providing confidence that no species were missed. The species accumulation curves indicated that the level of pit sampling for both traditional hand-sorting and eDNA soil sampling was appropriate for this study system, whilst the NMDS ordinations produced for all methodologies revealed that the earthworm communities in the ley and arable treatments had not yet become clearly distinct from one another, despite the ley treatment samples having consistently higher earthworm diversity. When looking at earthworm relative abundances and the site occupancy abundance proxy across the different methodologies, both eDNA methods were found to be consistent with each other but considerable variation was seen at the sample level when compared with the hand-sorting relative abundances.

Firstly, the results clearly show a positive increase in average earthworm species diversity in the samples that had been converted from arable to ley management. These findings lend support to the hypothesis that conversion to ley can restore earthworm populations in arable fields that have been intensively managed, corroborating the evidence put forward by Berdeni et al. (2021) and Postma-Blaauw et al. (2012). It is interesting that despite the higher species richness in the ley strips, the communities were not found to be distinct when analysed through NMDS and PERMANOVA. Visual inspection of the ordinations for the two eDNA methods may indicate some slight clustering of ley samples towards the bottom and bottom-right of the ordinations (Figure 4.4), but as there is sufficient overlap with samples in the arable treatment the groups are deemed not to be statistically different. This could suggest that the ley strip earthworm communities are transitioning away from the arable communities, but the conversion to ley is too recent (33 months) to show significant distinctions between them. The fact that the earthworm populations in the ley strips will have been seeded by the arable field populations (as the strips were directly sown within the arable fields) and the close proximity of the two treatments may also account for the similarities. In addition, some spill over of earthworms between the two treatment groups cannot be ruled out, especially given the estimates

of active dispersal rates that are reported in the literature for the species found here (for example Eijsackers, 2011; Butt et al., 2004; Marinissen and van den Bosch, 1992).

With regards to the performance of the different sampling methods, when compared with each other both eDNA primer pairs gave consistent results and captured similar pictures of earthworm diversity, so both could prove useful in wider scale monitoring programmes. As predicted by Bienert et al. (2012), ewB/ewE provided better species resolution but amplified a greater proportion of *Enchytraeidae* sequences. Although we chose to focus solely on earthworms in this study, enchytraeids also offer important contributions to soils and can be good indicators of soil health (Koutika et al., 2001; Marinissen and Didden, 1997; Pelosi and Römbke, 2016), so future surveyors employing eDNA sampling may wish to utilise the ewB/ewE primers in order to include them. However, caution would be needed as they may be less well represented in the BLAST database than earthworms. For surveys looking solely at earthworms the ewD/ewE primers are recommended, as these amplified more earthworm sequences overall.

Both eDNA primer pairs performed well in comparison to traditional hand-sorting, identifying more species on average across all the different treatment groups and field sites. This may be because eDNA sampling is not constrained by some of the limitations of hand-sorting mentioned earlier, particularly the exclusion of juvenile worms, which cannot be identified to species level morphologically. Given that the majority of earthworms collected through hand-sorting were juveniles (almost 83% of the total), this represents a significant potential reservoir of diversity that is excluded from the hand-sorting results. Another potential drawback that may explain the lower species diversity seen through traditional hand-sorting may be the issue of worms fleeing the pit area as the soil block is being excavated. Earthworms are sensitive to vibration and are known to retreat deeper into their soil burrows when disturbed, which can be an issue particularly when sampling larger vertical burrowing anecic worms whose burrows can extend well below the depth of the excavated pits (Pelosi et al., 2009; Singh et al., 2016). The site occupancy percentages reflect this, showing that the eDNA methods were markedly better at detecting the two larger anecic species *A. longa* and *L. terrestris* (for *A.longa*, site occupancy measured by hand-sorting was 18.8% compared with 46.9% and 43.8% for the eDNA methods; for *L. terrestris* hand-sorting site occupancy was just 6.3% compared with 37.5% and 53.1% for eDNA). It is worth noting that hand-sorting is often combined with the use of a chemical expellant like AITC or formaldehyde, which can improve the detection rate of anecic earthworms (for example Crittenden et al., 2015 and Holden et al., 2019). Employing this combination approach could have bought more fleeing earthworms back to the soil surface and improved anecic detection for the hand-sorting method, but this was deemed unfeasible in this study due to time and labour constraints.

The only species that was found to have equal or slightly lower site occupancy percentages when measured using eDNA compared with hand-sorting was the epigeic worm *S. mammalis* (6.3% and 18.8% for the eDNA primer pairs compared with 18.8% for hand-sorting). Given that *S. mammalis* was found by all methodologies to be relatively rare at the study site, this may simply be due to random variation or the heterogenous spatial distribution of earthworm populations in soils (Valckx et al., 2011). However, it is worth noting as Bienert et. al (2012) also reported underrepresentation of epigeic species by eDNA sampling. They attributed this to the limited number of samples taken and to not taking the first few centimetres of soil where epigeic earthworms would be most active. However, although our study was not limited by these factors, we still observed the same outcome, which may indicate other possible causes. It has been shown previously that numerous factors can affect eDNA stabilisation and degradation rates in soils, including moisture levels, pH, temperature, microbial activity, soil type and chemistry (Barnes and Turner, 2016; Harrison et al., 2019; Pietramellara et al., 2009; Sirois and Buckley, 2019). Given that many of these attributes also vary with soil depth, it is possible that the surface soil may be experiencing different conditions that increase eDNA degradation rates compared with the deeper soil, which could mean that surface-dwelling species are less represented in the final survey. The fact that it was only the longer ewB/ewE primer pair that reported lower site occupancy may support this, as we would expect longer DNA fragments to be more susceptible to degradation. Future studies may shed further light on whether the lower detection of epigeic species here is due to a random phenomenon or a result of wider eDNA sampling bias, but more research is needed on how eDNA stabilisation and degradation rates may be affected by soil depth. This is especially needed given the potential role of earthworm-mediated bioturbation in the transport of DNA molecules throughout the soil profile, as demonstrated by Prosser and Hedgpeth (2018).

Related to the issue of eDNA degradation, the capacity for eDNA to persist in the environment over time has led to ongoing debate over the extent to which eDNA sampling is measuring current or past populations (Sirois and Buckley, 2019; Thomsen and Willerslev, 2015). Our study shows clear differences in soil eDNA profiles after a few years under different management treatments, demonstrating similar patterns revealed by traditional sampling techniques. This shows that eDNA sampling can be sensitive enough to pick up fine-scale differences in earthworm communities in a relatively short time period, in spite of any 'background' eDNA that may be present. However, we cannot definitively rule out that some of the sequences in our totals may originate from background eDNA deposited by earthworms that were no longer present. It is possible, for example, that for the ewD/ewE primer pair the small numbers of sequences of *S. mammalis* and *O.cyaneum* in the arable samples may be due to eDNA from past populations, as these species were not picked up by hand-

sorting or the longer ewB/ewE primer pair (which would not have picked up shorter, more degraded eDNA fragments). This is just one potential explanation for the presence of these species in the arable controls and it is unlikely that any legacy eDNA present would significantly change the results in this case, but future research focused on the degradation rate of eDNA in soils and ways to account for background effects is essential (Thomsen and Willerslev, 2015). A recent study by Marshall et al. (2021) offers a useful starting point for this, which describes a method of estimating the age of eDNA based on accompanying eRNA sampling and analysis of the eDNA:eRNA ratio. Utilising techniques like this may prove useful if agricultural soil eDNA surveys become more widespread, as they would allow the surveyor to account for variations in eDNA degradation rates that may be bought about by different agricultural practises (Foucher et al., 2020; Sirois and Buckley, 2019).

Sampling intensity and effort is another important aspect to consider if earthworm eDNA sampling is to be taken up for use in more widespread soil monitoring schemes (Dickie et al., 2018). The species accumulation curves indicated that an appropriate level of sampling was carried out for this study system, but for accurate sampling of larger sites and whole farms, more samples are probably needed. For developing a standardised sampling regime, future soil health monitoring programmes should conduct further investigations across multiple sites and calculate appropriate eDNA soil sample numbers per unit area, similar to the work of Valckx et al. (2011) for hand-sorting and chemical extraction. Surveys should also calculate accompanying species accumulation curves to check for appropriate coverage, as performed in this study. With regards to sampling effort, the collection of soil samples for eDNA analysis was less time and labour intensive in the field when compared with hand-sorting, but this was traded off against more time spent in the lab preparing the samples for sequencing and subsequent bioinformatics. Future streamlining could be achieved which could further reduce the sampling effort needed, including through the use of faster decontamination techniques in the field (for example Foucher et al., 2020) or the utilisation of labs with dedicated eDNA facilities and protocols in place.

Achieving a reliable indicator of species abundances is a key challenge that remains for soil eDNA sampling. As well as diversity, earthworm abundance is very important for agricultural soil health as it affects soil functioning, how quickly earthworms can improve degraded soils and the rate at which they spread to new areas (Bertrand et al., 2015; Capowiez et al., 2014; Mathieu et al., 2010; Schon et al., 2017). For our study, inferring species abundance from the eDNA sequence numbers is problematic. Raw sequence numbers in this case should not be used as a proxy for abundance given that their proportions can be distorted during the PCR amplification, pooling and sequencing processes (Fonseca, 2018; Kebschull and Zador, 2015; Pinto and Raskin, 2012). A common solution in metabarcoding studies is to use relative abundance data instead, but this is also not without criticism

(Jian et al., 2020; Lovell et al., 2015; Pinto and Raskin, 2012). Our results suggest that caution should be taken when trying to infer actual abundance patterns from soil eDNA sequence numbers and relative abundance data, due to high within-site variability between the eDNA measures and hand-sorting abundances. Looking at site occupancy proportions has been suggested as a suitable alternative proxy for abundance in eDNA studies (Hänfling et al., 2016), which is slightly more consistent with the hand-sorting abundances obtained. However, this only gives an overall abundance estimate for each species across the whole sample area, and does not give any information on abundances within and between samples. Progress is being made in developing solutions that will allow more rigorous estimation of abundances from eDNA sampling, including through the use of new statistical techniques and quantitative PCR (Lovell et al., 2015; Spear et al., 2021; Yates et al., 2019). Utilising alternative techniques like these may help future surveys to obtain more reliable earthworm abundance estimates, but more development is needed before these can be widely adopted.

4.6 Conclusions

Environmental DNA sampling represents a promising alternative to the conventional ways of measuring earthworm diversity that could help to increase standardisation, reduce time spent in the field and tackle some of the existing sampling biases. Our results show that eDNA can be used to sample active agricultural fields and detect fine scale changes in earthworm diversity bought about by different management practises, making it a good candidate for use in wider soil health monitoring programmes. Future research could help to optimise and refine the technique further, by addressing some of the key unanswered questions surrounding eDNA deposition and degradation in soils, the contribution of legacy eDNA and obtaining reliable abundance information from sequence data. Based on the results of this study and the previous work described here, it is anticipated that eDNA sampling could become an important tool for monitoring earthworm diversity and soil health in agriculture, just as it has become a vital instrument for monitoring marine and aquatic habitats.

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4.8 Supplementary materials

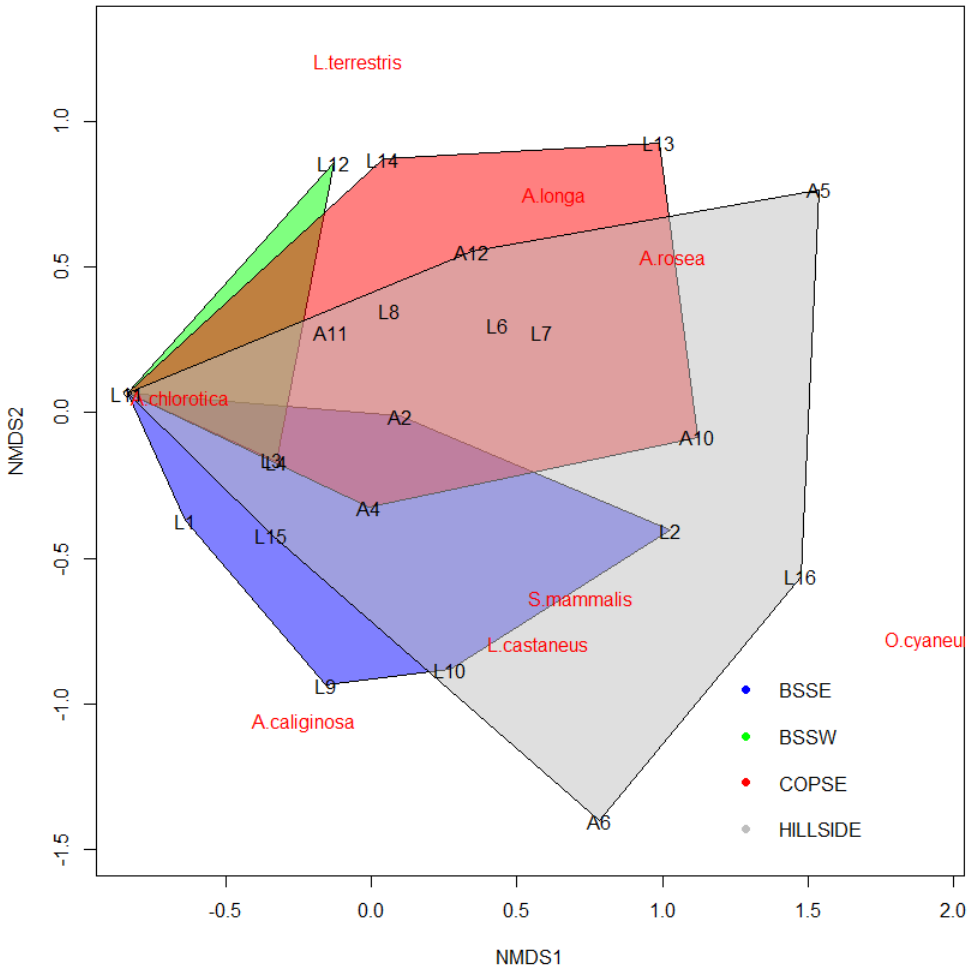

Figure S1. NMDS ordination for the results of hand-sorting overlayed by polygons connecting the vertices of points made by the communities in the different fields. The site scores in ordination space are represented by the site labels (arable sites = 'A1', 'A2' etc., ley sites = 'L1', 'L2' etc.), and the species labels are positioned at the weighted average of the site scores.

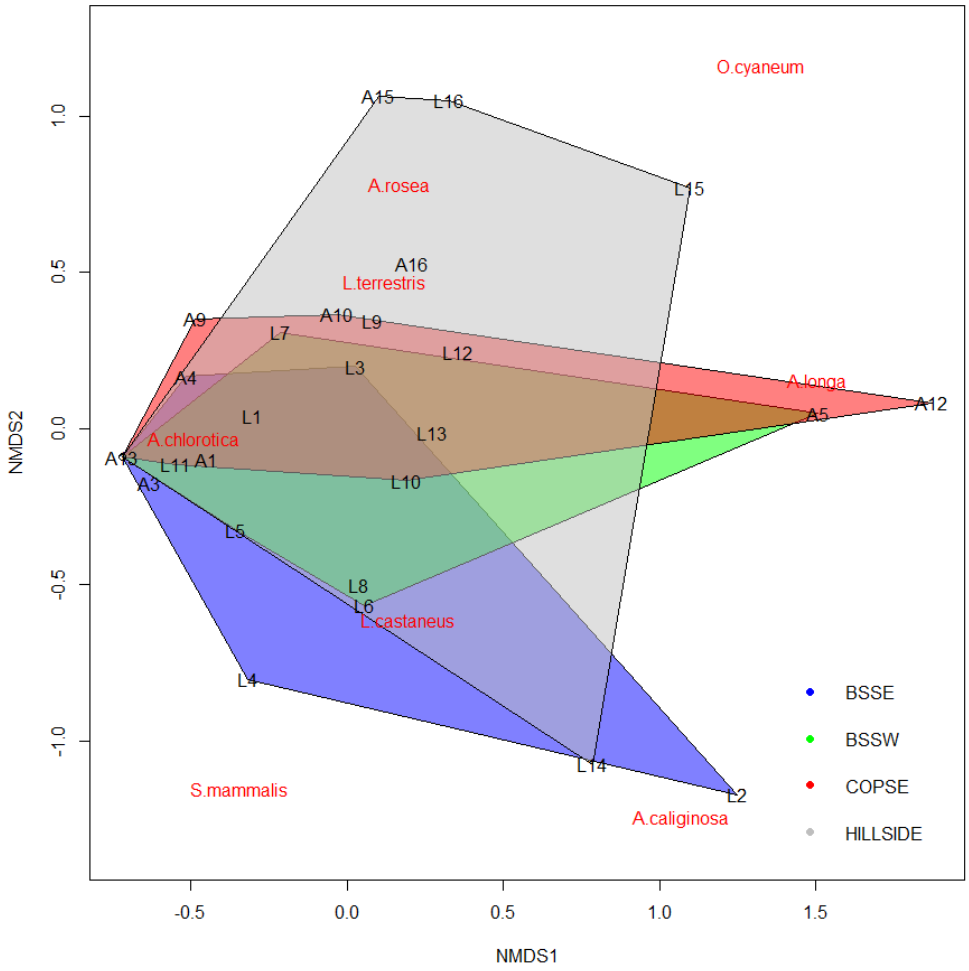

Figure S2. NMDS ordination for the results of eDNA sampling using ewB/ewE primers, overlayed by polygons connecting the vertices of points made by the communities in the different fields. The site scores in ordination space are represented by the site labels (arable sites = 'A1', 'A2' etc., ley sites = 'L1', 'L2' etc.), and the species labels are positioned at the weighted average of the site scores.

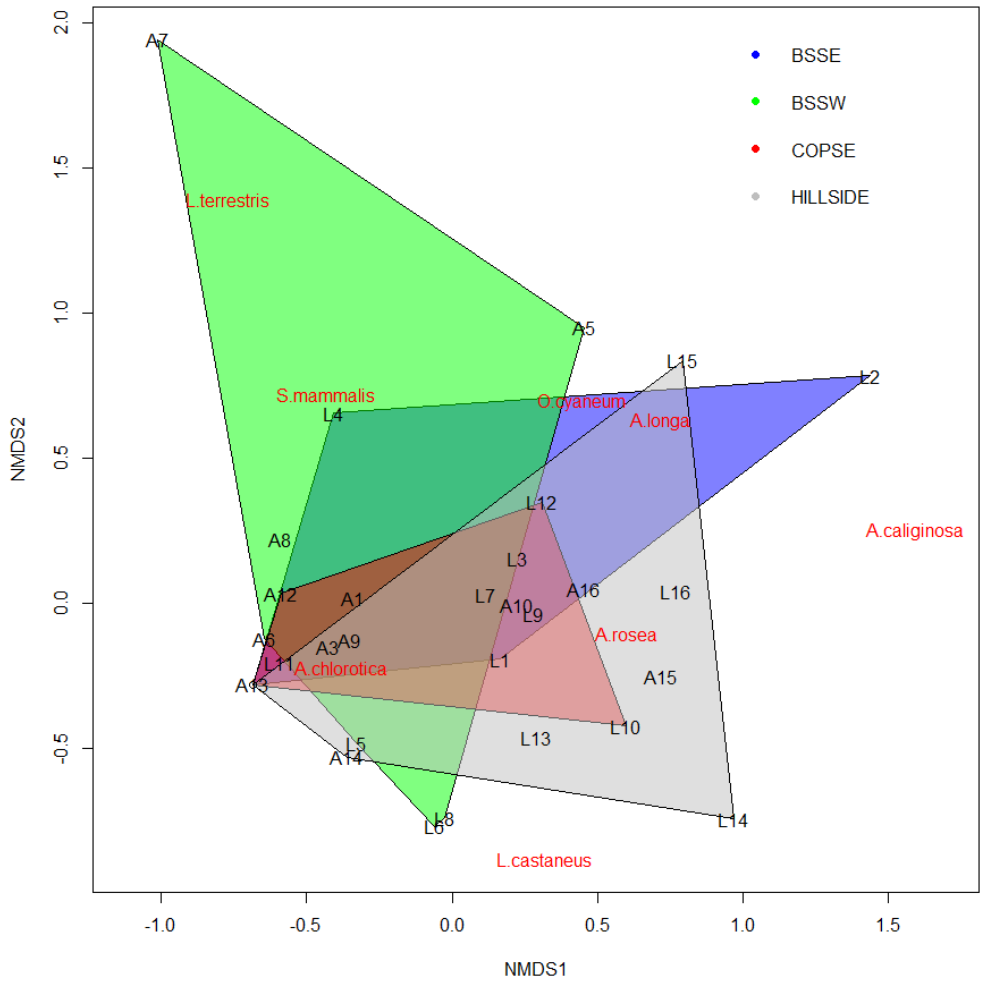

Figure S3. NMDS ordination for the results of eDNA sampling using ewD/ewE primers, overlayed by polygons connecting the vertices of points made by the communities in the different fields. The site scores in ordination space are represented by the site labels (arable sites = 'A1', 'A2' etc., ley sites = 'L1', 'L2' etc.), and the species labels are positioned at the weighted average of the site scores.

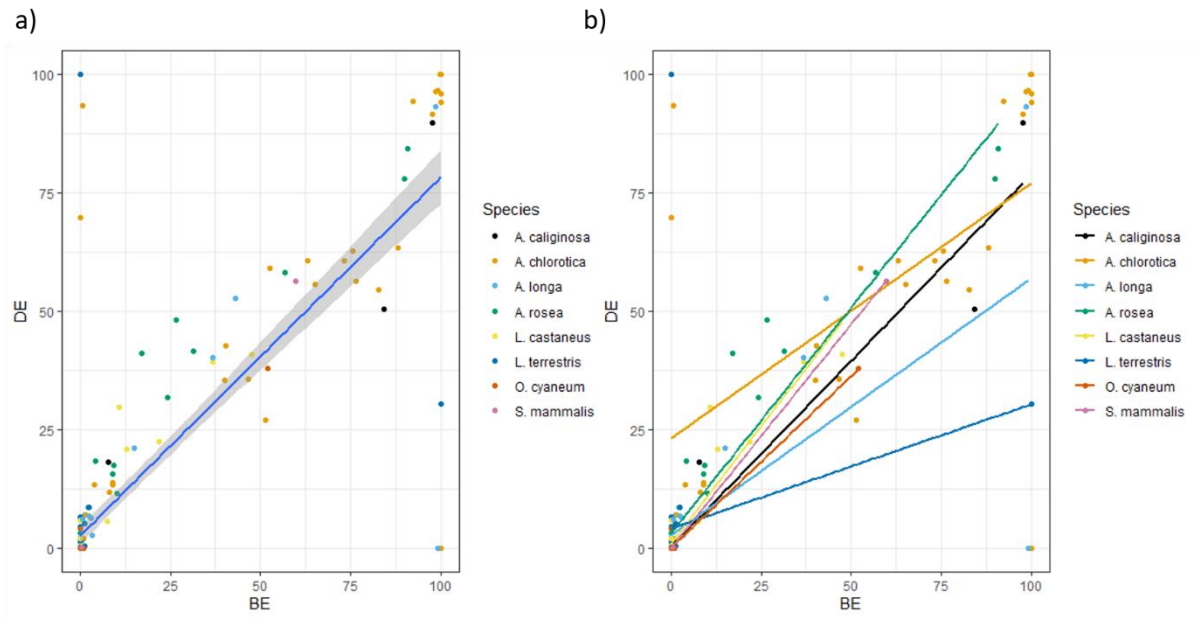

Figure S4. Comparing the relative abundances of different species in each sample reported by the ewB/ewE and ewD/ewE primer pairs. A) gives the overall trend with all species grouped together while b) shows the separate trends for each species. 'DE' = ewD/ewE and 'BE' = ewB/ewE.

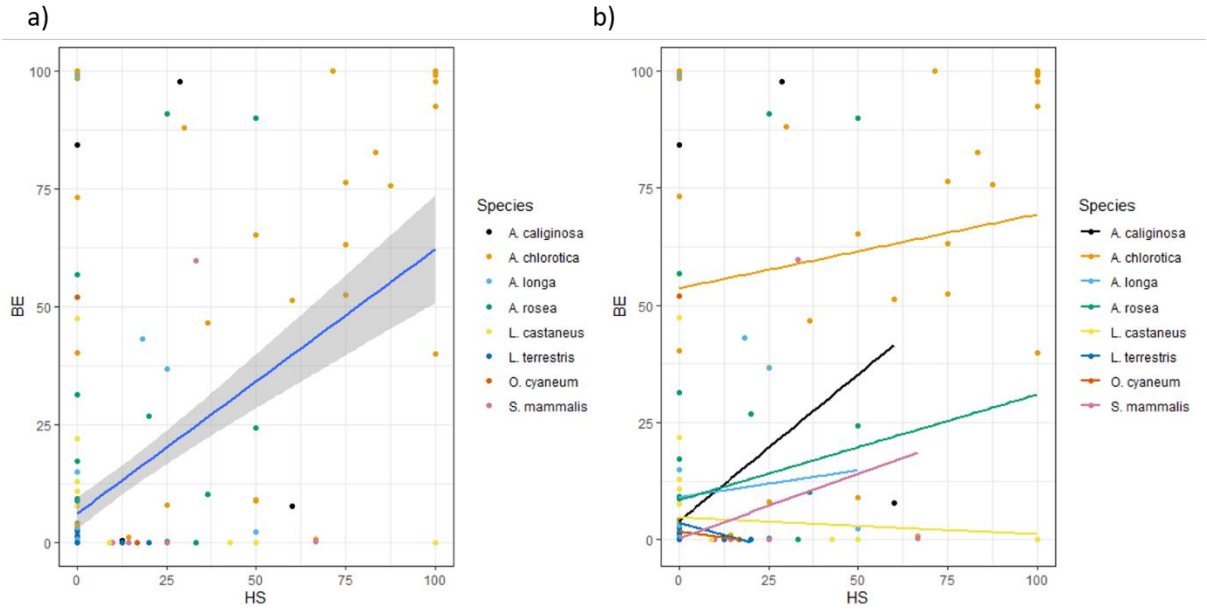

Figure S5. Comparing the relative abundances of different species in each sample reported by the ewB/ewE eDNA primer pair and hand-sorting. A) gives the overall trend with all species grouped together while b) shows the separate trends for each species. 'BE' = ewB/ewE and 'HS' = hand-sorting.

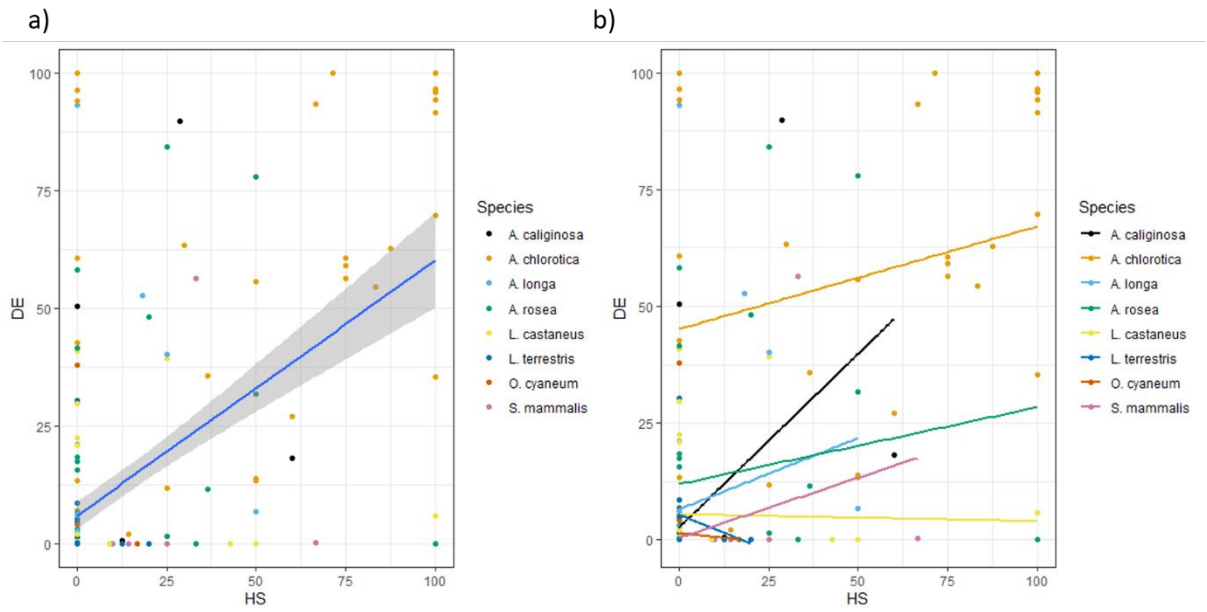

Figure S6. Comparing the relative abundances of different species in each sample reported by the ewD/ewE eDNA primer pair and hand-sorting. A) gives the overall trend with all species grouped together while b) shows the separate trends for each species. 'DE' = ewD/ewE and 'HS' = hand-sorting.

Chapter 5

Labile carbon, land use and earthworms: Investigating the associations between two important soil health indicators

5.1 Abstract

Declines in soil organic carbon (SOC) are being seen across many parts of the globe, due to a combination of factors including land use change, intensive agricultural practises and climate change. Finding ways to manage agricultural soils that can maintain and restore SOC levels is therefore a priority, but it can take a long time for changes in the SOC pool to be observed using conventional measurements. Measuring labile carbon, which is the active portion of SOC that has much shorter turnover times, has been proposed as an alternative indicator that could be useful for investigating responses in the carbon pool to recent land use change. Research has also shown interactions between labile carbon and earthworms that could have important repercussions for soil processes, so investigating potential relationships between them is important for understanding their wider contributions to agricultural soil health. Here, the labile carbon concentrations of soils taken from arable fields and temporary leys were measured through oxidation with potassium permanganate, to investigate how recent conversion to ley affected this carbon pool. The labile carbon levels were then correlated with earthworm species richness, abundance and biomass, to check for possible associations between them. Temporary conversion to ley was found to significantly increase labile carbon levels in under three years, and further differences between fields were also seen that may indicate lasting effects of previous management histories. Significant correlations were initially seen between labile carbon concentrations and earthworm population variables, but these disappeared when accounting for land use affects and were likely due to both labile carbon concentrations and earthworms responding positively to the conversion to ley. It was concluded that measuring labile carbon concentrations was an effective way to monitor short-term changes in soil carbon levels bought about by conversion to ley, that if maintained would likely lead to longer-term increases in the total SOC pool.

5.2 Introduction

Soil carbon is an essential resource that plays a fundamental role in soil functioning, food production and carbon sequestration (Jackson et al., 2017; Lal, 2004; Ostle et al., 2009). The soil carbon pool is the largest terrestrial carbon store and estimated to contain more carbon than both the biosphere and atmosphere combined (Lal, 2004), and is second only to the oceans in terms of quantity of carbon stored (Stockmann et al., 2013). The majority of carbon in the soil is categorised as Soil Organic Carbon (SOC), making up an estimated 1500 gigatons globally (Scharlemann et al., 2014). Declines in SOC levels across the globe, which are linked with land use change, soil degradation as a result of intensive agricultural practises and climate change (Lal et al., 2004; Muñoz-Rojas et al., 2017; Smith, 2008), highlight the need for continued soil carbon monitoring and a better understanding of the soil processes and mechanisms through which it acts. Traditionally, SOC has been measured using a variety of techniques, with some of the most well-established including loss on ignition, wet oxidation and the employment of dry combustion analysers (Hoogsteen et al., 2015; Mingorance et al., 2007; Wang and Anderson, 1998).

Despite the widespread use of these well-established SOC measures, researchers are increasingly recognising that taking measures of total SOC alone can lack sensitivity when assessing short-term changes in soil carbon, and presents an oversimplified picture of the carbon dynamics at play (Abdelrahman et al., 2020; Shen et al., 2021; Weil et al., 2003). This is because SOC can be further broken down into different functional pools, which can interact with other soil components in different ways and act over different time scales (Sherrod et al., 2005). For example, the non-labile 'passive' carbon pool makes up the bulk of total SOC and is highly recalcitrant, resistant to microbial decomposition and has very long turnover rates of decades to millennia (Mills et al., 2014). The smaller labile carbon pool, also referred to as the 'active' pool, is much more readily available to soil microbes and typically has a relatively short turnover time of weeks to years (Budge et al., 2011; Zou et al., 2005). Due to the larger size of the non-labile passive pool, focusing on total SOC can therefore hide important changes in the labile carbon fractions that may occur under much shorter time frames (Geraei et al., 2016; Malobane et al., 2020).

For agriculture, measuring the concentration of soil labile carbon separately from total SOC has been proposed as a potential valuable indicator of soil quality, given its sensitivity to changes in land use management and the important role that it plays in the soil food web (Zhang et al., 2020). Made up of a variety of materials that can be readily decomposed by microorganisms, including particulate organic matter from plants, soil carbohydrates, phenolic compounds and microbial biomass carbon (Abdelrahman et al., 2017; Saviozzi et al., 1999; Six et al., 2001; Zhang et al., 2020), many studies have

demonstrated the central role that labile fractions play in stimulating the soil microbial community. For example, increased labile carbon levels associated with changes in land use management and organic matter inputs have been shown to enhance microbial diversity, biomass and activity (Berthrong et al., 2013; Fang et al., 2020; Ng et al., 2014), which in turn lead to increases in carbon mineralisation and agricultural productivity (Garcia-Pausas and Paterson, 2011; Tautges et al., 2016). However, there has been less focus on the relationships between the labile carbon pool and other components of soil biodiversity, including macrofauna such as earthworms.

Numerous studies have previously shown significant effects of earthworms on total SOC, but their role in the carbon cycle is still considered under debate (Lemtiri et al., 2014; Lubbers et al., 2017). The effects of earthworms on soil organic carbon dynamics are often shown to be very context specific, leading to the conclusion that earthworms can both sequester and release carbon from soils, depending on the circumstances (de Graaff et al., 2015; Lubbers et al., 2013; Zhang et al., 2013). Furthering our understanding of earthworm-carbon dynamics could therefore be beneficial not only for maximising agricultural productivity, but also for helping to develop more effective climate change mitigation strategies. This requires taking a closer look into the interactions between earthworms and labile carbon in particular, since recent studies have suggested that there can be important interactions between the two. For example, Vidal et al., (2019) demonstrated that earthworms were more able to assimilate labile carbon derived from plant shoots as opposed to roots, but in the longer-term facilitated the microbial degradation of both. Another study by Angst et al. (2019) found that earthworms had minimal effects on overall SOC content, but they did act as 'biochemical reactors' and altered the molecular composition of labile carbon through their feeding behaviour, which significantly increased the resilience of soil carbon stocks to disturbance. Therefore, given the potential importance of earthworm-labile carbon interactions for soil processes, investigating potential relationships between them is important for enhancing our understanding of how they both contribute to wider agricultural soil health.

In this experiment, the labile carbon concentrations of soil samples previously used for eDNA analysis were measured through oxidisation with a potassium permanganate solution. Although a range of methodologies for measuring labile carbon concentrations have been developed (see for example McLauchlan and Hobbie, 2004; and Zakharova et al., 2014), oxidation with potassium permanganate was chosen as it is uses safe and readily available laboratory reagents, has been shown to associate well with other physical and biological indicators of soil health, and has been suggested as a sensitive indicator of potential fluctuations in soil carbon brought about by land use change (Bongiorno et al., 2019; Culman et al., 2012; Weil et al, 2003). The soil samples utilised for this study had been collected from agricultural fields that were being managed either as conventional arable fields or temporary

grass-clover leys, and earthworm population variables had been measured previously using eDNA and traditional hand-sorting in Chapter 4. The labile carbon concentrations of the different land use types were compared and correlations with earthworm population variables performed. The following questions were addressed: 1) Is labile carbon concentration an effective indicator of short-term changes in soil carbon stocks resulting from land use change? 2) Does temporary conversion to ley lead to a change in soil labile carbon concentrations? 3) Are clear relationships between labile carbon and earthworm populations observed? The answers to these questions are subsequently discussed with regards to their implications for monitoring and restoring agricultural soil health, and suggestions made for further investigation.

5.3 Methodology

Study site and soil sampling

The soil samples used in this labile carbon experiment were the same as those used for earthworm eDNA analysis in Chapter 4. Samples were collected from four fields at the University of Leeds Field Research Unit, which were referred to as 'Big Substation East', 'Big Substation West', 'Copse' and 'Hillside'. These fields had all undergone continuous annual cultivation since at least 2008 (Figure 5.1), but in 2015 each had a pair of 70-metre-long x 3-metre-wide grass-clover ley strips sown into them as part of the SoilBioHedge experiment. The goal of this experiment was to investigate the effects of temporary arable to ley conversion on a range of different physical, chemical and biological soil quality indicators.

In 2018, around 33 months after the ley strips had been established, a total of 96 soil cores were extracted from the four fields for eDNA and labile carbon analysis (24 per field, 12 from the ley strips and 12 from the surrounding arable field; Figure 5.2). The 5 x 5 cm cores were excavated using a pallet knife and small trowel to a depth of 15 cm, resulting in an average weight of around 175 g of soil per sample. These soil cores were placed in sealed plastic bags and taken back to the University of Sheffield, where they were frozen at -20°C prior to earthworm eDNA extraction. Alongside the soil cores taken for eDNA/labile carbon analysis, larger soil pits were also excavated in the same locations and hand-sorted for earthworms, which were counted and weighed to obtain measures of earthworm abundance, total biomass and average individual biomass. A total of 32 soil pits were excavated (8 per field). This meant that for each pit, there were three corresponding soil cores taken from that location

to be used for eDNA and labile carbon analysis. A full description of the soil sampling strategy can be found in Chapter 4.3.

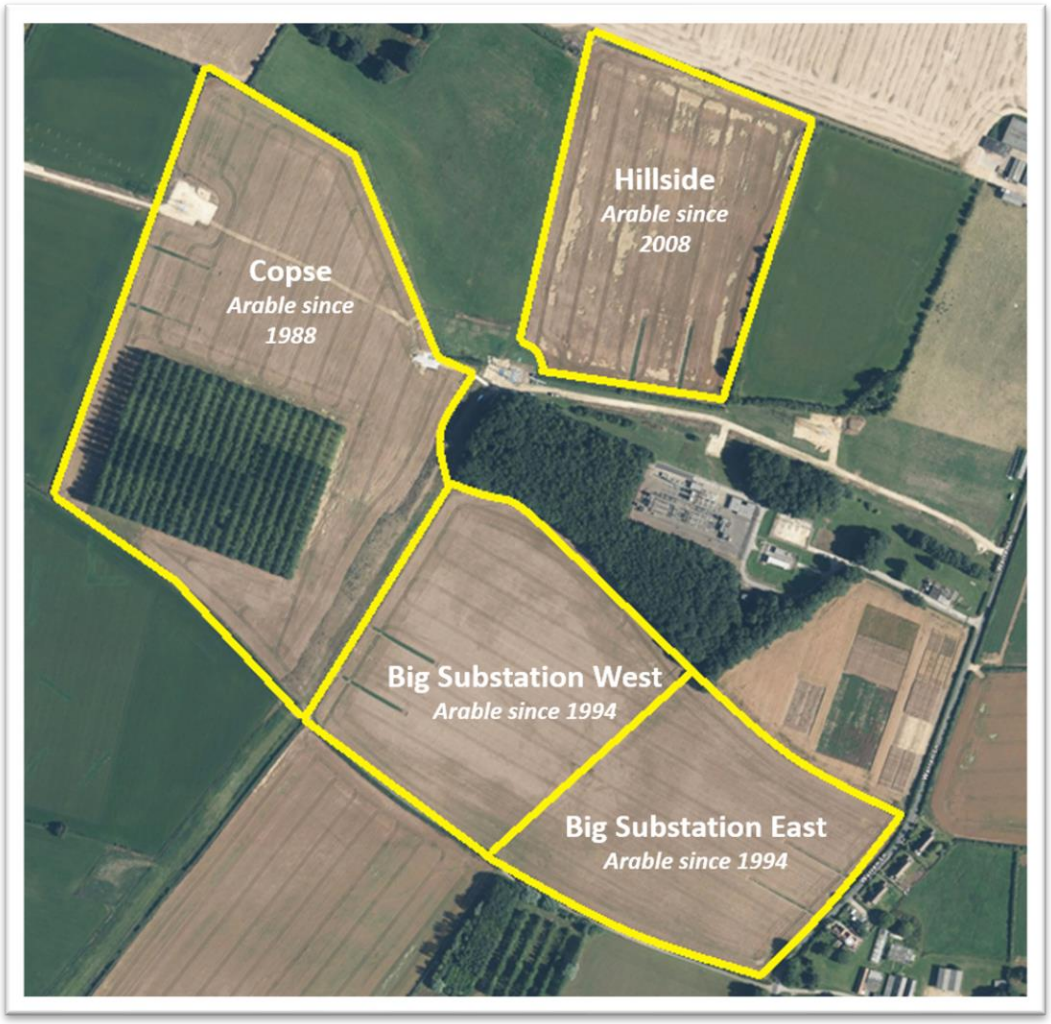

Figure 5.1. An aerial view of the four focal fields at the University of Leeds Field Research Unit. The ley strips are visible, extending inwards from the edge of the fields. Google Earth (2017), earth.google.com/web/.

Labile carbon measurements

After being used for eDNA analysis, the remaining soil in each sample was resealed in its plastic bag and stored at -20°C, before being defrosted for the labile carbon experiment. The defrosted soils, which had previously been homogenised within the plastic bag during eDNA extraction, were then placed in separately labelled aluminium weighing dishes and left to air dry in a fume cupboard for 48 hours. After drying, each sample was passed through a 1 mm sieve and returned to a labelled weighing dish. To increase accuracy in the subsequent labile carbon tests, two replicates from each original soil sample were taken, each weighing 5 g. These replicates were labelled accordingly and treated as

separate samples during the potassium permanganate oxidation stage, giving a total of 192 labile carbon measurements on completion. The mean of the two replicates was then taken for each sample (n = 96) and the resulting concentration used in further statistical analysis.

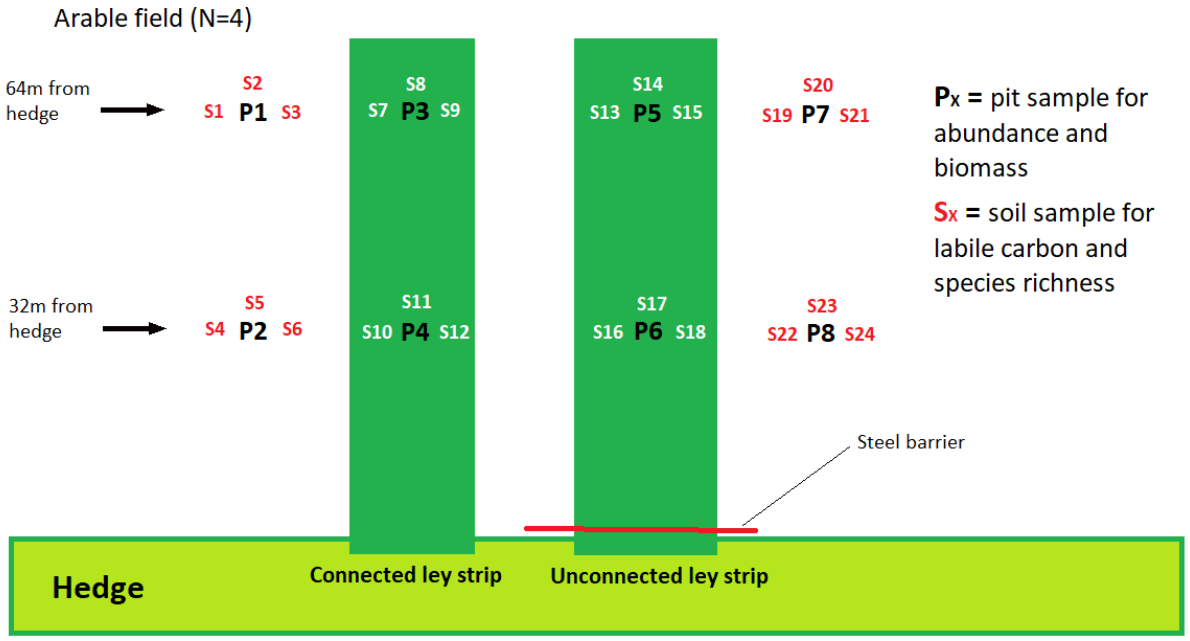

Figure 5.2. The number and positions of samples taken from each field. A total of 32 pit samples ('Px') were excavated to obtain earthworm abundances and biomass, whilst 96 soil samples ('Sx') were taken for eDNA and labile carbon analysis.

Labile carbon concentrations were determined by calculating the concentration of potassium permanganate ($KMnO_4$) oxidisable carbon, using the protocol developed by Weil et al. (2003) but modified for use in the laboratory. A stock solution of 0.2 M $KMnO_4$ was prepared by mixing 31.61 g $KMnO_4$ in 1 L of distilled water, followed by the gradual addition of 10% hydrochloric acid to bring the pH to 7.2. The stock solution was stored in a dark bottle and kept covered with aluminium foil when not in use. Batches of eight 5 g soil samples were processed at a time. For each 5 g sample, 2 ml of the 0.2 M $KMnO_4$ stock solution was measured out using a disposable bulb pipette and placed in a 50 ml centrifuge tube wrapped in foil, topped up to 20 ml using distilled water and mixed thoroughly. The 5 g soil sample was added to the centrifuge tube, placed on a horizontal shaker and shook at 120 rpm for two minutes. The tubes were taken off the horizontal shaker, shaken by hand for a further 10 seconds and centrifuged at 3800 rpm for five minutes. At this point standard solutions containing distilled water only, 0.005 M $KMnO_4$, 0.01 M $KMnO_4$ and 0.02 M $KMnO_4$ were pipetted into clean 5 ml

glass cuvettes and the absorbance at 550 nm recorded for each using a spectrophotometer. These standards were freshly made for each batch and were used to plot a standard curve, which was necessary for calculating the labile carbon concentrations from the absorbance readings later on. After recording the absorbance readings for the standards, the sample tubes containing the soil – $KmnO_4$ suspensions were removed from the centrifuge and 0.5 ml of each was pipetted into 49.5 ml of distilled water. The diluted suspensions were shaken well, pipetted into clean glass cuvettes and placed into the spectrophotometer, where the absorbance for each was recorded at 550 nm.

Once the absorbance readings had been measured for all samples, the labile carbon concentration of each was calculated using the following equation described in Weil et al. (2003):

$$Labile\ carbon\ (mg\ kg^{-1}) =$$

$$[0.02\ mol\ l^{-1} - (a + b\ \times absorbance)] \times 9000\ mg\ C\ mol^{-1} \times (\frac{0.02\ l\ solution}{0.005\ kg\ soil})$$

In the equation, $0.02\ mol\ l^{-1}$ is the initial solution concentration, $a + b$ is the intercept plus the slope of the standard curve, $absorbance$ is the 550 nm absorbance reading for the sample, $9000\ mg\ C\ mol^{-1}$ is the amount of carbon oxidised by 1 mol of MnO_4, $0.02\ l\ solution$ is the volume of $KMnO_4$ solution used in the reaction and $0.005\ kg\ soil$ is the amount of soil.

Statistical analysis

All statistical analyses were performed in R Studio using R version 4.0.3 (R Core Team, 2020). To test whether soil labile carbon concentrations differed between arable controls and ley strips, a two-way ANOVA was performed using the function 'lm' with labile carbon concentration as the response and land use as the predictor. Field ID was also included as a predictor in the model in order to test for any location-specific effects that may impact labile carbon concentrations. The 'autoplot' function in the package 'ggplot2' (Wickham, 2011) was used to check that the model satisfied the ANOVA assumptions. Post-hoc Tukey Honest Significant Difference tests were carried out using the 'TukeyHSD' function.

To test for possible relationships between labile carbon and earthworm population variables, correlations were performed between labile carbon concentrations and earthworm species richness, abundance, total biomass and average individual biomass. Species richness was calculated from the results of eDNA analysis using the same 96 soil samples that were also used to measure labile carbon concentration. The data for the remaining earthworm variables were gathered from the traditional hand-sorting of earthworms from the 32 soil pits. In order to perform correlations using the latter

variables, a labile carbon concentration for each of the 32 pit samples was determined by calculating the mean of the three associated soil samples (i.e. in Figure 5.2 the mean of S1, S2, and S3 gave the labile carbon concentration associated with P1). As none of the four earthworm population variables were normally distributed, Kendall rank correlations were carried out using the function 'cor.test'. The correlations were performed on the overall data and then repeated for separate fields grouped by land use, in order to account for potential effects of these factors on the variables being correlated. All graphs were plotted using ggplot2 (Wickham, 2011).

5.4 Results

Land use effects on soil labile carbon concentration

Land use had a statistically significant effect on labile carbon concentrations (ANOVA: $F = 100.45$, d.f. = 1, $p < 0.001$), with higher concentrations in the ley strip soils compared with the arable controls (Figure 5.3). The mean concentration of soil labile carbon measured in the arable control soil samples was 440.61 mg kg^{-1}, compared to a mean of 506 mg kg^{-1} in the ley samples. Using the arable soils as a baseline, this represents an overall labile carbon increase of 14.8% in soils that had been managed as leys for 33-months. In absolute terms, conversion to ley increased the labile carbon concentrations by an average of 23.80 mg kg^{-1} per year. In addition to land use, the field that the samples were taken from also had a significant effect on labile carbon concentrations ($F = 51.00$, d.f. = 3, $p < 0.001$). Samples taken from the 'Hillside' field had significantly higher labile carbon levels than 'Big Substation East', 'Big Substation West' and 'Copse' fields (Tukey tests, all $p < 0.001$; Figure 5.3), which did not differ significantly from each other (Tukey tests, $p = 0.91$ or greater). With a mean labile carbon concentration of 543.16 mg kg^{-1}, 'Hillside' labile carbon levels were 19.8% higher than those in 'Big Substation East' (453.59 mg kg^{-1}; Table 5.1), 20.94% higher than 'Big substation West' (449.10 mg kg^{-1}) and 21.40% higher than 'Copse' (447.45 mg kg^{-1}). Despite the higher general concentration of labile carbon in 'Hillside' field, concentrations were still significantly higher in the 'Hillside' ley strips compared to the arable control soil samples ($p = 0.001$), indicating a significant effect of land use regardless of the baseline labile carbon level.

Table 5.1. Mean labile carbon concentrations across the different field and land use types, including the percentage increase between the arable and ley samples for each field.

	Mean labile carbon concentration (mg kg $^{-1}$)			
Field	Overall	Arable control	Ley	% increase arable-ley
Big substation East	453.59	422.66	484.51	14.63
Big substation West	449.10	412.15	486.06	17.93
Copse	447.45	411.94	482.97	17.24
Hillside	543.16	515.67	570.65	10.66

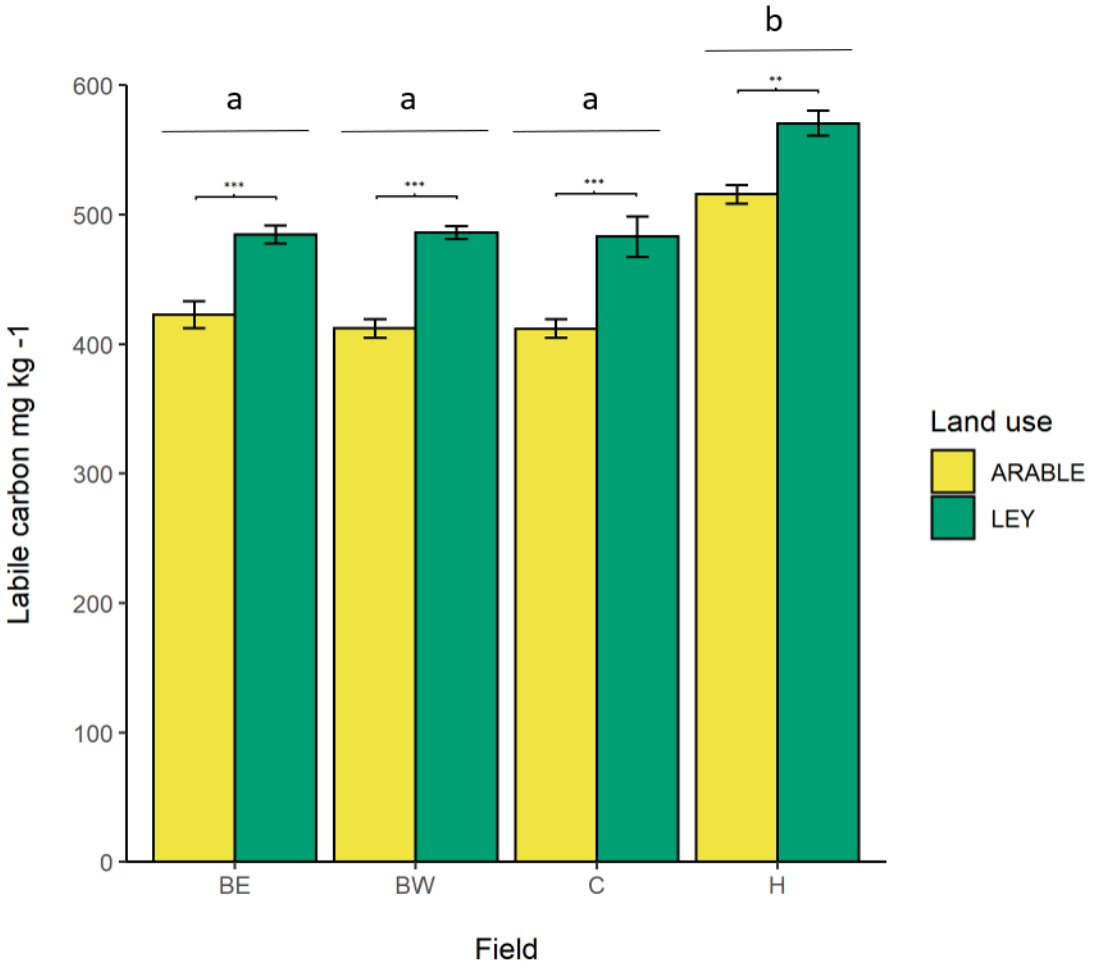

Figure 5.3. Mean labile carbon concentrations across the different fields and grouped by land use (+/- standard error). Different lowercase letters indicate significant differences between fields ($p < 0.001$) and asterisks indicate differences between arable controls and ley strips in post-hoc Tukey tests ('***' for $p < 0.001$, '**' for $p = 0.001$). For field, 'BE' = Big Substation East, 'BW' = Big Substation West, 'C' = Copse and 'H' = Hillside.

Relationship between labile carbon and earthworms

There was an overall significant positive correlation between labile carbon concentration and earthworm species richness (Kendall's rank correlation: $\tau = 0.36$, $p < 0.001$; Figure 5.4a), as was expected given that both labile carbon concentrations and earthworm species richness were higher in the ley strips and Hillside field. However, when the effects of land use and field ID were taken into account, no further correlations were observed (Figure 5.4b and Table 5.2). Overall positive correlations were also observed between labile carbon and both earthworm abundance ($\tau = 0.33$, $p = 0.01$; Figure 5.5a) and total biomass ($\tau = 0.25$, $p = 0.04$), but again, when land use and field were taken into account no significant correlations remained (Figure 5.5a and Table 5.2). No significant correlation was found between labile concentration and mean individual earthworm biomass ($\tau = 0.03$, $p = 0.78$).

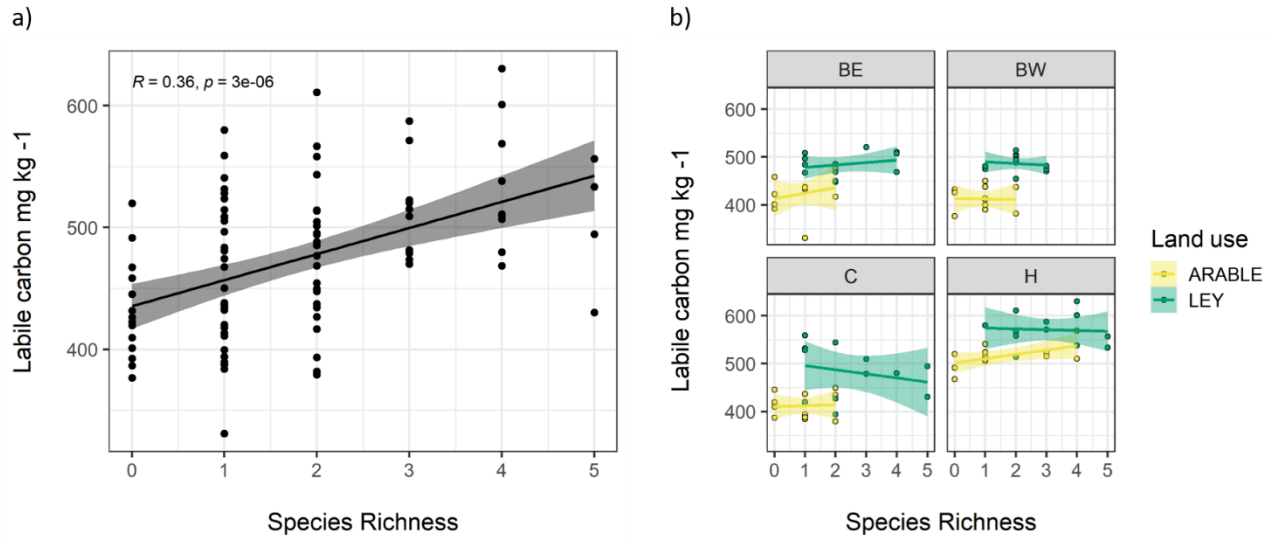

Figure 5.4. The relationship between labile carbon concentration and earthworm species richness when looking at a) all samples combined and b) samples grouped by field and land use. Kendall's correlations were performed to calculate Kendall's Tau and p-values for each line, which are included on the graph if significant. 'BE' = Big Substation East, 'BW' = Big Substation West, 'C' = Copse and 'H' = Hillside.

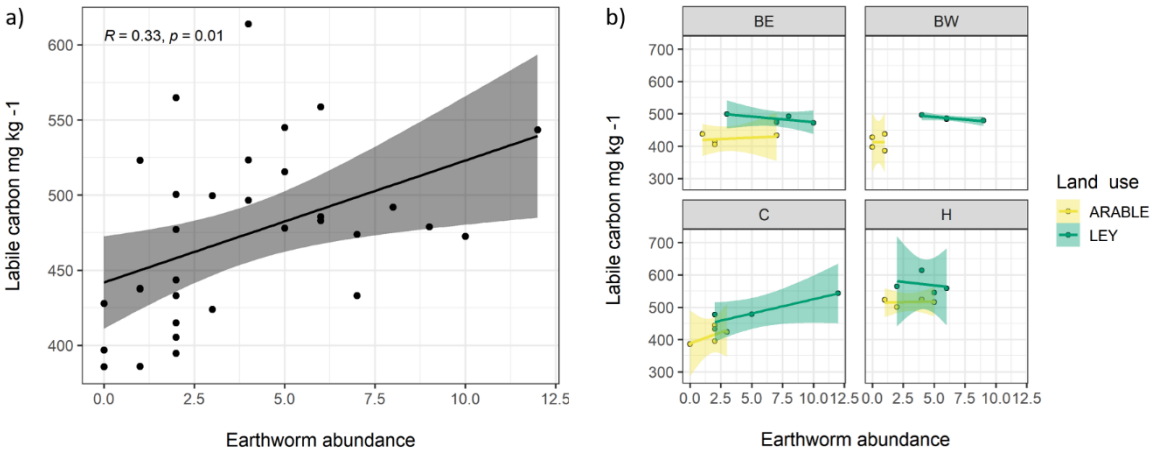

Figure 5.5. The relationships between labile carbon concentration and earthworm abundance per pit when looking at a) all samples combined and b) samples grouped by field and land use. Kendall's correlations were performed to calculate Kendall's Tau and p-values for each line, which are included on the graph if significant. For field, 'BE' = Big Substation East, 'BW' = Big Substation West, 'C' = Copse and 'H' = Hillside.

Table 5.2. Results of Kendall's rank correlation tests between labile carbon concentration and earthworm species richness, abundance and total biomass, overall and when land use and field ID were taken into account. Significant p-values are in bold. Tau = test statistic, N = sample size. For field, 'BE' = Big Substation East, 'BW' = Big Substation West, 'C' = Copse and 'H' = Hillside.

		SPECIES RICHNESS			**ABUNDANCE**			**TOTAL BIOMASS**		
		Tau	p-value	N	Tau	p-value	N	Tau	p-value	N
	Overall	0.36	**2.97E-06**	96	0.33	**0.01**	32	0.25	**0.04**	32
Field	Land use	Tau	p-value	N	Tau	p-value	N	Tau	p-value	N
BE	Arable	0.22	0.36	12	-0.18	0.72	4	-0.33	0.50	4
	Ley	0.14	0.56	12	-0.67	0.17	4	-0.67	0.17	4
BW	Arable	-0.02	0.94	12	0.00	1.00	4	0.18	0.72	4
	Ley	-0.13	0.58	12	-0.91	0.07	4	-0.67	0.17	4
C	Arable	-0.02	0.94	12	0.55	0.28	4	0.33	0.50	4
	Ley	-0.18	0.43	12	0.91	0.07	4	0.33	0.50	4
H	Arable	0.39	0.09	12	0.00	1.00	4	0.00	1.00	4
	Ley	-0.08	0.72	12	-0.33	0.50	4	-0.33	0.50	4

5.5 Discussion

In this experiment clear effects of land use on labile carbon concentrations were observed, demonstrating that temporary conversion of arable fields to grass-clover ley can significantly increase soil labile carbon concentrations in under three years. Labile carbon concentrations were also found to show field-specific differences, indicating potential lasting effects of variable management histories. With regards to potential relationships between labile carbon concentrations and earthworm populations, positive correlations were found between labile carbon concentrations and species richness, abundance and total biomass. However, in Chapters 2 and 4, earthworm species richness, abundance and biomass were found to increase in soils that were converted to ley and also varied by field, which is likely to explain the overall positive relationships observed. When accounting for these field effects and the differences between ley and arable samples that were previously observed, no further positive correlations were seen between labile carbon concentration and earthworms.

Firstly, the finding that soil labile carbon concentration is clearly higher in the ley strips compared to the surrounding arable soils demonstrates another benefit of temporary conversion to ley, in addition to the boosts in earthworm populations previously described. The significant changes in the labile carbon soil pool found here contrast with the findings of Berdeni et al. (2021), who looked at total soil organic carbon within the same study system and found no significant increases in the leys when compared with the arable. The lack of a significant change was attributed to a combination of factors including inherent soil variability, the relatively small increases seen in comparison to the size of the total carbon pool and the short time period in which the increases were given to accrue. Other studies that have looked for possible effects of land use on total soil carbon concentrations over relatively short time periods have reported similar results (Gosling et al., 2017; Loaiza Puerta et al., 2018), and often changes in the larger passive carbon pool only become visible many years after the initial change in management (Johnston et al., 2017; Poeplau and Don, 2015). The highly significant changes in labile carbon concentrations found in this experiment therefore support previous findings that measuring labile carbon can be a more sensitive indicator of changes in soil carbon in the short-term, which may otherwise be missed if using conventional total SOC measurements. The fact that soil carbon had a further 13 months to accumulate from the time that Berdeni et al. (2021) measured total SOC may account for some of the differences seen between the labile carbon and total SOC measurements. However, given the rate at which total SOC is reported to accumulate (Berdeni et al., 2021; Poeplau and Don, 2015; Prade et al., 2017) and the highly significant differences that were observed in this

study, it is still likely that these findings indicate labile carbon concentrations are a more sensitive measure of changes in soil carbon bought about by land use in the short-term, supporting similar conclusions made by other studies utilising this method (Malobane et al., 2020; Shen et al., 2021; Weil et al., 2003).

The significant effect of field ID on labile carbon concentration was an interesting and unexpected finding of the analysis. Labile carbon concentrations were significantly higher in the Hillside field when compared with all the other fields (which did not differ from each other), indicating location-specific differences despite the close proximity and identical cropping histories over the last decade. One possible explanation for the variation in labile carbon between fields could be the difference in management of Hillside compared to the other three fields prior to 2008. In contrast with Big substation East, Big substation West and Copse, which had all experienced conventional tillage and continuous cropping since at least 1995, Hillside was taken out of production in 1998 and managed as pasture for 10 years, before being returned to production and then managed in a similar way to the others up until the soil samples were taken for analysis. Therefore, the high labile carbon concentrations seen in Hillside could be an enduring product of this pasture management period, during which increases in soil carbon and other soil quality improvements would likely have occurred, as observed in the leys. The ability of higher soil carbon levels to persist for several years after being taken out of pasture and returned to cropping is supported by Studdert et al. (1997), who measured soil organic carbon levels in a continuously cropped system after a five-year ley. Studdert et al. (1997) found that although organic carbon levels declined in the years of cropping following the ley, they still remained higher than those observed before the ley had been implemented. Taken together, the research presented here and previous findings suggest that the beneficial effects of temporary arable to ley conversion can still be seen many years after the leys are taken back into cultivation, with longer leys increasing both the magnitude and longevity of increases in soil organic carbon (Franzluebbers et al., 2014; Lemaire et al., 2015; Panettieri et al., 2017).

After land use and field effects had been accounted for, no correlations between labile carbon concentrations and earthworm population variables were observed. It is difficult to draw conclusions at this stage as the small number of data points makes significant correlations unlikely, but the lack of association could suggest that the parallel increases in labile carbon and earthworm species richness, abundance and biomass seen are not directly related to each other, and more likely due to other factors related to the change in land use management. There are a number of reasons why both labile carbon levels and earthworm population variables could be increased with temporary conversion to ley, including through reduced tillage-related soil disturbance and as a result of the benefits associated with the continuous cover provided by the grass-clover sward. Tillage is known to break-up the soil

structures that protect labile carbon fractions, increasing their exposure to the atmosphere and accelerating carbon losses (Bongiorno et al., 2019; Olson, 2013), whilst also leading to higher earthworm mortality (Chan, 2001). Therefore, the lack of tillage in the leys, combined with the continual addition of labile carbon from the unharvested plant biomass and the extensive grass and clover root systems (Conant et al., 2001), can promote favourable conditions for the reversal of labile carbon losses that are usually associated with conventional arable systems. Furthermore, for earthworms it is well documented that reductions in tillage intensity can reduce the direct and indirect mortality associated with ploughing (Briones and Schmidt, 2017; Chan, 2001; Curry et al., 2002), whilst the cover provided by the year-round grass-clover sward can provide them with suitable protective habitat and a high-quality, nitrogen rich food supply (Edwards, 2004; Euteneuer et al., 2020; Roarty et al., 2017).

Whilst previous studies have shown that labile carbon concentration can show clear associations with other physical and biological indicators of soil quality (Bongiorno et al., 2019; Weil et al., 2003), the results described here suggest that the relationships between earthworm populations and labile carbon are more complex. However, as previously mentioned caution must be taken when drawing wider conclusions from the correlations between labile carbon and the earthworm variables, especially abundance and biomass, since these had particularly low sample sizes when split into separate fields and grouped by land use. More research is needed to explore possible associations and causal links between the labile carbon pool and earthworms, which could include wider field sampling of both labile carbon and earthworms concurrently, and behavioural preference testing in the laboratory.

5.6 Conclusions

In conclusion, the results in this chapter show that measuring labile carbon concentrations could represent an effective way to discover short-term changes in soil carbon levels bought about by changes in management, that if maintained are likely to lead to significant increases in total organic carbon in the long-term. Monitoring labile carbon could give an early indication of the success of recently implemented land use changes, and can easily be carried out in combination with the measurement of other soil quality indicators (such as earthworm eDNA sampling). The potential of temporary arable to ley conversion for restoring soil health in degraded arable land has also been highlighted again, this time through its ability to boost soil labile carbon. Whilst no direct associations

were found between earthworm populations and labile carbon after land use differences had been accounted for, more research is needed to examine the possible interactions between them. Laboratory preference tests that investigate the response of earthworms to soils with varying carbon levels could prove particularly beneficial, allowing more insight into the potential mechanisms through which these two important soil quality indicators may influence each other.

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Chapter 6

General Discussion

6.1 Introduction

This thesis set out to address some of the knowledge gaps surrounding the effects of different land use practises on agricultural earthworm populations, and to develop and test an environmental DNA (eDNA) based sampling method to facilitate further population monitoring. To do this, experiments were carried out utilising the SoilBioHedge study system at the University of Leeds Field Research Unit, which was set up to investigate the consequences of temporary arable to ley conversion for a range of different soil properties and soil health indicators. A novel semi-controlled mesocosm experiment, described in Chapter 2, was used to investigate earthworm casting activity, abundance, biomass and diversity in soil monoliths that had been under arable or ley management, with the latter monoliths coming from ley strips that were either connected or unconnected to an adjacent hedgerow. The effects of weather stress on the earthworm populations within these monoliths was also investigated, given that drought and flooding events are forecast to become more common in the coming decades as a result of climate change (DEFRA, 2018; Rahmstorf and Coumou, 2011). In Chapter 3, an eDNA sampling technique was developed and refined, using laboratory testing of various eDNA extraction, amplification and sequencing procedures. The resulting eDNA methodology was then applied in Chapter 4, in which soil samples for eDNA analysis were collected from the arable fields and ley strips described earlier, and concurrent traditional hand-sorting performed alongside. The ability of the eDNA sampling approach to detect fine-scale differences in earthworm communities resulting from conversion to ley was explored, and quantitative comparisons made with the traditional hand-sorting method to assess its effectiveness. In Chapter 5, labile carbon concentrations in the arable and ley soils were measured, using the soil samples previously utilised for eDNA analysis. The resulting labile carbon values were then correlated with earthworm species richness, abundance and biomass, to investigate possible associations between these soil health indicators.

The aim of this chapter is to discuss the overall findings of the work described above, specifically with regards to answering the research questions posed in Chapter 1, which are included below. Potential implications of these findings for future land use management and earthworm population monitoring are also explored further. Following on from this, some of the limitations of the studies and suggestions for further work are discussed, and overall conclusions are drawn.

1) How do different land use practices affect earthworm populations and behaviour?
2) Can earthworm populations in agroecosystems be distinguished using eDNA sampling?
3) How sensitive is eDNA to changes in communities bought about by land use changes?
4) How does eDNA sampling compare with traditional earthworm sampling approaches?
5) Are changes in other soil health indicators associated with changes in earthworm populations?

6.2 Land use effects on earthworm populations

The results presented in this thesis clearly show that temporary arable to ley conversion can be beneficial for agricultural earthworm populations. Significant increases in earthworm diversity, abundance and biomass were seen in Chapters 2 and 4, whilst Chapter 5 also demonstrated the benefits of ley conversion for soil labile carbon levels. It is likely that the increases in earthworm population variables observed under ley management are due to a combination of factors, including the lack of tillage-related soil disturbance, the increased surface cover from the grass-clover sward and increased soil organic matter inputs from the plants and their root systems, which the earthworms rely on for food (Briones and Schmidt, 2017; Edwards, 2004; Euteneuer et al., 2020). The conditions created by the temporary leys are similar to those in permanent grasslands (Berdeni et al., 2021; van Eekeren et al., 2008), which are known to be favourable environments for earthworms which can support large and diverse communities (Rutgers et al., 2009; Singh et al., 2021; Spurgeon et al., 2013). The conclusion that leys can restore earthworm populations in degraded arable land and lead to the earthworm community transitioning towards those typically seen in permanent grassland soils is supported by other studies on temporary leys. For example, Lamandé et al. (2003) sampled earthworm communities in fields that were managed as either continuous maize crop, maize crop in rotation with a three-year rye-grass ley, or as long-term grassland, and found intermediate earthworm abundances and functional diversity within the crop-ley rotation when compared with the continuous maize or long-term grassland. Similar findings were reported by van Eekeren et al. (2008), who found that earthworm abundance in arable land was restored to the equivalent levels seen in permanent grassland after three years of a grass ley. However, earthworm biomass still remained lower in the ley treatments and the anecic earthworm population in particular was not restored to the same levels seen in the permanent grassland. Alongside the results presented here, these studies suggest that temporary arable to ley conversion can bring multiple benefits to earthworm populations in the short term, but longer-term ley periods (i.e., three years plus) may be needed to fully restore the functional diversity typically seen in grasslands.

As well as highlighting the success of temporary arable to ley conversion for increasing earthworm populations, the results of this thesis suggest that attaining benefits from ley conversion does not necessarily require the presence of additional landscape factors, in this case hedgerows, to reseed the earthworm populations within the fields. This is because no evidence was found for increased earthworm abundances or diversity in the leys connected to hedgerows when compared with those that were unconnected, suggesting that the proliferation of surviving earthworm populations already present in the arable fields was the more likely source of the observed population increases. Although further work is needed to establish how earthworms utilise hedgerows given the limitations of the monolith methodology described earlier and the inconclusive results of previous studies (Frazão et al., 2017; Hoeffner et al., 2021; Roarty and Schmidt, 2013), the lack of evidence for any refugia effects could be seen as a positive as it implies that ley farming can still be suitable for farm systems that may not have existing hedgerows present. This is good news for the wider deployment of this type of land management and could make it more attractive for inclusion in agricultural policy schemes going forward. However, it is important to note that encouraging the uptake of leys in these schemes may require financial incentives or subsidies given the potential for short-term yield reductions, and further work is needed to fully quantify the costs and gains of widespread adoption of ley cropping (Martin et al., 2020).

Significant effects of field ID on earthworm population variables and labile carbon levels were surprising but consistent findings observed in Chapters 2, 4 and 5. Despite the sampled fields originally being picked due to their similarities, which included similar 10-year cropping histories, soil types and close proximities, the emergence of clear differences between the earthworm populations and carbon levels in the fields suggests previous management histories and/or location-specific biotic differences can still be important. Clear differences in earthworm populations resulting from geographic separation alone have been reported in the literature (Maggia et al., 2021), and it is possible that the inherent heterogeneity and 'patchiness' of earthworm populations may also be influencing the results given the relatively small spatial areas from which samples were taken (Hodson et al., 2020; Rossi, 2003; Valckx et al., 2009). However, the fact that one particular field ('Hillside') consistently stood out from the others for both higher earthworm population variables and labile carbon levels, suggests that previous management stretching back longer than ten years may be responsible, as discussed further in Chapter 5. As well as strengthening the case for the positive soil health impacts associated with ley farming, this highlights the need for more studies looking at the longer-term consequences of arable to ley conversion for earthworm populations and other important soil properties.

The beneficial effects of temporary arable to ley conversion for earthworm populations have been highlighted throughout this thesis, suggesting that incorporating this management practise into policy

schemes focused on restoring soil health could be an effective strategy. However, this work has not explored the potential wider ramifications of increased adoption of arable to ley conversion, beyond those affecting earthworms and soils. For example, ley farming systems have traditionally been strongly associated with livestock, which can either graze directly on the ley pasture or eat the hay produced by them (Martin et al., 2020). Mixed arable and livestock systems like this were particularly common pre-1950, before the widespread availability of chemical fertilisers and pesticides caused a shift towards more specialised and intensive production systems (Knox et al., 2011). If the proliferation of arable to ley conversion were to lead to the transition towards mixed-farming systems once again, this could lead to significant changes in the UK's agricultural sector and wider countryside. On the one hand, a move towards mixed-farming systems could encourage the spread of regenerative farming practises more widely, reducing the reliance on pesticide and fertiliser inputs and increasing soil health and sustainability further (LaCanne and Lundgren, 2018; Lal, 2020). However, it is also important that any transition towards mixed-farming systems does not lead to the overall increase in livestock production, as this would be detrimental to reducing meat consumption and the carbon footprint associated with UK diets. Such an outcome is possible, but it would require a significant shift in policy thinking, and farmers and land managers would need to be supported through the transition (Lymbery, 2021). Although beyond the scope of this thesis, further work is needed to explore the potentially significant ecological, social, and cultural ramifications that the transition to mixed-farming systems could bring.

6.3 eDNA sampling for monitoring earthworm populations

With regards to answering research questions 2, 3 and 4, the findings of this thesis suggest that eDNA sampling can be used to assess earthworm diversity in agricultural ecosystems, it can detect changes in the earthworm communities resulting from recent changes in land use, and for measuring earthworm diversity it performs favourably when compared to traditional methods. However, this thesis also shows that further work is needed before eDNA sampling can be used to obtain reliable quantitative measures of earthworm abundances or biomass. To our knowledge, the research presented here is the first quantitative analysis comparing earthworm hand-sorting with eDNA sampling, answering the call to action for more quantitative comparisons between eDNA and traditional sampling methods issued by Fediajevaite et al. (2021). Another novel finding emerging from this thesis was the suitability of a new primer combination discussed in Chapter 3, which was originally proposed by Bienert et al. (2012) but until now remained untested.

As reported in Chapter 4, demonstrating the sensitivity of the eDNA sampling approach for detecting fine-scale differences in earthworm communities bought about by in-field land use change represents an important step towards utilising eDNA sampling for more widespread population monitoring. Previous studies have shown that eDNA sampling can be used to detect differences in earthworm communities across sites at the landscape level (Pansu et al., 2015), but until now there has been little consideration of how management changes within the same site may influence the community composition results measured using eDNA. There have been concerns that the continued presence of detectable eDNA left behind after organisms have vacated the area may lead to soil eDNA giving readings of past communities (Sirois and Buckley, 2019; von Ammon et al., 2019; Zaiko et al., 2018), which would erode the sensitivity of this approach and compromise its ability to pick up changes in community composition occurring across short temporal and spatial scales (Barnes and Turner, 2016). Although this thesis did not directly investigate the potential influence of 'background' eDNA on the overall results and therefore cannot rule out its presence, the fact that clear differences in eDNA profiles were seen in soil samples taken metres apart, which had only been under different treatment regimens for less than three years, suggests that eDNA sampling can pick up fine-scale community differences in space and time. However, further work is still needed to fully understand the rate of eDNA degradation in soils and how persistent eDNA may influence earthworm population monitoring. Suggestions for follow up work to achieve this are discussed in section 6.4.

Overall, the results presented in Chapters 3 and 4 could facilitate the uptake of wider earthworm eDNA sampling, as they provide empirical evidence that it performs at least as well as traditional methods when measuring diversity. In general, the uptake of novel eDNA approaches in official monitoring schemes has been relatively slow and tried and tested conventional methods are still largely favoured (Fediajevaite et al., 2021). However, the use of eDNA approaches is now beginning to percolate through, particularly for marine and aquatic population monitoring. For example, in 2014 Natural England certified eDNA sampling to be an accepted technique for monitoring the protected Great Crested Newt (*Triturus cristatus*), and it has now become an established survey tool that can offer improved detection rates when compared to other conventional methods (Rees et al., 2017, 2014). In a similar way, the results presented in this thesis indicate eDNA sampling could satisfy the requirements needed to become a verified approach for sampling earthworms in wider monitoring schemes, which several authors have reported as urgently needed (Carpenter et al., 2012; Phillips et al., 2021; Rutgers et al., 2016). Any eDNA sampling approach based on the techniques developed in Chapters 3 and 4 of this thesis should focus on elucidating earthworm species diversity or presence/absence, until more accurate abundance proxies can be developed.

As well as the other advantages of eDNA sampling described earlier, it is also worth noting that if eDNA monitoring is employed more widely it would also mean that earthworm sampling would be less affected by preceding weather conditions. This is because the results of traditional pit sampling have been shown to be significantly influenced by the weather conditions in the week prior to sampling taking place (Prendergast-Miller et al., 2021), with surveys that take place after recent dry weather typically yielding lower earthworm abundances. This could be particularly problematic for widespread monitoring programmes where sampling takes place over several months, and would make direct comparisons between data that had been collected with different antecedent weather conditions difficult. Although care was taken to conduct fieldwork and hand-sorting under broadly similar weather conditions when it was carried out for this thesis, the possibility that differences in antecedent weather conditions may account for some of the variation seen between hand-sorted samples cannot be ruled out. Further work is needed to explore how soil eDNA signatures change over time, but based on the work presented here it is thought that this sampling approach will be less affected by antecedent weather conditions, improving the standardisation of earthworm sampling further.

Given that chapter 5 indicated no direct correlation between eDNA-measured earthworm diversity and labile carbon concentrations, this suggests that if eDNA sampling is rolled out for use in wider soil health monitoring schemes it may not be a suitable proxy for other soil health indicators. Despite this, the methodology used does demonstrate that multiple soil health indicators can be accurately assessed using the same soil sample. This could prove useful for soil health monitoring schemes going forward as it would avoid the need for different methodologies to be deployed in the field, and allow one collected sample to give measurements for separate biological and chemical soil health indicators. Getting additional measures for soil physical health using the same sample may prove more difficult, but this could be achieved if prior to homogenisation a subsample was taken and used to assess a soil physical health indicator. A good candidate for this could be macroaggregate stability, which is a widely used physical indicator that can be measured from disturbed soil samples using wet sieving or a rainfall simulator (Barthès and Roose, 2002; Moebius et al., 2007). This would allow a set of biological (earthworm eDNA), chemical (labile carbon) and physical (macroaggregate stability) soil health indicators to be measured using a single sample, which could greatly increase efficiency. Given the ongoing piloting of the new Environmental Land Management Scheme and the recent announcement of the Soil Health Action Plan for England, such a development is extremely timely.

6.4 Limitations and further work

Some of the limitations of the work presented in this thesis have already been discussed in previous chapters, but in this section a few of the key limitations are outlined and suggestions made for further work to address them.

Firstly, the data presented in this thesis originated from a single study system, as all soil and earthworm samples were collected from the SoilBioHedge experimental fields at the University of Leeds Field Research Unit. Whilst this allowed greater control and manipulation of the treatment groups and conditions, it also means that caution should be exercised when generalising to other farm systems. This is because as touched upon previously, outcomes of both arable to ley conversion and the effectiveness of eDNA sampling may be influenced by different soil types, previous management practises and climatic conditions that could affect soil property recovery or eDNA degradation rates (Jacinthe et al., 2004; Pietramellara et al., 2009; Sirois and Buckley, 2019). The existing earthworm community present in the arable fields prior to ley conversion may be particularly important for the ability of leys to restore earthworm populations, as if extensive cultivation has reduced earthworm numbers to near zero then recoveries may take much longer (Curry et al., 2002). In the SoilBioHedge system, earthworm numbers in the arable fields were very low, but most samples did find at least one individual. To further strengthen our understanding of both the effectiveness of leys for restoring earthworm populations and the utility of earthworm eDNA sampling, it is therefore recommended that more eDNA sampling and ley conversion experiments are carried out in other systems with varying soil types, cropping histories and climatic variables.

In Chapter 2, the monolith mesocosm approach enabled soil processes and earthworm activity to be closely observed, whilst also allowing weather stress manipulations that would be near impossible to achieve in the field. However, some sampling biases arising from the way the monoliths were extracted mean that is a less effective approach for measuring earthworm diversity, so care must be taken when interpreting the overall diversity patterns from the monoliths. This is because the excavation of the monoliths was a lengthy process, which likely produced a lot of vibration and would have provided anecic earthworms in particular with ample time to escape away from the area (Čoja et al., 2008; Singh et al., 2016). In addition, the treatment of the surface of the monoliths to remove the grass-clover sward and transplant wheat would have likely disturbed epigeic earthworms disproportionally. The fact that both of these ecological categories were found to be relatively rare in the monoliths supports this, so it is concluded that the earthworm diversity results presented in chapter 4 are a more reliable measure of the populations at the study site. Despite this, the use of the

monolith approach is still justified given the higher levels of control and the improved ability to measure earthworm casting activity in real-time that it allowed.

The results of Chapter 2 also revealed intriguing changes in the behaviour of earthworms as a response to the simulated weather stress conditions, although no lasting effects on overall population variables were observed at the end of the experimental period. As discussed in Section 2.5, limitations with the monolith approach prevented the flood treatments in particular from adequately replicating field-realistic flood conditions, so further work is needed to make more robust conclusions on the possible wider impacts of flooding on earthworm populations. Recent studies do indicate that flooding events can have substantial impacts on earthworm community composition and abundance, and the effects of flooding can be amplified when populations are already stressed due to other land management practises (Kiss et al., 2021a; Sánchez-Rodríguez et al., 2019). Intriguingly, Kiss et al. (2021b) also show that different earthworm species show varying survival rates when exposed to the depleted oxygen levels associated with flooding events, with larger anecic earthworms like *L. terrestris* showing particularly high mortality even at low levels of oxygen depletion, whilst endogeic earthworms – particularly *A. chlorotica* – were able to survive throughout. This corroborates the findings in Chapter 2 that indicated *A. chlorotica* populations seemed to do particularly well in the flooded monoliths treatments, and also suggests that the very small number of anecic earthworms present in the monoliths initially may be a reason for the lack of observed flooding impacts on overall earthworm population variables. Whilst the results presented in this thesis provide a useful glimpse into earthworm behavioural responses to weather stress, they also highlight the need for more field studies to look at the impact of floods on earthworms further. The eDNA sampling approach described in Chapters 3 and 4 could help to facilitate these studies, as it will allow earthworm populations in flooded areas to be more easily sampled during and after flooding events take place.

This thesis has contributed to the field by demonstrating the potential of eDNA for sampling earthworms and for use in soil health monitoring. It has also raised a number of avenues and prospects for further development and research. In particular, many unanswered questions remain surrounding the rate of eDNA degradation in soils and how 'background' eDNA levels may impact the diversity results of present-day communities. Studies have begun to address some of these questions, such as the recent controlled laboratory experiment that showed the concentration of synthetic eDNA reduced by more than 99% within one week of being added into soils, but small amounts of eDNA did become stable and remained detectable for the 80-day period (Sirois and Buckley, 2019). Whilst important, questions surrounding eDNA degradation were not the focus of this thesis and more work is needed to establish how these factors may impact future monitoring schemes utilising the eDNA approach (Pietramellara et al., 2009). If significant differences are found between the degradation

rates of eDNA in different soil types for instance, methodological or statistical adjustments may need to be made in order to account for this when comparing the soil health results of different systems (Barnes et al., 2014).

In addition to soil eDNA degradation rates, developing a quantitative measure of earthworm abundance from eDNA sampling is an area that would benefit from further research. The results presented in Chapter 4 suggest that relative abundances of different species calculated from eDNA were a poor approximation for actual abundance, whilst absolute sequence numbers are already considered to be an unsuitable abundance proxy due to distortions bought about by PCR amplification (Kebschull and Zador, 2015). There are studies that have developed quantitative approaches that may allow abundances to be more accurately estimated from eDNA sampling, including the promising techniques of real-time quantitative PCR and the application of eRNA:eDNA ratios (Marshall et al., 2021; Spear et al., 2021). However, further research is needed before these methods can be more widely applied to soil eDNA and earthworms. This research should also consider other factors that may influence potential abundance measures generated using eDNA sequence numbers, including the potential for variable eDNA deposition rates across different earthworm species, as differences in behaviour, ecology and body size can affect the amount of eDNA an organism sheds into the environment (Stewart, 2019; Yates et al., 2020).

Finally, this thesis highlights the need for future studies that directly investigate earthworm behaviour, which is an often neglected but important part of agricultural soil regulation and functioning (Blouin et al., 2013). These future studies could be used to address some important questions that were not definitively answered by this thesis, including the question of whether or not hedgerows act as refugia for earthworms explored in Chapter 2. For example, earthworm dispersal behaviour between the hedgerow and arable soils could be investigated in the field by performing capture-mark-recapture with visible implant elastomer tags, as described by Butt and Lowe (2007). In the laboratory, earthworm choice chambers and habitat preference tests could also be utilised to measure earthworm responses to hedgerow and arable soils (using arenas similar to those described in Jones et al., 2016 and Mathieu et al., 2010). These methods could also prove useful for investigating the relationships between earthworms and labile carbon more closely, as mentioned previously.

6.5 Overall conclusions

Addressing the global issues of soil degradation and food insecurity will require innovative solutions, and radical changes to the way we grow our food are urgently needed. The essential services that

earthworms provide have been shown to play a crucial role in the promotion, maintenance and restoration of agricultural soil health, which makes earthworm-focused land management a promising option for boosting both crop yields and the wider sustainability of agriculture. This thesis has demonstrated the benefits that traditional land use practises can bring for earthworms in agroecosystems, adding weight to the evidence that shows temporary arable to ley conversion to be an effective way to increase soil health. Throughout the different experiments presented here conversion to ley was consistently found to increase earthworm activity, abundance, biomass and diversity in relatively short time periods, with benefits for other soil health indicators like labile carbon concentrations also being observed. These results therefore suggest the uptake of ley farming could bring substantial benefits to agroecosystems in the UK and elsewhere, justifying its inclusion in future agricultural policy schemes. As well as the beneficial effects of changing land use practises, this thesis has shown that novel eDNA sampling can be a feasible way to monitor earthworms in agroecosystems, which could help towards the management of their populations going forward. It is essential that earthworm population monitoring is improved if we are to fully utilise the many benefits they provide, and the results presented here show for the first time that eDNA sampling can outperform traditional sampling methods when measuring earthworm species diversity. More work is needed before this new technology can be used to gain accurate measures of other population variables, such as abundance and biomass, but the findings in this thesis do show that it is sensitive enough to detect fine-scale changes in earthworm diversity across relatively short time and spatial scales.

In conclusion, it is clear that greater consideration of the role of earthworms in agriculture could bring many benefits. In the words of Charles Darwin: “It may be doubted whether there are many other animals which have played such an important part in the history of the world, as have these lowly organised creatures.” It is hoped that as a result of the work presented in this thesis, earthworms will have more of a chance to play an important part in our future too.

6.6 References

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