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How do earthworms and soil microbial communities interact to determine the fate of organic matter amendments to soil?

By

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Declaration

This work was carried out by me and the use of material from other sources has been fully and properly acknowledged.

Signed: Henny O Omosigho

Dedication

I dedicate this thesis to my loving husband, Dr Osa Omosigho, who has offered staunch support and encouragement during these years of my doctoral journey. He has been my rock, made me laugh, wiped my tears, and given me confidence so that I could see this work through to the end. I can never forget how proud I was when he was able to name three different earthworm species. He had been paying attention. Thank you, Osa.

I also want to offer many thanks to my family and friends in general, whose love, reassurance and prayers day and night were unwavering.

To my siblings, Blessing, Goodness and Mercy Folorunso, this is just the beginning.

To my parents, thank you for your prayers.

To mother, Olabimpe, this is a special acknowledgement. We girls rule.

Finally, I dedicate this to me.

'The future belongs to those who believe in the beauty of their dreams'

Eleanor Roosevelt.

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Abstract

The soil ecosystem harbours most of the global ecosystem's biodiversity. The soil ecosystem contains a dynamic complex of microorganisms such as bacteria, microfauna (such as nematodes) and macrofauna (such as earthworms) and the non-living environment interacting as one functional unit. The biodiversity of this ecosystem plays a critical role in the provision of ecosystem functions and services. These ecosystem functions and services benefit humans. However, the rise in global challenges due to the rapid increase in human population, food shortage, the increase in our ecological footprint, rapid changes in the earth's climate, the introduction of invasive exotic organisms capable of changing ecosystem biodiversity and the increasing incidences of droughts and other natural disasters, has resulted in a 'trade off' of ecosystem services and a reduction in soil biodiversity that is pivotal to the ecosystem functions and services.

Earthworms are dominant members of soil invertebrate communities that play a key role in soil ecosystems' functioning directly through impacts on soil structure and through the stimulation of soil microbial decompositional activities in bipores and as a result of soil ingestion and gut passage. The earthworm gut microbiome, mainly derived from ingested soil, is hypothesised to influence host physiology, for example, by enhancing nutrition through the provision of assimilable nutrients via depolymerisation. However, few studies have examined the nature of the relationship between earthworm health and function and their soil-derived gut microbiome's diversity and composition. Also, we must improve our understanding of soil functioning especially given the pressures on the soil to deliver the services related to addressing the global challenges and the potential feedback between soil

and the climate system into the future. The overall aim of this thesis was to increase our understanding about the importance of the earthworm - earthworm microbiome relationship in supporting critical soil ecosystem process such as organic matter mineralisation, explicitly paying attention to the feedback of the presence of the earthworm microbiome to the health and function of earthworms in supporting ecosystem processes.

I used a novel antibiotic-based procedure to suppress *Lumbricus terrestris* earthworms' gut microbiome in the pilot study. The use of antibiotics significantly reduced the abundance of *L. terrestris*-associated culturable microorganisms ($P < 0.05$), but 16S rRNA gene amplicon analysis showed no effect on earthworm microbiome alpha diversity and only subtle effects on beta diversity despite the pronounced knockdown of bacterial colony-forming units.

The influence of the earthworm microbiome (antibiotic-treated or intact) and the soil microbiome (autoclaved or intact), and their interaction on *L. terrestris* feeding on, and preference for, three plant species litters (*Lolium multiflorum* (ryegrass), *Quercus robur* (oak) and *Fraxinus excelsior*(ash)) was then investigated. Across all earthworm microbiome x soil microbiome treatments, *L. terrestris* showed a greater preference for ash litter ($P < 0.05$) when compared to ryegrass and oak litter: a preference that may relate to differences in litter quality parameters (C: N and polyphenol content). However, disruption of either the soil microbiome, earthworm microbiome or soil and earthworm microbiome resulted in significantly ($P < 0.05$) reduced overall consumption of litter and a shift in litter preference to consume less oak litter.

Finally, in a soil microbial diversity manipulation experiment, the research attempted to examine whether an increase in OM mineralisation in the presence of earthworms would depend on soil microbial diversity and could be linked to microbial diversity impacts on earthworm health. The aim to use the created soil with a gradient of microbial richness and *L.*

terrestris as a test species to examine whether changes in soil microbial richness influence the effect of earthworms on organic matter decomposition to respired CO_2 and whether any effects were linked to earthworm health status that may feedback to ecosystem functioning. Health status was evaluated by assessment of earthworm energy reserves through lipid, carbohydrate, and protein analysis. There was no effect of microbial diversity on earthworm functional impacts on SOM mineralization or earthworm health and therefore does not support the overall hypothesis for this thesis. Taken together, the findings currently suggest that the correlated properties of the soil microbiome that are important for earthworm health and functional role are abundance (biomass) and activity rather than the richness of species present or the presence of specific species or combinations of species. The insensitivity of both earthworm health and function to bacterial species loss suggests that the nature of the function provided by the ingested soil bacterial microbiome to its host is a functionally redundant one, because the functions provided are very generic, as many other microbial groups can provide similar function.

Acronym glossary

ANOVA	Analysis of variance
C	Carbon
CO2	Carbon dioxide
SOM	Soil organic matter
GES	Geography and Environmental Science
SAPD	
K_2SO_4	potassium sulphate
TOC	Total organic carbon
TC	Total carbon
IC	Inorganic carbon
FDA	Fluorescein diacetate
DHA	Dehydrogenase activity
	2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium
INT	chloride
INTF	p-iodonitrotetrazolium formazan

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Chapter 1 General Introduction

1.1 Introduction

The soil ecosystem harbours most of the global ecosystem's biodiversity. It contains a dynamic complex of microorganisms such as bacteria, microfauna (such as nematodes) and macrofauna (such as earthworms), as well as the non-living environment interacting as one functional unit (Guerra *et al.*, 2020; Ritz and van der Putten, 2012). This ecosystem biodiversity plays a critical role in the provision of ecosystem functions and services. Ecosystem services are the benefits that humans obtain from the ecosystem, such as regulating, supporting, provisioning and cultural services. These services include nutrient cycling (Brickhill, 2015; Millennium Ecosystem Assessment, 2005), organic matter mineralisation (Maron *et al.*, 2018), water flow regulation (Esquivel *et al.*, 2020), provision of food (FAO, 2019), and climate regulation (Bardgett and Hefin Jones, 2012; Phillips *et al.*, 2019).

The rise in global challenges due to the rapid increase in the human population, food shortage, the increase in our ecological footprint, rapid changes in the earth's climate, the introduction of invasive exotic organisms capable of changing ecosystem biodiversity and the increasing incidence of droughts and other natural disasters (Bronselaer *et al.*, 2018; Dai, 2013; Geisen, Wall and van der Putten, 2019; Kharin *et al.*, 2007; Popp *et al.*, 2017; Titeux *et al.*, 2016; Wall, Nielsen and Six, 2015) has resulted in a 'trade off' of ecosystem services such as using land mining to support us with energy, agriculture for bioenergy (Geisen, Wall and van der Putten, 2019), and a reduction in soil biodiversity that is pivotal to the ecosystem functions and services (Bardgett and Van Der Putten, 2014; Wagg *et al.*, 2014). All this has highlighted the importance of improving our understanding of soil functioning especially given the pressures on the soil to deliver the services related to addressing the global challenges and the potential feedback between soil and the climate system into the future.

Earthworms are an integral part of soil ecosystems (Kodama *et al.*, 2014)and are often referred to as ecosystem engineers (Edwards, 2004). They have a vital role in soil formation and functioning and essential interactions with microorganisms to regulate soil biochemistry. At the same time as interacting with soil microorganisms to influence soil functions, earthworms also host, and functionally interact with, an active "microbiome" that is mainly soil-derived. The earthworm gut contains an enormous number of microbes, and these gut microbes (gut microbiome) may form a complex symbiotic relationship with their host (Byzov *et al.*, 2009; Knapp *et al.*, 2009). Among these are earthworm impacts on soil OM and in determining the fate of OM amendments to soils to build soil OM content and therefore soil health. Earthworm activity results in OM incorporation and fragmentation in soils, increasing its initial susceptibility to microbial attack. Earthworms also secrete mucus into their gut that primes microbial activity, enhancing decomposition of ingested OM during gut passage (Edwards, 2004). However, earthworms casting activity also stimulates soil aggregation, potentially stabilising soil OM long-term through the occlusion. Through these effects on the bioavailability of microbial substrates, earthworms have a significant role in shaping the structure and function of soil microbial communities and the overall OM dynamics of soils (Blouin *et al.*, 2013; Edwards and Bohlen, 1996).

In recent years, and primarily due to the increased accessibility of high-throughput sequencing, there has been a growing interest in the gut microbiome due to the report of their functionalities and importance for their hosts (Drake and Horn, 2007; Liu *et al.*, 2018). The human gut has one of the most studied microbiomes, with research highlighting the importance of the microbiome in performing several functions that heavily influence host physiology. These studies highlight the established link between the gut microbiome and its role in modulating the host's immune defence (Belkaid and Hand, 2014) and regulating host metabolism (Brubaker, 2018; Fabbiano *et al.*, 2018; Molinaro *et al.*, 2017).

The microbiome for invertebrates is not as well studied in comparison to humans. However, research has indicated that the invertebrate gut microbiome also plays a critical role in the health and functioning of the hosts (Kostic, Howitt and Garrett, 2013). For example, the gut microbiome of the *Drosophila melanogaster* (fruit fly) is known to have inducible responses that enable it to confer local immunity especially following exposure to pathogens (Engel and Moran, 2013; Ha *et al.*, 2005; Schneider and Ayres, 2008). Another example of the important role of the gut microbiome can be demonstrated in termites, where the host's guts have nutritional symbioses with the microorganisms. Lignocellulose is a complex polymer made from cellulose, lignin and hemicellulose and is the most abundant complex of polymers found on earth. It is present in wood and not digestible by humans and animals. However, some termites are remarkably efficient in degrading this polymer due to the endogenous cellulases produced by their microbiome (Lo, Tokuda and Watanabe, 2011; Warnecke *et al.*, 2007). The symbiotic microbiome present in the hindgut of the host is crucial for the degradation of lignocellulose that ultimately leads to the release of compounds that are easily accessible to the host for nutrition (Brune, 2014; Köhler *et al.*, 2012). The important presence of these symbionts for termite nutrition is seen when the loss of symbionts reduces the cellulolytic potential of termite guts (Peterson, Stewart and Scharf, 2015) and a significant reduction in the host's fecundity, weight, and longevity (Rosengaus *et al.*, 2011).

Reciprocally, and as noted previously in other organisms, it is thought that the earthworm gut microbiome forms a symbiotic nutritional relationship with earthworms. It helps with the digestion and the acquisition of nutrients from plant litter for nutritional benefit (Fujii, Ikeda and Yoshida, 2012). In addition to the soil-derived transient microbiome in the gut, we also know that earthworms harbour vertically transmitted and specific symbionts in their nephridia (excretory organs) and that this symbiosis has been demonstrated to influence the reproduction and maturation of the host (Viana *et al.*, 2018).

However, further to these hypothesised or demonstrated roles in nutrition, reproduction, and maturation, it is not clear if the earthworms' microbiome confers any additional functionalities, such as in immunity (Sansone *et al.*, 2015; Zhang *et al.*, 2019) and resistance to pest-induced stress (Kikuchi *et al.*, 2012) as established in other animals.

Whilst there have been numerous studies examining earthworm impacts on soil microbial communities and their activities, there is a lack of research aiming to understand the impact of ingested soil microorganisms on their earthworm host and the potential for general and more exquisite functional roles of earthworm transient microbiomes. The known high microbial diversity in the soil and high levels of microbial functional redundancy has opened a knowledge gap for further research into the role of this diversity for the earthworm microbiome's greater functionality and its support of ecosystem processes, as well as earthworm's health.

1.2 General aim

This PhD project aims to further our understanding of the importance of the earthworm - earthworm microbiome relationship in supporting critical soil ecosystem process such as organic matter mineralisation, explicitly paying attention to the feedback of the presence of the earthworm microbiome to the health and function of earthworms in supporting ecosystem processes.

1.3 Overall hypothesis

Earthworm health and function with respect to the decomposition of added organic matter sources depends on soil microbial biodiversity.

1.4 Specific hypotheses addressed in each research chapter

Chapter 3 An antibiotic -based procedure can be used to produce earthworms with a suppressed microbiome/ near- axenic earthworm.

Chapter 4 Earthworm litter feeding behaviour depends on the soil's and the earthworm's microbial status.

Chapter 5 Soil with a gradient of microbial richness can be constructed using a dilution-to extinction approach.

Chapter 6 The extent of the earthworm-induced increase in OM mineralisation will depend on soil microbial diversity and can be linked to microbial diversity impacts on earthworm health.

1.5 Outline of the thesis

Chapter 2 explores the relevant literature on soil functions, microbial diversity, and the earthworm microbiome to establish a knowledge base for the research's motivation.

Chapter 3 presents a laboratory experiment conducted to assess the efficacy of antibiotic treatment to produce earthworms with a suppressed microbiome/ near- axenic earthworm. The overall objective was to develop and evaluate an antibiotic-based procedure for producing near-axenic specimens of earthworms/ earthworms with suppressed microbiome belonging to epi-anecic (*Lumbricus terrestris*), epi-endo anecic (*Allolobophora chlorotica*) and epigeic species (*Eisenia fetida*) as a first step towards understanding the importance of

the earthworm's gut wall-associated microbiome for earthworm health and ecological function roles. This chapter has been submitted for publication in the European Journal of Soil Biology. It is currently under review and is available as a pre-print at: https://www.biorxiv.org/content/10.1101/2021.04.16.440126v1.

Chapter 4 presents a laboratory experiment using near-axenic epi- anecic (*L. terrestris*) earthworm individuals, created using the method described in chapter 3, to assess the role of the earthworm's tightly-associated microbiome and the soil microbiome in influencing earthworm's feeding activity and choice. It specifically examines the earthworm's response to the lack of microbial community in influencing the earthworm's feeding preferences and behaviour in a food choice chamber containing three plant litter species (oak, ryegrass and ash litter) with different chemical and physical properties. This chapter is formatted for journal submission. Because of the format, there are some repetitions in the methodology.

Chapter 5 presents a laboratory experiment aimed at producing soil with a gradient of microbial richness using the dilution-to-extinction approach and an autoclaving sterilisation process. This chapter also verifies whether the dilution-to-extinction soil series could recover to reach a comparable biomass and activity level during a 27-week equilibration period following its creation. This was carried forward in Chapter 6, where the role of soil biodiversity (richness) was determined whilst considering the potential confounding effects due to unequal biomass.

Chapter 6 uses the soil with a gradient of microbial richness created in Chapter 5 and *L. terrestris* as a test species to examine whether changes in soil microbial richness influences the effect of earthworms on organic matter decomposition to respired CO_2 and whether any effects were linked to earthworm health status that may feedback to ecosystem functioning.

Health status was evaluated by assessment of earthworm energy reserves through lipid, carbohydrate, and protein analysis.

Chapter 7 discusses the key findings of this research, makes general conclusions and suggestions for future work.

Chapter 2 Literature review

2.1 Soil functions and global challenges

The world is faced with some big challenges mainly linked to the rapid growth in the human population (Hinrichsen and Robey, 2000), food security (FAO, 2019; Myers *et al.*, 2014), and climate change (Feulner, 2017). More land is being cultivated for food (Tilman *et al.*, 2011), agricultural practices have intensified to support growing populations (Tilman *et al.*, 2011), increased use of chemical fertilisers and heavy machinery that have adverse effects on the soil structure, plant and soil biodiversity (Song *et al.*, 2017; Tsiafouli *et al.*, 2015; Wardle *et al.*, 1999). For example, herbicides and pesticides remain in the soil for years and impact non-targeted organisms, reducing soil biodiversity (Bünemann, Schwenke and Van Zwieten, 2006). Also, pollution from machinery can run onto the soil, killing microorganisms and changing the soil community structure (Giller, Witter and Mcgrath, 1998). There are also the impacts of anthropogenic changes on global climate change that affect the soil biodiversity due to increases in extreme events such as droughts, heavy rainfall, increases in CO_2, and high temperatures (Eisenhauer *et al.*, 2012; Thakur *et al.*, 2019). All of which is affecting the ecosystem, and if this is not corrected, the loss of biodiversity will proceed at a rate that eventually leads to mass extinction (Barnosky *et al.*, 2011).

The soil ecosystem harbours most of the global ecosystem's biodiversity, which contains a dynamic complex of microorganisms such as bacteria, microfauna (such as nematodes) and macrofauna (such as earthworms) as well as the non-living environment interacting as one functional unit (Guerra *et al.*, 2020). Biodiversity plays a critical role in the provision and functioning of ecosystem services. According to (Millennium Ecosystem Assessment, 2005), ecosystem services refer to the services provided by the ecosystem that benefit humanity. Examples of these essential supporting services are soil formation, nutrient cycling, and water

cycling. There are also provisioning services, which are the products obtained from ecosystems such as food, regulating services such as climate and carbon sequestration, and cultural services. Out of the soil ecosystem services and functions, those determining the fate of organic matter inputs in the soil carbon cycle are key to the soil C and nutrient balance.

2.2 Soil microorganisms and earthworms as key players in organic matter decomposition and stabilisation

2.2.1 The role of microorganisms in OM decomposition and soil C dynamics

Microorganisms play a key role in OM decomposition and soil C dynamics. They are significant agents in decomposition, mineralisation, and organic C protection through soil structure formation (Ghaley, Porter and Sandhu, 2014; Li *et al.*, 2002; Patil *et al.*, 2019; Smith and Smith, 2015).

Soil organic matter (SOM) is a complex of living organisms, fresh organic residues, stabilised organic matter, and decomposing material that influences many biological, physical and chemical properties of the soil (Patil *et al.*, 2019; Paul, 2014), its decomposition is a key process in recycling nutrients as well as determining the amount of soil organic carbon (SOC) within the soil ecosystem (Coleman, Crossley and Hendrix, 2004; Olson, 1963; Smith and Smith, 2015), and influences the biogeochemical cycle and gas emissions that affect other ecosystem services that support humans (Ghaley, Porter and Sandhu, 2014). Decomposition is a complex of several processes that includes fragmentation, changes in physical and chemical structure and ingestion but ultimately involves the conversion of organic compounds to inorganic nutrients (Smith and Smith, 2015; Stuart Chapin, Matson and Vitousek, 2012). A variety of decomposers organisms drives the process of soil OM decomposition. Decomposers are primarily bacteria and fungi that feed on dead OM (Castellano *et al.*, 2015; Stuart Chapin, Matson and Vitousek, 2012). This decomposition

process is largely an enzymatic process (Patil *et al.*, 2019). Both bacteria and fungi secrete enzymes into plant and animal tissues to facilitate the breakdown of the complex organic compounds (Patil *et al.*, 2019; Smith and Smith, 2015).

Soil structure is a key factor in soil functioning, with aggregate stability an indicator of soil structure (Six *et al.*, 2000). Although soil structure and soil aggregates are often used synonymously, soil aggregates are the basic unit of soil structure. They are clusters of soil particles that adhere to each other more strongly than their surrounding particles. They are stabilised by an adhesive agent strong enough to resist a disruptive force such as physical disruption (Dexter, 1988; Lynch and Bragg, 1985; Wilpiszeski *et al.*, 2019). Soil aggregate mediates many physical and chemical processes in the soil (Albalasmeh *et al.*, 2013; Cates *et al.*, 2016; Gupta and Germida, 2015; Six, Elliott and Paustian, 2000; Trivedi *et al.*, 2015). For example, soil nutrient cycling (Wang, Yost and Linquist, 2001), reduced soil erosion (Barthès and Roose, 2002), organic matter protection (Tisdall and Oades, 1982), water regulation (Prove *et al.*, 1990) and soil compaction (Bronick and Lal, 2005), all of which affect soil organic matter dynamics and nutrient cycling (Six *et al.*, 2004).

The link between soil microbial activity and soil aggregate formation and stabilisation has been extensively reviewed (Degens, 1997; Oades, 1993). The varying degree of connection between aggregation and microbial activity is often dependent on the different scales of influence by either fungi or bacteria or if it is a micro-aggregate or a macro-aggregate. For example, the fungal mycelium often referred to as a ‘sticky string bag’ and, in accordance to aggregate hierarchy theory and the pore exclusive principle, is a major factor for the formation of macroaggregates (Bossuyt *et al.*, 2001; Tisdall and Oades, 1982; Tisdall, Smith and Rengasamy, 1997). Fungi and bacteria can also secrete mucilage, a thick, gluey-like substance to enhance the formation of micro-aggregates (Bossuyt *et al.*, 2001; Oades, 1993; Six *et al.*, 2004; Tisdall, Smith and Rengasamy, 1997). This formation of soil aggregates

influence soil carbon dynamics in soil by promoting the storage of C in soil, protect SOC from mineralisation and decrease the loss of soil organic C through erosion (Balesdent, Chenu and Balabane, 2000; Chevallier *et al.*, 2004).

2.2.2 The role of earthworms in OM decomposition and soil C dynamics

Although soil OM decomposition is primarily carried out by decomposers such as microorganisms (Lavelle and Spain, 2001), the process is also facilitated by other decomposer biotas such as invertebrate fauna like earthworms (Ball *et al.*, 2009; Gómez-Brandón *et al.*, 2011). Earthworms play a significant role in soil OM decomposition by significantly accelerating decomposition and nutrient turnover (Lavelle and Spain, 2001). Earthworms indirectly contribute to the decomposition of OM by consuming and mixing fresh litter and pre-existing surface SOM into mineral soil and alteration of the physical structure of the OM through comminution to increase the surface attack by microorganisms (Angst *et al.*, 2019; Edwards and Bohlen, 1996; Jouquet *et al.*, 2006; Ravindran, Contreras-Ramos and Sekaran, 2015; Van Veen and Kuikman, 1990), and reducing the storage of soil C. Conversely, earthworms can also stabilise SOM by promoting the formation of aggregates and stabilisation of SOM (Bossuyt, Six and Hendrix, 2004; Fahey *et al.*, 2013; Martin, 1991; Tisdall and Oades, 1982).

Earthworms mediate soil aggregates through burrowing and cast formation (Brown, Barois and Lavelle, 2000). When earthworms burrow, they exert pressure on the surrounding soil, and the mucus from the earthworms mixes with the oriented clay that lines the surface of the burrow to form a stable structure (Edwards and Bohlen, 1996; Six *et al.*, 2004). When earthworms ingest OM mixed with inorganic soil material, the mixture passes through the earthworms' guts and is excreted as casts (Bossuyt, Six and Hendrix, 2004). The stability of casts depends on the age and dryness, and the quality of the ingested organic matter (Marinissen and Dexter, 1990; Shipitalo and Protz, 1988). Apart from the mediation of

macroaggregates through cast formation, earthworms are also involved in the mediation of micro-aggregate formation (Angst *et al.*, 2019; Barois *et al.*, 1993; Bossuyt, Six and Hendrix, 2005). Many studies have shown that during gut transit, the organic material mixes intimately with mucus to create new nuclei micro-aggregates (Barois *et al.*, 1993), protecting labile SOM and increasing the stabilisation of C in soil (Bossuyt, Six and Hendrix, 2004; Jongmans, Pulleman and Marinissen, 2001; Six *et al.*, 2004).

2.3 Microbial-earthworm interactions in OM decomposition

2.3.1 Direct earthworm stimulation of microbial activity during gut passage and through drilosphere' effects

Apart from increasing the access of OM to microbial decomposers through the mixing and fragmentation of ingested litter through muscular action (Lazcano, Gómez-Brandón and Domínguez, 2008; Ravindran, Contreras-Ramos and Sekaran, 2015; Van Veen and Kuikman, 1990), earthworms contribute towards OM decomposition by stimulating the activity of the ingested soil-derived earthworm gut microbiome. When earthworms ingest SOM, many changes occur to its chemical, physical, and biological properties as the ingested OM transits through the gut until the undigested substance is deposited as casts. These casts are hotspots for intensive microbial activity; they increase OM decomposition and enhance carbon mineralisation (Abail, Sampedro and Whalen, 2017; Brown, Barois and Lavelle, 2000; Tiunov and Scheu, 2000).

In the gut, the fast-growing bacteria and specialised catabolic capabilities are enhanced and contribute to the increase in SOM mineralisation. This promotion of fast-growing bacteria in the gut is referred to as 'the sleeping beauty paradox' (Brown, Barois and Lavelle, 2000; Lavelle *et al.*, 1995). It involves the production of intestinal C-rich mucus ('the kiss') by the earthworm ('Prince Charming'). This process awakens ingested dormant microflora

('sleeping beauties'), thereby increasing the decomposition of ingested OM because of a 'priming' effect (Abail, Sampedro and Whalen, 2017; Hoang *et al.*, 2017; Lavelle *et al.*, 1995).

2.4 The earthworm microbiome

The interest in the earthworms gut associate microbiome has been growing in recent years due to the report that their composition and activity is vital to the host (Sapkota *et al.*, 2020). So far, only two types of gut-associated microbiomes have been described in detail. These include the gut microbial community and the nephridial microbial community (Liu *et al.*, 2018; Singleton *et al.*, 2003; Thakuria *et al.*, 2010).

2.4.1 The gut microbial community

When considering that earthworms species vary broadly based on their burrowing and feeding habits, it is assumed that the earthworms would be exposed to different compositions of food (Aira *et al.*, 2015; Medina-Sauza *et al.*, 2019), which would in effect affect their gut microbiome diversity (Horn, Drake and Schramm, 2006; Thakuria *et al.*, 2010). The gut microbiome can be subdivided into two types, the transient gut microbiome, which includes the most researched types of gut microbiomes and is primarily composed of transient microbes (Drake and Horn, 2007; Zeibich, Schmidt and Drake, 2019), or the resident gut microbiome, based mainly on the microbes that are tightly associated with the gut wall and not egested with the cast (Thakuria *et al.*, 2010; Zeibich, Schmidt and Drake, 2019). As aforementioned, the food source is the major determinant of gut microbial composition. However, characteristically the microbial composition in the gut is different to the surrounding soil or the food eaten (Aira *et al.*, 2015; Knapp *et al.*, 2009; Medina-Sauza *et al.*, 2019). This difference in the microbial composition is based on the selective and filtering ability imposed on the microbes in the earthworm gut. This is largely facilitated by the stable

conditions of the mucus, pH and nutrient pool present in the gut (Drake and Horn, 2007; Lavelle and Spain, 2001; Sapkota *et al.*, 2020), which causes the bacterial community to change as it transits through the gut (Pass, 2015; Sampedro and Whalen, 2007). Also, the unique anaerobic environment of the earthworm gut due to lower oxygen provides the micro-environment that facilitates the activation of anaerobic bacteria that can perform fermentation reactions (Meier, Hunger and Drake, 2018; Pass, 2015; Sun *et al.*, 2020) and results in a significant variation in the microbial diversity when compared to the surrounding soil. Apart from the fermentation products, the unique gut conditions facilitate the stimulation of the denitrifying bacteria essential to produce N_2O and make the gut an ideal environment for the denitrification process (Drake and Horn, 2007; Wüst *et al.*, 2009). Consequently, the environment of the gut ensures that the gut plays a vital role in nutrient cycling and the chemical transformation of the ingested SOM.

Although there are some studies on resident gut microbiomes that are tightly associated with the earthworm gut wall (Thakuria *et al.*, 2010), the research is not as extensive as the transit gut microbiome. However, some research like that of Jolly *et al.*, (1993) indicated the existence of resident gut microbiome. Here using scanning electron microscopic examination of the gut surface of two different earthworm species (*Lumbricus terrestris* and *Octolasion cyaneum*) indicated the presence of similar gut-associated organisms present in all regions of the gut. They were less numerous and less morphologically diverse despite the two earthworms species deriving their food from different sources and therefore supposedly should have different microbial communities as explained before. Another evidence of resident gut microbiome was observed by Singleton *et al.*, (2003) using direct count, culturability studies, 16S rRNA gene clone libraries and fluorescent in situ hybridisation (FISH) on the bacteria associated with the intestine and casts of *L. terrestris*. They observed that many prokaryotes that remained in the intestine after casting were tightly associated with

the earthworm intestine wall. Also, using the bacterial 16S rRNA gene libraries, most of the retrieved phylotypes were either absent or in low abundance in the casts, indicating that the community composition of the resident microbes that are tightly associated with the earthworm guts are opportunist microbes that colonise the intestine under certain conditions and are mostly different to the transient microbes that form the majority found in the cast.

2.4.2 The nephridial microbial community

Although many earthworm microbiome studies have mainly focused on microbiome transit through the earthworm gut, tightly associated with the gut wall and cast (Aira *et al.*, 2015; Thakuria *et al.*, 2010), some studies have suggested the presence of another type of microbiome associated with earthworms. These are nephridia microbiomes that are based in the nephridia. The nephridium is an excretory organ located laterally along the length of the earthworm (Davidson, Powell and James, 2013; Lund, Davidson, *et al.*, 2010; Pinel, Davidson and Stahl, 2008; Schramm *et al.*, 2003; Viana *et al.*, 2018). The nephridium **Figure 2-1** consists of a continuous winding tube that wraps around to form three major loops and a ciliated portion that drives the current through more intricate winding tubes to the bladder, where the fluid gets excreted to the exterior through the nephridiopore. The second loop contains a narrow tube that widens into the ampulla, packed with bacterial cells. This ampulla varies in length depending on the earthworm, with bacterial colonisation restricted to this section of the nephridia (Knop, 1926; Schramm *et al.*, 2003; Villaro *et al.*, 1985).

One of the most studied nephridial microbial communities is found in the lumbricid earthworms. These earthworms harbour specific bacteria (Verminephrobacter; genus) in their excretory nephridia. The *Verminephrobacter* is species-specific, and it occurs throughout the Lumbricidae (Davidson, Powell and Stahl, 2010; Lund, Holmstrup, *et al.*, 2010; Lund, Kjeldsen and Schramm, 2014; Pinel, Davidson and Stahl, 2008; Schramm *et al.*, 2003).

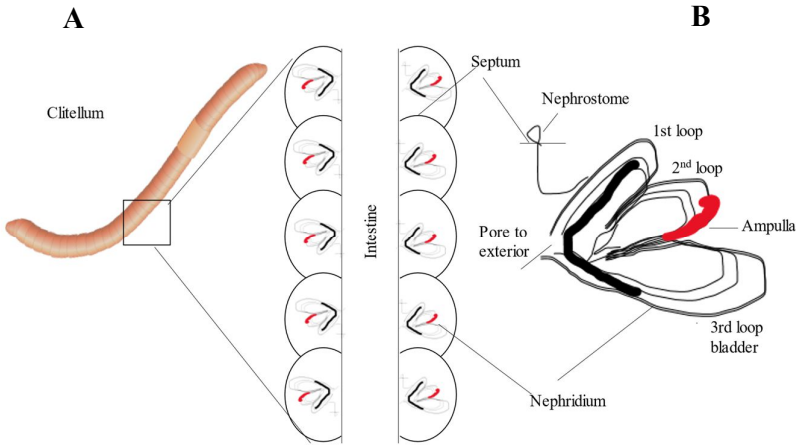

Figure 2-1. (A) Schematic outline of nephridia in an earthworm showing the dissected earthworm with pair of nephridia attached to the body wall in each segment. (B)The detail of a single nephridium showing the three major loops. The symbionts are restricted to the ampulla alone (diagram modified from Schramm *et al.*, 2003; Lund, Kjeldsen and Schramm, 2014)

The *Verminephrobacter* symbionts are passed on directly from parents to offspring through vertical transmission (Davidson and Stahl, 2006). The earthworms are hermaphrodite but still require mating to cross-fertilise by exchanging spermatozoa (Butt and Nuutinen, 1998). The cocoons are formed when the clitellum secretes precapsule. The worm then deposits the eggs cells, stored spermatozoa, and albumin into the precapsule before the earthworm crawls out of the capsule, where fertilisation occurs (Lund, Kjeldsen and Schramm, 2014). The *Verminephrobacter* symbionts of earthworms are deposited into the precapsule, presumably through nephridiopore from the parent earthworm (Lund, Kjeldsen and Schramm, 2014), and during the embryonic development, the symbionts colonise the nephridia (Davidson and Stahl, 2006; Dulla *et al.*, 2012). The colonisation only takes place at the embryonic stage.

This claim is supported in Davidson and Stahl, (2006), where the symbionts were removed using antibiotics, and the juvenile produced were cleared of their symbionts.

The function is of symbionts is still not noticeably clear, but studies that have attempted to understand their importance (Davidson, Powell and Stahl, 2010; Viana *et al.*, 2018) showed that aposymbiotic worms have slower maturity rates and reduced reproductive output. However, in cases such as in Viana *et al.*, (2018), the loss of symbionts did not affect the riboflavin content of the earthworm and therefore is most likely not involved in vitamin B12 provision. Also, in Lund *et al.*, (2010), there were no significant differences in the total organic carbon, total nitrogen, and amino acid content in the cocoons with or without symbionts. These uncertainties found in studies question the hypothesised role of symbionts in their hosts.

2.4.3 The earthworm microbiome and its function

Many studies have shown that host (invertebrate) associated microbiomes play a significant role in their host functioning (Engel and Moran, 2013; Kwong, Mancenido and Moran, 2017). The host-associated microbes have been known to optimise nutrients, when the earthworm has a poor diet, they assist in digesting recalcitrant food components (Hosokawa *et al.*, 2010; Peterson, Stewart and Scharf, 2015). For example, termites rely on the degradation of lignocellulose from wood as a food source. This food provision is only possible because of the presence of microbial communities in their hindguts, which can break down the lignocellulose and release compounds easily accessible by the host. Any disruption to this gut microbiome may lead to reduced host longevity and ability to reproduce (Rosengaus *et al.*, 2011). Some microbes also provide a protective capacity for their host by

providing resistance against bacterial infection (Kim *et al.*, 2015; Weiss, Wang and Aksoy, 2011).

The earthworm microbiome is well described, as seen in **section 2.4**, with the resident and transient microbial communities as well as the nephridial microbial communities playing a significant role in the functioning of the earthworms. Pertaining to the resident and transient microbiomes, some studies have highlighted the significance of the earthworm microbiome in aiding digestion and nutrition (Zeibich, Schmidt and Drake, 2019), mainly since the soil microbiome influences the earthworm microbiome with the help of the gut's favourable conditions. However, it is not noticeably clear if the function is also dependent on the microbiome's diversity.

2.5 Biodiversity and ecosystem function

2.5.1 Hypothetical B-EF relationships

Many years of debates have tried to theorise and experimentally determine the role that biodiversity plays in determining ecosystem functioning (Thompson and Kao-Kniffin, 2017). The review by Nielsen *et al.*, (2011) attempted to determine whether there was a link between species diversity richness and ecosystem process such as C cycling (supporting services). The review used 85 experimental observations from 26 published papers and concluded that the relationship between the species richness and C cycling differs. It can either be a negative, positive or neutral relationship. When considering a possible relationship (negative or positive), there are three forms possible: linear, asymptotic (redundancy) and idiosyncratic **Figure 2-2**.

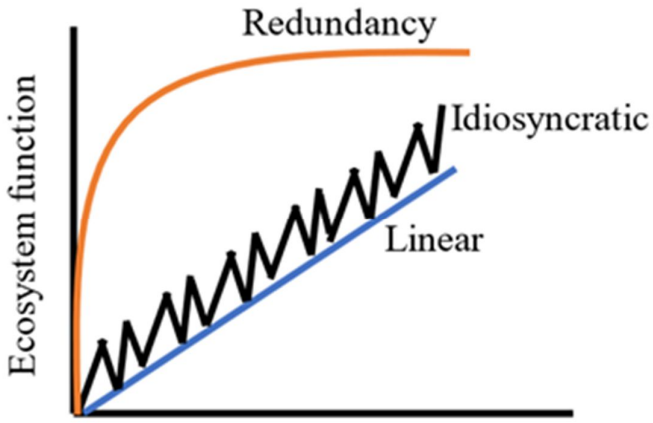

Figure 2-2. Hypothesis of biodiversity and Ecosystem Function (B-EF) relationship postulated redundancy, linear and idiosyncratic responses in ecosystem processes to declining biodiversity. Modified from Nielsen *et al.*, (2011).

A linear relationship indicates a direct relationship between the total number of species and the ecosystem functioning, any loss in biodiversity would imply a loss in ecosystem function. In a redundant relationship, the model assumes that many species have a similar effect on the ecosystem functioning. Any further increase in species richness will result in a diminishing response and make no significant changes to the response (Lawton and Brown, 1994). In an idiosyncratic relationship, the model indicates that the effect of the relationship is not entirely dependent on the species richness because the presence of one unique species has a more significant influence on the ecological process when compared to other species in that community, the composition of the community structure is much more important than the species richness (Nielsen *et al.*, 2011; Philippot, Spor, *et al.*, 2013; Thompson and Kao-Kniffin, 2017)

2.5.2 Microbial biodiversity and soil ecosystem function

So far, this literature review has highlighted a few supporting services of soil microorganisms, such as stabilisation of soil structure, decomposition of OM and nutrient cycling. However, not all microorganisms contribute to each functioning (Coleman, Crossley and Hendrix, 2004); hence, soil microbes can be categorised by their functional groups, a set of microbial species known to perform similar functions (Joseph *et al.*, 2003).

A healthy ecosystem is dependent on the presence of all the functional groups working together, and the loss of a functional group may negatively influence the microbial diversity and impair the provision of the ecosystem supporting services. This is an important but complex argument that involves the link between microbial diversity and ecosystem functioning when discussing the maintenance and conservation of the ecosystem. It is argued that species differ in their influence on ecosystem functioning and that some species are advantageous when present compared to others. Given the estimated 10^4 microbial species present in a 1g sample of soil (Nielsen *et al.*, 2011), it is hypothesised that due to this high soil microbial diversity, there is a chance that for any given ecological function, most of the species performing the function will be redundant and so removing some of the species may not have an adverse effect on the provision of that ecosystem's function. However, it is almost impossible to identify all the species involved in ecosystem functions or that the primary provider of the function does not depend on other unknown species that are not currently identifiable with the methods available. This uncertainty over the effect of biodiversity on ecosystem functioning means that we need a better understanding of the relationship between species diversity and ecosystem functioning.

This literature review has also demonstrated the importance of interactions between soil microbiome and earthworm in carrying out role in nutrition, reproduction, and maturation. What is not very clear if the earthworms' microbiome confers any additional functionalities,

such as in immunity (Sansone *et al.*, 2015; Zhang *et al.*, 2019) and resistance to pest-induced stress (Kikuchi *et al.*, 2012) as established in other animals.

Whilst there have been numerous studies examining earthworm impacts on soil microbial communities and their activities, there is a lack of research aiming to understand the impact of ingested soil microorganisms diversity on their earthworm host and the potential for general and more exquisite functional roles of earthworm transient microbiomes. The known high microbial diversity in the soil and high levels of microbial functional redundancy has opened a knowledge gap for further research into the role of this diversity for the earthworm microbiome's greater functionality and its support of ecosystem processes, as well as earthworm's health.

Author's statement of contributions

Chapter 3

HO, **TPS** and **LJS** conceived the original idea and methodology. This was discussed with **DJS** and **CS**. All authors discussed and agreed with the main focus and ideas of the paper.

The experiment was performed by **HO**, as well as the formal analysis and data curation. **ES** helped with genomic analysis, formal analysis, and the initial data curation.

LJS and **TPS** supervised, visualised, and helped with the writing- review and editing. **DJS** and **CS** also helped with the writing- review and editing. **HO** wrote the main text of the paper.

Chapter 3 Assessing the efficacy of antibiotic treatment to produce earthworms with a suppressed microbiome.

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3.1 Abstract

Earthworms are an integral part of soil ecosystems, especially for their role in soil functions such as organic matter (OM) decomposition and nutrient cycling. Earthworms and microorganisms are interdependent, and a considerable portion of the contribution earthworms make to influencing OM fate is through interactions with microorganisms. However, the importance of the earthworm-associated microbiome is not fully understood, because it is difficult to separate the direct influence of the earthworms from the indirect influence of their microbiome. Here, we evaluated an antibiotic-based procedure to suppress the microbiome of individuals of ecologically-contrasting earthworm species (*Eisenia fetida*, *Lumbricus terrestris, Allolobophora chlorotica*) as the first step towards soil studies aimed at understanding the importance of the earthworm microbiome for host health and function. Individual earthworms were exposed to antibiotics: cycloheximide (150 μg ml^{-1}), ampicillin (100 μg ml^{-1}), ciprofloxacin (50 μg ml^{-1}), nalidixic acid (50 μg ml^{-1}), and gentamicin (50 μg ml^{-1}) either singly or in a cocktail via culture (96 h) in a semi-solid agar carrier. Compared to the non-antibiotic treated control, the cocktail (for all three species) and ciprofloxacin (for *E. fetida* and *A. chlorotica*) treatments significantly reduced ($P<0.05$) culturable microbial abundance on nutrient agar and potato dextrose agar. The microbial counts were reduced to below detection (<50 CFU $individual^{-1}$) for *E. fetida* and *A. chlorotica* receiving the cocktail. Illumina 16S rDNA amplicon sequence analysis of culturable *L. terrestris* -associated bacteria showed that antibiotic treatment influenced community composition revealing putative sensitive (*Comomonas*, *Kosakonia* and *Sphingobacterium)* and insensitive *(Aeromonas, Pseudochrobactrum)* taxa. Overall, we report a rapid, with minimal earthworm-handling, process of creating suppressed-microbiome *E. fetida*, *A. chlorotica* and *L. terrestris*

as a tool to be used in future ecological studies of earthworm microbial interactions affecting host health and function.

3.2 Introduction

Earthworms are one of the most dominant soil invertebrates in terms of biomass (Curry, 1994; Lee, 1985) and are frequently referred to as 'ecosystem engineers' due to their effects on soil structure and nutrient availability (Brown, Barois and Lavelle, 2000). Earthworms have been classified into three main ecological categories (epigeic, endogeic and anecic groups) by Bouché, (1977) based on ecological and morphological characteristics as well as their vertical distribution in the soil profile (Bottinelli *et al.*, 2020; Bouché, 1977; Thakuria *et al.*, 2010). Epigeic species are surface dwelling, non-burrowing and consume decaying plant residues on the soil surface. Anecic worms build permanent vertical burrows but feed on plant litters at the surface or dragged into burrows to be pre-decomposed by microorganisms; endogeic worms inhabit and feed in organo-mineral and deeper mineral horizons (Bouché, 1977; Lee, 1985). Recently, Bottinelli *et al.*, (2020) applied a numerical approach to the classification of earthworms to the ecological categories. This approach enabled a given species to be defined by three dimensions of membership to the three main categories and allowed for species to belong to supplemental intermediary categories (e.g., epi-anecic or epi-endo-anecic).

Earthworms are major players in determining soil organic matter (SOM) dynamics (Bohlen *et al.*, 2004; Ferlian *et al.*, 2018). Earthworms not only stimulate organic matter (OM) decomposition, but they also promote SOM stabilization within soil aggregates (Angst *et al.*, 2019; Bossuyt, Six and Hendrix, 2005). Decomposition is enhanced both by increasing the access of microbial decomposers to OM substrates through mixing and fragmentation of litter (Angst *et al.*, 2019; Jouquet *et al.*, 2006; Ravindran, Contreras-Ramos and Sekaran, 2015;

Van Veen and Kuikman, 1990; Wessels Perelo, Jimenez and Munch, 2006) and by stimulating the activity of the ingested soil-derived earthworm gut microbiome, which accelerates the breakdown of earthworm-ingested OM during gut passage. This latter is referred to as 'the sleeping beauty paradox' (Brown, Barois and Lavelle, 2000; Lavelle *et al.*, 1995). It involves the production of intestinal C-rich mucus ('the kiss') by the earthworm ('Prince Charming'). This process awakens ingested dormant microflora ('sleeping beauties') and thereby increases the decomposition of ingested organic matter because of a 'priming' effect (Abail, Sampedro and Whalen, 2017; Hoang *et al.*, 2017; Jenkinson, 1966; Lavelle *et al.*, 1995).

It has long been suggested that most earthworm species are not capable of secreting the full set of enzymes that are required for the depolymerization of plant-derived polymers. Whilst the possession of endogenous endocellulase genes by some earthworm species has been reported (Nozaki *et al.*, 2009), indicating the ability to digest cellulose, it is thought that even when earthworms can produce endocellulase, their ability to digest and acquire nutrients from plant litter lies fundamentally in their relationship with microorganisms (Fujii, Ikeda and Yoshida, 2012). This is because efficient degradation of a complex polymer such as lignocellulose requires the synergistic action of suites of enzymes, such as hemicellulase, endocellulase, lignin peroxidase and exocellulase, that are primarily secreted by microorganisms (Lynd *et al.*, 2002). The role of the aforementioned 'kiss' may therefore be to stimulate microbial depolymerase production during gut passage to aid acquisition of nutrients from ingested plant litter. However, depolymerase activity in soil is a function of recently secreted enzymes, and those produced in the past and stabilized through association with the soil matrix (Garbuz, Yaroslavtseva and Kholodov, 2016; Tabatabai and Dick, 2002). Therefore, it is not clear if earthworms rely on the microbial production of enzymes during gut transit, or, if already produced enzymes (before ingestion) are sufficient for complete

depolymerisation. In the latter case, earthworms would not depend on ingested microorganisms themselves, but only on their pre-produced enzymes that were obtained through ingestion.

In addition to a role of an active, soil-derived, gut microbiome for host nutrition, it is possible that the earthworm microbiome is also vital for other purposes. For example, many studies have suggested that gut microbiomes of various hosts such as humans, *Drosophila melanogaster* (fruit fly), *Riptortus pedetris* (bean bug) and termites, play essential roles in different physiological processes. This includes immunity (Artis, 2008; Sansone *et al.*, 2015; Tian *et al.*, 2015; Zhang *et al.*, 2019), reproduction (Rosengaus *et al.*, 2011), and resistance to pesticide-induced stress (Kikuchi *et al.*, 2012). The earthworm gut microbiome, and indeed the microbiome associated with the other organs (such as skin and the nephridia), may confer additional functions that extend beyond roles in digestion and provision of nutrients to the host such as functions that affect host sexual maturity and reproduction (Lund, Davidson, *et al.*, 2010; Viana *et al.*, 2018).

Despite the uncertainties regarding the role of the earthworm microbiome in providing nutritional and non-nutritional benefits to the host, comprehensive studies on this topic, and on the role played by the earthworm host-microbiome interaction for ecosystem processes, are lacking. These uncertainties are due to our inability to separate the contribution of the microbiome to host processes. Whilst microorganisms associated with ingested soil and plant material that are transient during gut passage might be removed via depuration of earthworm individuals prior to experiments, distinct microbiome components known to be more tightly host-associated (for example, with the intestinal wall; (Singleton *et al.*, (2003); Thakuria *et al.*, (2010); Gupta, (2013) and Pass, (2015)) would not be removed in this way. Therefore, we need a method to eliminate the non-transient microbiome to allow the understanding of

the contributions of the host, the microbiome (and host x microbiome interactions) to functional effects.

Previous studies have attempted to produce suppressed-microbiome or 'axenic' (where 'axenic' was used as the term to describe earthworm individuals that harbour no cultivable microorganisms as detectable by the method employed) earthworm cultures through the passage of individual animals via sterile solutions or suspensions containing antibiotics, both single antibiotics and cocktail of antibiotics (Hand and Hayes, 1987; Whiston and Seal, 1988). These studies used *Eisenia fetida* as the 'model' organism; presumably because it can easily be reared on a variety of organic substrates (Yasmin and D'Souza, 2010) using standard protocols (ISO, 2012). However, *E. fetida,* an epigeic species, is not a typical soil dwelling earthworm species (ISO, 2012), preferring organic-rich habitats. Hence to understand microbiome effects, there is a need to extend studies to other species of earthworms occupying different niches within the soil.

In this present study, we developed and evaluated an antibiotic-based procedure for producing suppressed-microbiome specimens of earthworms belonging to the epi- anecic (*L. terrestris*) and epi-endo-anecic (*A. chlorotica*) groupings as well as *E. fetida* as a comparatively well-studied comparison. The study, thus, provides a first step towards understanding the importance of the earthworm microbiome for earthworm health and ecological functional roles. We evaluated the effects of antifungal and anti-bacterial antibiotic treatments (individually and in a cocktail) on culturable earthworm-associated colony forming units (CFUs). To further understand how antibiotic exposure influenced the *L. terrestris*-associated culturable bacterial diversity, we used 16S rDNA amplicon sequencing. This provided insights into the taxa specific changes associated with specific treatment knockdowns.

3.3 Materials and methods

3.3.1 Earthworm collection and culture

E. fetida and *L. terrestris* were purchased from Worms Direct (Essex, UK). *A. chlorotica* specimens were collected from the University of Reading dairy farm at Shinfield (grid reference 51.408580, -0.927353) by hand sorting for adult *A. chlorotica*, identified using the guide of Sherlock (Sherlock, 2012). Identified earthworms were washed with autoclaved de-ionised water before transport back to the laboratory in a cool box. Each earthworm species was acclimated to laboratory conditions in the dark at 20 ± 2 ^{o}C for two weeks (Fründ *et al.*, 2010; Sizmur *et al.*, 2017) prior to the start of the experiment in a culture made from Kettering loam and Irish moss peat (2:1 ratio) (Sizmur *et al.*, 2011) and the earthworms were fed Irish moss peat at approximately 1 g earthworm^{-1} week^{-1} after one week of acclimation (Sizmur *et al.*, 2017).

3.3.2 Antibiotic exposure

The adult earthworm individuals selected for antibiotic exposure were responsive to stimuli and visibly healthy. Selected individuals were of similar sizes and weights (within the same species) to avoid the potential for size-specific and weight-specific effects. Following initial depuration (48 h on moist filter paper in the dark), single earthworm specimens were incubated in Duran bottles of either 250 ml (*E. fetida* and *A. chlorotica*) or 500 ml (*L. terrestris*) in volume, containing either 50 ml (*E. fetida* and *A. chlorotica*) or 150 ml (*L. terrestris*) of sterile 0.65 % (m/v) technical agar medium (Fisher Scientific, UK) made with deionised water. The technical agar concentration used resulted in a medium that, as determined in a preliminary experiment, was of a consistency that allowed the earthworms to burrow within the agar. The agar volume ensured that there was an agar depth of at least 10 cm, as this was found to be a suitable depth, especially for the anecic earthworms, to form

vertical burrows(Lowe and Butt, 2005). The agar medium was supplemented with antibiotics (Sigma-Aldrich) added individually or as a cocktail of the five antibiotics in the concentrations shown in **Table 3-1**. The concentration of each antibiotic in the cocktail was the same as the concentration used when a single antibiotic was applied. Hence when combined this treatment provides both a more complex and greater total antibiotic exposure treatment. The anti-bacterial antibiotics belonged to the classes beta-lactam (ampicillin), (fluro)quinolone (ciprofloxacin, nalidixic acid) and aminoglycoside (gentamicin) and were chosen considering: (i) their reported bactericidal, as opposed to bacteriostatic, activity (Baquero and Levin, 2021); to eliminate the possibility of the resumption of bacterial growth following removal from antibiotic exposure): and, (ii) broad spectrum of activity, targeting both Gram negative and Gram positive bacterial species (ampicillin, ciprofloxacin, gentamicin; (Battista, 2015); (iii) their usage in previous regimes for antibiotic treatment of earthworms (Whiston and Seal, (1988), nalidixic acid, gentamicin). Cycloheximide was chosen as the antifungal antibiotic as also based on Whiston and Seal (Whiston and Seal, 1988).

Antibiotics that were not purchased as already-made solutions but in solid form were dissolved in either 0.1 M hydrochloric acid (ciprofloxacin) or distilled water (nalidixic acid) as required to make up stock solutions.

For each earthworm species, triplicate samples for each antibiotic treatment were incubated at 20 ± 2 ^{o}C in darkness for 96 hours following earthworm addition. Control samples with technical agar but without antibiotics added were included (n = 3). The bottles were covered with aluminium foil to prevent earthworm escape, with pin holes in the cover to ensure aeration.

Table 3-1. Antibiotic types and concentrations used to amend agar media for the production of 'axenic' earthworms.

Antibiotic	Antibiotic concentration (µg ml^{-1} agar)
Cycloheximide	150[a]
Ampicillin	100
Ciprofloxacin	50
Nalidixic acid	50 [a]
Gentamicin	50 [a]

[a] Antibiotic concentration used in Whiston and Seal (1988)

3.3.3 Assessment of the abundance and diversity of earthworm-associated culturable microorganisms

3.3.3.1 *Microbial biomass*

After 96-hours of antibiotic exposure, the earthworms were removed from the agar medium with sterile tweezers. No earthworm mortality was recorded during the incubation period and all earthworms had burrowed and were responsive to a physical stimulus. Following removal from the antibiotic medium, earthworms were washed with autoclaved de-ionised water and placed in 10 ml sterile centrifuge tubes. Earthworms were placed in a 4°C cold room for 1 hr and then crushed using sterile glass rods. This 4°C will not kill the earthworms but make them less active and easier to euthanised. One ml of autoclaved de-ionised water was added to the tube, followed by vigorous shaking (250-rev min^{-1} for 2 min). The resulting suspension was serially diluted in triplicate with autoclaved de-ionised water in a ten-fold dilution series (10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}). To determine the number of colony-

forming units (CFUs) of culturable earthworm-associated microorganisms, 20 µl (*E. fetida* and *A. chlorotica*) or 200 µl (*L. terrestris*) of the dilutions were plated on to agar plates following the Miles and Misra method (Miles, Misra and Irwin, 1938) or using the spread plate method, respectively. The differences in the volume plated were due to the drop/spread plating method adopted. The spread plate method was used for *L. terrestris* to facilitate the extraction of DNA from colonies in subsequent analysis (**section 3.3.3.2**). Nutrient agar (NA), that predominantly favours bacterial growth, and potato dextrose agar (PDA), normally used for culturing fungi, were the agar media used. The agar plates were incubated in the dark at 26 °C under oxic conditions. The emerging colonies were observed after 24 hrs and then at two weeks when the colonies were counted. The limit of detection of the plate count method was determined using the volume plated and the dilution factor (Sutton, 2011).

3.3.3.2 *DNA extraction, 16S rDNA sequencing*

Out of the three earthworm species studied, *L. terrestris* (as the only species that had CFUs above detection limits for all antibiotic treatments and both agars) was carried forward for DNA-based analysis of associated microorganisms that were cultured on plates arising from the dilution spread plate estimation of microbial abundance.

For each antibiotic treatment, earthworm individual and agar type, colonies growing across all dilutions were harvested using a sterilised cell scraper. Harvested cells from each plate were initially suspended in 1 ml sterile de-ionised water in a 2 ml centrifuge tube and then the different dilutions of the same replicates were pooled and stored at -20 °C prior to DNA extraction.

Total genomic DNA was extracted from the samples using DNeasy Ultraclean Microbial Kit (Qiagen) according to the manufacturer's protocol. The quality and concentration of the

extracted DNA sample was measured using a NanoDrop spectrophotometer (ND-2000/2000c, NanoDrop Technologies).

A ~550 bp fragment of the V3-V4 hypervariable region of the bacterial 16S-rRNA gene was amplified by PCR with 5'-CCTACGGGAGGCAGCAG-3' as the forward primer and 5'-GGACTACHVGGGTWTCTAAT-3' as the reverse primer. Each reaction was done in a 50 µl reaction using four ng of genomic DNA. Each sample was dual index barcoded following Kozich *et al.*, (2013). The amplification thermal cycling consisted of an initial denaturing step at 95 °C for 2 minutes, followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 55 °C for 15 seconds and extension at 72 °C for 40 seconds, with a final extension step at 72 °C for 10 minutes. All PCR reactions were performed using Q5® High-Fidelity DNA Polymerase (New England BioLabs, USA). Quality and verification of fragment size was performed using gel electrophoresis. Samples were normalised using a SequalPrep Normalisation Plate Kit (Thermo Fisher Scientific, UK) and subsequently pooled. The pooled samples were subsequently run on a 1.2% agarose gel and a ~550 bp fragment was gel extracted using a QIAquick Gel Extraction Kit (Qiagen, the Netherlands). The gel extracted samples were quantified using a Qubit HS DNA Kit (Thermo Fisher Scientific, USA) and diluted to 7 pM using HT buffer. The final library was the run with 10% PhiX using a MiSeq Reagent Kit v3 (600 cycles) on a MiSeq (Illumina, USA). Nucleotide sequence data have been submitted to NCBI and are available under submission number SUB9306713 as part of bioproject ID PRJNA715719.

3.4 Statistical and bioinformatics analyses

The effect of antibiotic treatment on the number of CFUs for each earthworm species (*E. fetida, A. chlorotica,* and *L. terrestris*) was assessed using one-way analysis of variance (ANOVA) followed by post hoc Tukey comparisons, where appropriate ($P<0.05$). Normality

of residuals (Anderson-Darling test) and equal variance (Levine's test) assumptions were verified, and data was square root transformed where required. All analyses for the plate count data were performed with Minitab 19.1.1.

MiSeq reads were demultiplexed using BaseSpace (Illumina, USA). Amplicon sequence variant (ASV) tables were generated using the DADA2 pipeline (Callahan *et al.*, 2016). Briefly, in this procedure, the forward and the reverse reads were inspected for quality. The Phred quality score of the reverse reads was below 30 from 200 bases onwards. This prevented sufficient merging of the forward and reversed reads, and hence only the forward reads were used in further analysis. The last ten bases of the forward reads were trimmed, and trimmed reads were subsequently filtered applying a maxN, maxEE and truncQ value of 0, 2 and 2, respectively. After sample inference, reads were subjected to chimera removal. Filtering of low-quality reads and removal of chimera led to removal of on average 18% of the forward reads per sample. Taxonomy was assigned using the Silva version 132 dataset (Callahan, 2018).

ASVs assigned to eukaryotes, archaea, chloroplasts, and mitochondria or to an unknown phylum or kingdom were removed from the dataset.

All statistical analyses of ASVs data and data visualisations were performed in R v.3.6.3 (R Core Team, 2020).The diversity analysis was carried out using the packages 'phyloseq' (McMurdie and Holmes, 2013) and 'vegan' (Oksanen *et al.*, 2018). Observed and Chao1 richness and phylogenetic diversity measures were used to estimate the alpha diversity. The normality of the dataset was checked using Shapiro-Wilk normality test and the significance of differences between alpha diversity and relative abundance of taxa was evaluated using analysis of variance (ANOVA). For the beta diversity, the principal coordinate analysis (PCoA) based on Jaccard distances using the binary data was used to visualise the similarity

of individual replicates based on the presence and absence of ASVs. The effect of antibiotic treatment on bacterial community patterns were further analysed by permutation analysis of variance (PERMANOVA) based on Jaccard distances with the Adonis function (999 permutations) of the 'vegan' package. The effect of antibiotic treatment on bacterial community patterns was also examined using ANOSIM. 'VennDiagram' was used to construct a logical visualisation of relationships between the bacterial genera present in the antibiotic treatments (Chen *et al.*, 2011).

3.5 Results

3.5.1 Effect of antibiotic treatment on earthworm-associated culturable microbial abundance

The aim was to develop and evaluate an antibiotic-based procedure to eradicate earthworm-associate microorganisms and create 'axenic' earthworms, as far as could be verified using culture-based methods. For the NA plates (**Figure 3-1**a, c, e), although, some individual ANOVA analysis of the antibiotic treatment did not show any effect on the earthworms, an overall ANOVA revealed a significant effect of antibiotic treatments on the number of colonies forming for *E. fetida* ($P < 0.001$), *A. chlorotica* ($P < 0.001$) and *L. terrestris* ($P < 0.001$). The post hoc Tukey test showed that when comparing the effect of the individual antibiotic treatments on earthworm- associated microbial abundance across all three earthworm species, cycloheximide and ampicillin had no significant effect on colony formation compared to non-antibiotic-amended control. All other antibiotic treatments, however, did significantly reduce the microbial burden for at least one earthworm species. The cocktail treatment was the most effective with CFUs on NA reduced to below the limit of detection (<50 CFU/worm) for *E. fetida* and *A. chlorotica* and by more than 2 orders of magnitude for *L. terrestris*. Although the cocktail of antibiotics resulted in the lowest number

CFUs, it did not result in statistically different CFUs when compared to the ciprofloxacin-only treatment in *E. fetida* and *A. chlorotica* (PDA), although this difference between the cocktail and ciprofloxacin-only treatment was significant for *L. terrestris*. For the PDA plates (**Figure 3-1**b, d, f), ANOVA indicated a significant effect of antibiotic treatment on the number of CFUs for E. fetida ($P < 0.001$), *A. chlorotica* ($P < 0.001$), and *L. terrestris* ($P < 0.011$). Post hoc Tukey test indicated that it was only the cocktail treatment that reduced CFUs compared to control consistently across species. CFU numbers for the cocktail were, however, not statistically different when compared to ciprofloxacin, gentamycin, and (for *E. fetida* and *L. terrestris*) nalidixic acid treatments.

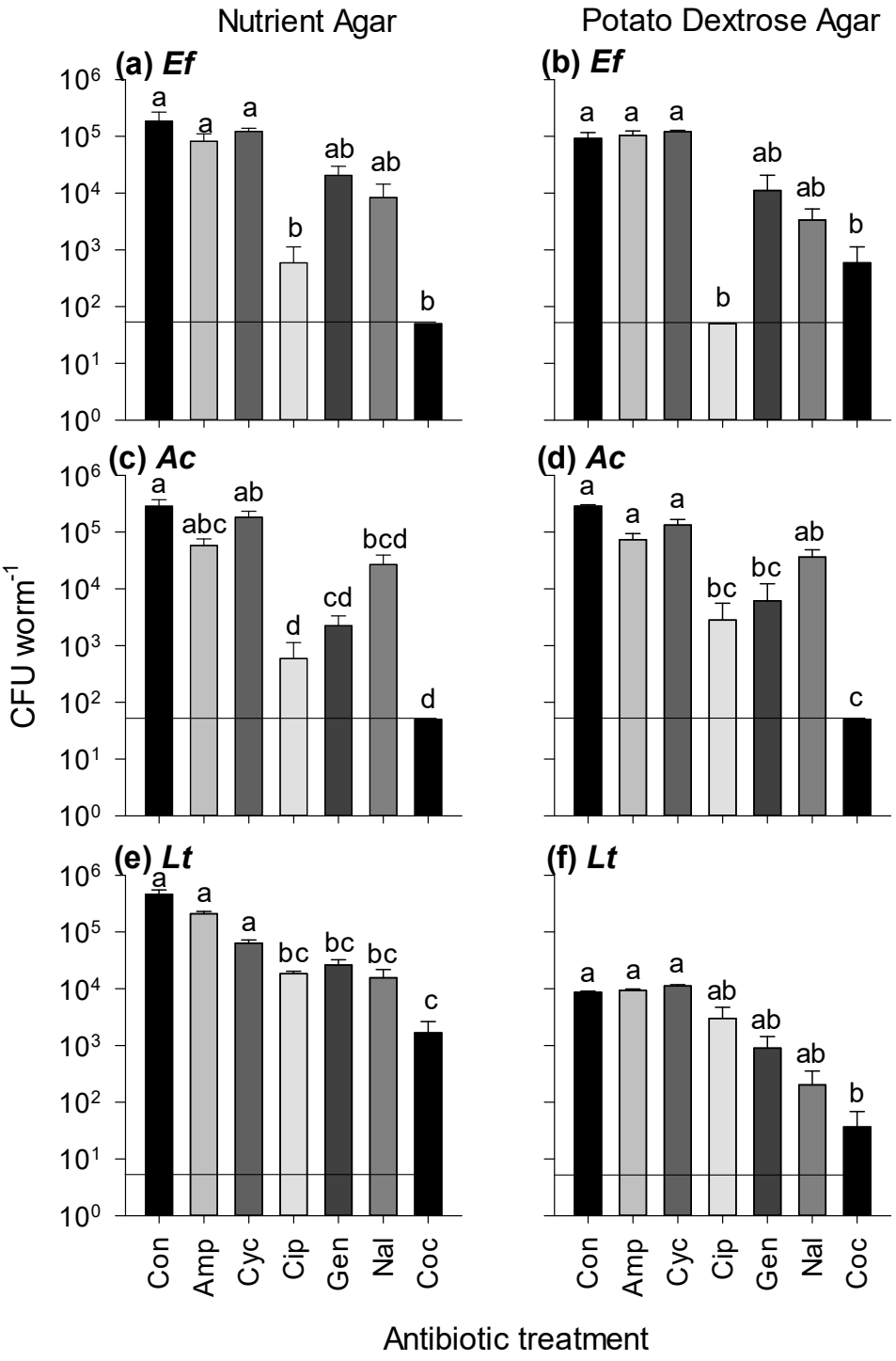

Figure 3-1. The effect of antibiotic treatment on culturable microbial abundance (Colony Forming Units; CFU) associated with: (a,b) *E. fetida* (Ef); (c,d) *A. chlorotica* (Ac); and, (e,f) *L. terrestris* (Lt) on nutrient agar and potato dextrose agar plates. Con = control; Amp = ampicillin; Cyc = cycloheximide; Cip = ciprofloxacin; Gen = gentamicin; Nal = nalidixic

acid; Coc = Cocktail. Values are means ± SE (n = 3). Different letters indicate significant differences between antibiotic treatments applied to the same agar media and the same earthworm species (Tukey HSD test; $\alpha = 0.05$). The horizontal line represents the limit of detection for the method of 50 CFU worm^{-1} (*E. fetida* and *A. chlorotica*) or 5 CFU worm^{-1} (*L. terrestris*).

3.6 Effect of amplicon treatment on *L. terrestris* associated culturable microbial activity

3.6.1 16S rDNA amplicon sequencing

Illumina 16S rDNA amplicon sequencing of DNA extracted from colony forming units harvested from NA and PDA dilution series plates from *L. terrestris* generated 1044826 high quality forward reads with an average of 24965 reads per sample. In total 524 ASVs were identified with an average of 31.5 ASVs per sample. Taxonomy was assigned using Silva database version 132 which resulted in the detected bacteria being classed into 10 phyla, 17 classes, 45 orders, 71 families and 143 genera.

3.6.2 Alpha diversity

The observed and estimated (Chao1) ASV richness between individual *L. terrestris* replicates had a large variation for control (e.g., for Chao1, the coefficient of variation was 82.31 % for NA plates and 39.5% for PDA plates) and some antibiotic-amended treatments (**Figure 3-2**a-d). Against this variable background, one way ANOVA revealed that these alpha diversity measures were not significantly influenced by antibiotic treatment ($P>0.05$; **Figure 3-2**a-d). Similarly, there was no overall effect of antibiotic treatment on Faith's phylogenetic diversity ($P>0.05$; **Figure 3-2**e, f).

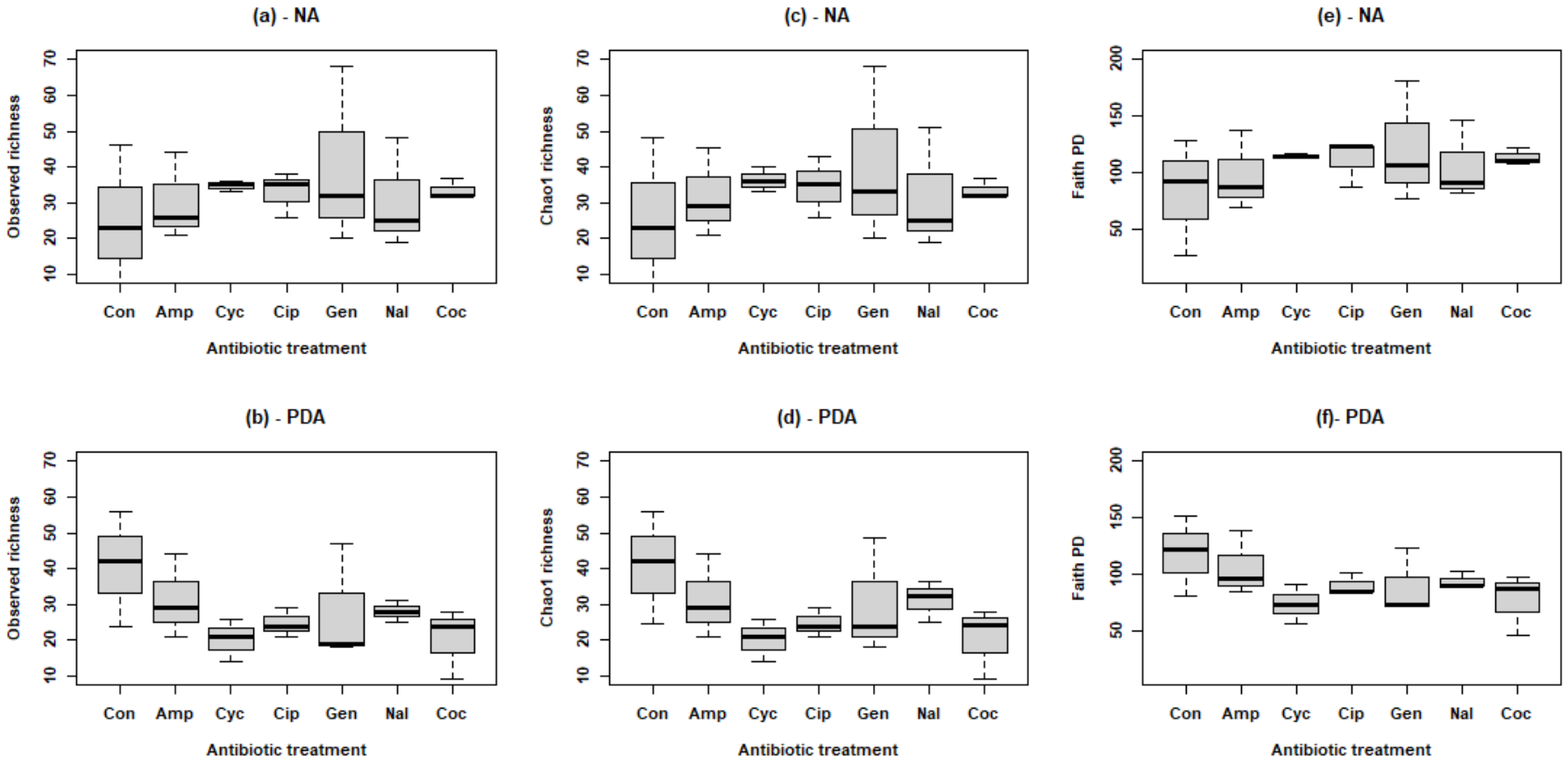

Figure 3-2 Box plots of Chao1 estimated (a, b) and observed (c, d) Amplicon Sequence Variant (ASV) richness and Faith's Phylogenetic Diversity (e, f) for L. terrestris-associated culturable bacterial communities for control and antibiotic-treated earthworm individuals (n=3) as

cultured on nutrient agar (NA; a, c, e) and potato dextrose agar (PDA; b, d, f). Con = control; Amp = ampicillin; Cyc = cycloheximide; Cip = ciprofloxacin; Gen = gentamicin; Nal = nalidixic acid; Coc = Cocktail.

3.6.3 Beta diversity

PCoA based on Jaccard distances was used to visualise the similarity in the data from bacterial community composition for *L. terrestris* samples subjected to the different antibiotic treatments (***Figure* 3-3**a, b). For bacterial communities culturable on NA, the non-antibiotic-treated control samples overlapped with those in the ampicillin- and cycloheximide- treated samples. These clusters appeared distinct from other antibiotic treatment clusters (**Figure 3-3**; NA). The PERMANOVA analysis (P = 0.037; [Adonis]) and weakly, the ANOSIM analysis (P = 0.053) supported that the NA-culturable *L. terrestris* bacterial communities were significantly affected by the antibiotic treatments. The data from the PDA-cultured bacterial community (***Figure* 3-3**; PDA), also showed that communities from control, ampicillin- and cycloheximide-treated *L. terrestris* clustered together and were separated from the clusters of bacterial communities from *L. terrestris* treated with nalidixic acid, ciprofloxacin, gentamicin, and cocktail. Both PERMANOVA ($P = 0.024$) and ANOSIM analysis ($P = 0.009$) revealed an overall significant difference between treatment groups. However, for both NA and PDA it is notable that, with few exceptions (ampicillin and control for PDA), individual within-treatment replicates did not group closely together within the ordination space.

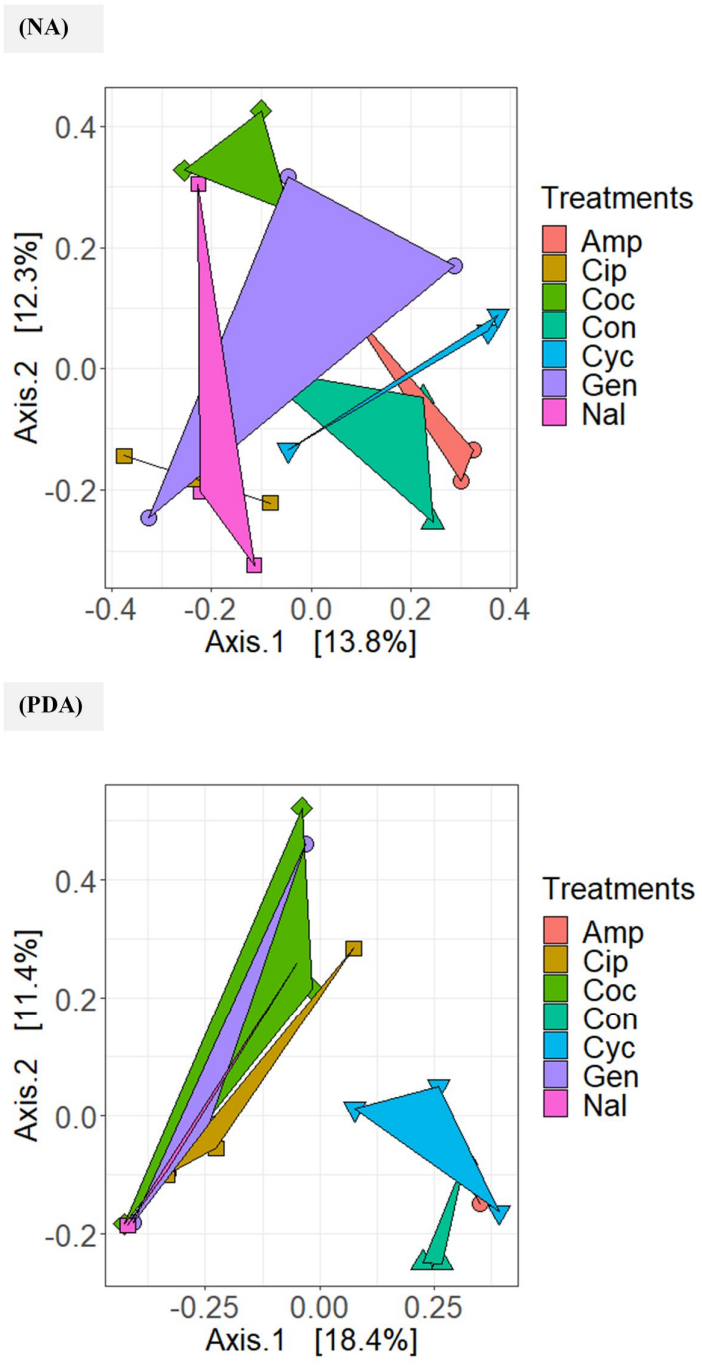

Figure 3-3. Principal coordinates analysis ordination based on Jaccard distances examining the similarity of composition of culturable bacterial communities for control and antibiotic-treated L. terrestris individuals (n=3) as determined by 16S rDNA amplicon sequencing of

colonies cultured on NA and PDA. Con = control; Amp = ampicillin; Cyc = cycloheximide; Cip = ciprofloxacin; Gen = gentamicin; Nal = nalidixic acid; Coc = Cocktail.

3.6.4 Cultivable shared and unique genera of *L. terrestris* individuals

Given the variability in both alpha and beta diversity at the individual *L. terrestris* (***Figure 3-2***; **Figure 3-3**), Venn diagrams were used to visualise genera that were unique or common to more than one *L. terrestris* individual within the same treatment, with a focus on the non-antibiotic-treated control [to understand the initial variability in the culturable *L. terrestris* microbiome **(Figure 3-4**a, b)] and the cocktail-treated (**Figure 3-4**c, d) *L. terrestris* individuals [as the treatment that most significantly impacted culturable *L. terrestris*-associated bacterial abundance (**Figure 3-1**e, f)]. For nutrient agar, one genera (*Lelliottia*), was consistently detected across control *L. terrestris* individuals (**Figure 3-4**a). Whilst *Lelliottia* could still be detected in 2 out of 3 cocktail-treated individuals (**Figure 3-4**c), two other genera, Aeromonas and *Pseudochrobactrum*, were core in cocktail-treated individuals (**Figure 3-4**c). Whilst *Aeromonas* was present in the microbiome of two of the NA controls (**Figure 3-4**a) (and in all individuals for both control and cocktail treatments for PDA, **Figure 3-4**b, d), *Pseudochrobactrum* was not present in any other situation. In addition to *Aeromonas*, 7 other genera were core to control *L. terrestris* individuals on PDA (**Figure 3-4**b). Out of these, *Pseudomonas, Raoultella* and *Verminephrobacter* were still detected in two of the individuals treated with the antibiotic cocktail (**Figure 3-4**d). However, *Comomonas, Kosakonia, Pedobacter* and *Sphingobacterium* could not be detected in cocktail PDA plates (**Figure 3-4**d), and, except for *Pedobacter,* were similarly not present in the cocktail treatment for NA plates when they were detected in at least one NA control individual (**Figure 3-4**a, c).

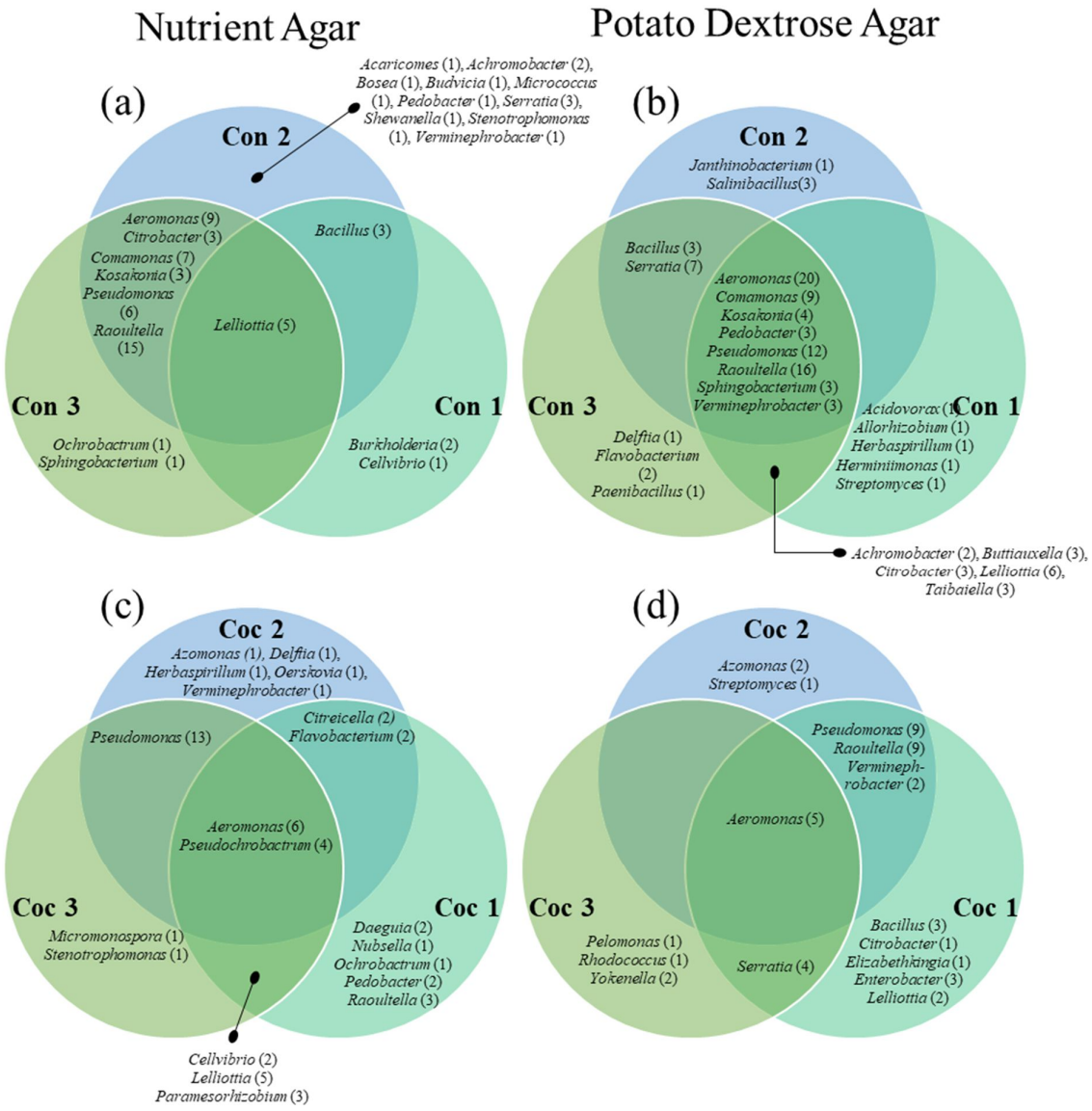

Figure 3-4. Venn diagram visualisation of genera that are unique or common to more than one *L. terrestris* replicate individual within the control (a, b; replicates Con 1, Con 2, Con 3) and cocktail (c, d; replicates Coc 1, Coc 2, Coc 3) treatments on nutrient agar (NA; a, c) and potato dextrose agar (PDA; b, d). The numbers in the brackets are the number of ASV representatives within each genera

3.7 Discussion and Conclusion

Earthworms are key soil organisms contributing to ecosystems processes and associated services (Angst *et al.*, 2020). It is recognised that earthworms harbour an abundant and diverse microbiome (Medina-Sauza *et al.*, 2019). However, there are considerable uncertainties regarding the role of the earthworm microbiome in providing nutritional and non-nutritional benefits to the host and the consequences of the earthworm host-microbiome interaction for ecosystem processes such as OM decomposition. In this study we investigated the potential of antibiotics to create suppressed-microbiome earthworms for subsequent use in experiments aiming to improve our understanding of the role that the earthworm microbiome plays in host health and function. Previous studies have been carried out to produce 'axenic' *Eisenia fetida* (Hand and Hayes, 1987; Whiston and Seal, 1988). However, the applicability of this method to species that can be considered more typical soil inhabitants was uncertain. Accordingly, here we extend the previous studies to consider species representing different earthworm ecotypes covering epi-anecic (*L. terrestris*) and epi-endo-anecic ('intermediate'; *A. chlorotica*) ecological groups (according to Bottinelli et al.'s (Bottinelli *et al.*, 2020) re- classification).

As well as examining the impact of the various antibiotic treatments on the earthworm-associated microbial abundance, we additionally report on the diversity (richness) and composition of the culturable microbiome of *L. terrestris* and its response to antibiotic treatment.

Overall, we have shown that is it possible to significantly reduce the abundance of earthworm-associated culturable microorganisms through the treatment of earthworm individuals with antibiotics or antibiotic cocktail. Our approach is suitable for use in *E. fetida* and the soil dwelling species *L. terrestris* and *A. chlorotica*. However, the efficacy of

antibiotic treatment depended upon the antibiotic(s) used and the earthworm species. Evaluation of the efficacy of antibiotic treatment also depended on the agar medium used for microbial enumeration. In relation to the agar medium, we noted that colonies forming on PDA, like those for NA, were small and smooth resembling bacterial growth. Although PDA is associated with the cultivation of fungi (not bacteria), the composition of the medium (potato extract, glucose) does not select against bacterial growth. It contains glucose as a readily utilised C source. Given this colony appearance and also the observation that CFU abundance on PDA was not affected by the antifungal cycloheximide treatment (**Figure 3-1**), we assumed that colonies forming on PDA were bacterial.

Only the cocktail of five antibiotics (ampicillin, ciprofloxacin, cycloheximide, gentamicin and nalidixic acid) resulted in culturable numbers significantly lower than the control for both NA and PDA agar across all earthworm species (**Figure 3-1**), whilst ampicillin and cycloheximide mostly showed no significant differences when compared to the control. Resistance to ampicillin, a beta-lactam antibiotic, is known to be naturally prevalent among soil bacteria(Armalytė *et al.*, 2019; Demanèche *et al.*, 2008), and cycloheximide, an antifungal, is expected not to be effective on most bacteria (Badalucco *et al.*, 1994).

It was possible, however, through the treatment of *E. fetida* (NA) and *A. chlorotica* with the antibiotic cocktail to reduce the abundance of earthworm-associated microorganisms from ≥ 10^5 CFU per earthworm individual to below the limit of detection (50 CFU/ earthworm in our study). This agrees with previous studies by Hand and Hayes, (1987) and Whiston and Seal, (1988) that have also applied antibiotics to create earthworms (*E. fetida*) deemed 'axenic' with no associated microorganisms detectable by culture or $<10^1$ of microorganisms per worm. Although some antibiotics used in this study are similar to their studies, the media used differ, as well period of handling.

Whilst the application of the antibiotic cocktail [and ciprofloxacin applied singly for *E. fetida* (PDA)] reduced culturable microbial abundance to below detection in *E. fetida* and *A. chlorotica*, microbial numbers were not reduced to below detection limits for *L. terrestris*, although a significant >100-fold knockdown was recorded in this species for the cocktail. To be exposed to antibiotics, through both dermal and gut contact, earthworm individuals needed to burrow and ingest agar. Differences in burrowing behaviour between species may influence the degree to which earthworms are exposed to antibiotics, and therefore the effectiveness of the antibiotic treatment. Also, there may also be different exposure levels in different bacterial populations. Bacteria in the gut are likely to receive a high dose, whereas the nephridial symbionts may be more 'protected' against antibiotics due to their embedment in an organ that may be more 'sealed' from antibiotics. *L. terrestris*'s natural behaviour is to create permanent vertical burrows, travelling to the surface to feed on partially decomposed plant litters and other organic matters (Nuutinen and Butt, 2003; Potvin and Lilleskov, 2017). Although we scaled up agar volumes to accommodate the larger *L. terrestris* size and burrowing behaviour, it is possible that *L. terrestris* individuals did not explore and ingest the antibiotic-containing agar to the same extent, resulting in reduced exposure. In this case, increasing the concentration of antibiotics in the agar or the time of exposure might have improved the effectiveness of the antibiotic treatment. Alternatively, the *L. terrestris* microbiome may harbour a larger number of culturable antibiotic-resistant microorganisms (Li *et al.*, 2011; Zhu *et al.*, 2019). Earthworms are known to produce their own antimicrobial agents (Zhu *et al.*, 2019) which might lead to earthworm species-specific selection of antibiotic resistance traits within the microbiome.

Although based on methods of Hand & Hayes (1987) and Whiston and Seal (1988), our approach differed from previously published work in terms of the spectrum of antibiotics applied. Nalidixic acid, gentamicin, a penicillin (ampicillin) and cycloheximide (Whiston and

Seal, 1988) or cycloheximide (Hand and Hayes, 1987)were in common with the previous studies, but, additionally ciprofloxacin (a fluoroquinolone) was included as an antimicrobial not tested previously. In most cases ciprofloxacin, when applied alone, was just as effective in reducing culturable numbers as the cocktail treatment. This effectiveness may be related to its broad-spectrum DNA gyrase inhibitory activity against both Gram-negative and Gram-positive bacteria (Campoli-Richards *et al.*, 1988). Nalidixic acid similarly inhibits bacterial DNA gyrase (Bourguignon, Levitt and Sternglanz, 1973; Goss and Cook, 1975) whereas gentamicin has a different mode of action making it effective only towards Gram-negative bacteria (Lin *et al.*, 2011).

As well as differences in the choice of antibiotics used, our method also varied from previously published work in terms of the methodology and duration of antibiotic exposure. We used sterile semi-solid technical agar as the 'carrier' for antibiotic exposure. In contrast, previous studies used aqueous solutions (Hand and Hayes, 1987) or sterile suspensions of microcrystalline cellulose (Whiston and Seal, 1988). Our exposure period (4 days) was comparable to that employed by Whiston and Seal, (1988) (5 days), but shorter than the 35 days adopted by Hand and Hayes, (1987) and consisted of a single exposure step as opposed to one (Whiston and Seal, 1988) or several (Hand and Hayes, 1987) transfers of earthworm individuals between different antibiotic-containing media. Reducing the timescale of exposure and the degree of earthworm handling reduces the risk of earthworm mortality. In our trial, all earthworm specimens survived after the exposure to the antibiotic when using response to touch stimuli as a superficial measurement of health condition. The lack of mortality indicates low acute stress (but chronic impacts may have occurred undetected) and provides viable earthworm individuals for use in future experiments.

For *L. terrestris*, 16S rDNA amplicon sequencing of the NA- and PDA- grown bacterial communities was applied to characterise the richness and composition of the culturable

microbiome of *L. terrestris* and its response to antibiotic treatment. For reasons previously discussed, PDA-grown colonies were assumed to be bacterial and were included in the 16S rDNA-based sequencing effort. This enabled the characterisation of potentially different, agar specific, microbiomes due to the selective nature of bacterial growth (Bonnet *et al.*, 2020).

Whilst cognisant that the bacteria that can be cultured on laboratory media are only a very small proportion of the total diversity and therefore that we have not captured what might be a significant non-culturable fraction (Walsh and Duffy, 2013), we focussed on culturable microbiomes (i.e., amplicon sequencing from colony-extracted DNA). This was because we were concerned that amplification of bacterial DNA directly extracted from earthworm tissues would not be able to distinguish between DNA from living bacterial cells surviving the biocidal treatments and those that had been recently killed (Emerson *et al.*, 2017). Amplification of DNA from dead microorganisms would undermine the identification of bacterial taxa that escaped the effect of the antibiotic treatment. Since this culture-based approach will mean that the relative read abundance of a given ASV in a sample will depend not only on the original cell abundance in the earthworm sample but also confounded by the subsequent rate of multiplication on agar, the subsequent analysis of diversity and taxonomic composition was based on presence/absence, not relative abundance.

Comparison of estimated Chao1 ASV richness and observed richness suggested that the sequencing depth covered the richness of ASVs present. However, there was substantial within-treatment variation in ASV richness, including for the non-antibiotic-treated controls. Due to the destructive nature of sampling, it was not possible to examine the impact of antibiotic treatment on the microbiome for a given earthworm individual (i.e., before and after treatment). That there was no significant effect of antibiotic treatment on either ASV richness (Observed and Chao1) or phylogenetic richness, even for antibiotic treatments that significantly reduced the number of culturable bacteria (Cocktail (NA & PDA) and

ciprofloxacin (NA); **Figure 3-2**), might be partly due to initial variability in bacterial richness between earthworm individuals (**Figure 3-4**) going into the incubation. This variability is in agreement with other reports of high variability in host -associated microbiomes (Sapkota *et al.*, 2020; Swart *et al.*, 2020; Thompson *et al.*, 2017). When compared to other studies on earthworm-associated bacterial richness (Aira, Pérez-Losada and Domínguez, 2018; Sapkota *et al.*, 2020; Swart *et al.*, 2020), our analysis revealed a low number of ASVs per worm (e.g. ~30 ASVs for the NA control). However, this is expected due to the focus on only those bacteria that formed colonies on the NA and PDA medium. In addition, the *L. terrestris* individuals in the current trial were depurated before the plating of earthworm samples. This means that the culturable microbiome in our study was likely not dominated by diverse transient microbes associated with the ingested loam: peat substrate but those more tightly-associated with the gut and other organs (Swart *et al.*, 2020).

Whilst there was no significant impact on the richness of ASVs, PERMANOVA and ANOSIM analysis suggested an impact of antibiotic treatment on community composition. The PCoA (**Figure 3-3**) highlighted the variability between within-treatment replicates but suggested that the bacterial community compositions for the antibiotic treatments (cocktail, ciprofloxacin) that caused the most significant reduction in culturable abundance (**Figure 3-1**) were among the most dissimilar to the control.

Genera common to more than one *L. terrestris* individual within the same treatment were visualized by Venn diagrams (**Figure 3-4**) to identify core members of the culturable microbiome and those genera sensitive or tolerant to antibiotic treatment. The core bacterial diversity (phyla level) of the *L. terrestris* culturable microbiome composed of members of the *Proteobacteria* (*Aeromonas, Comomonas, Kosakonia, Lelliottia, Pseudomonas, Raoultella, Verminephrobacter spp.*) and *Bacteroidetes* (*Pedobacter, Sphingobacterium spp.*). This composition is in broad agreement with the earthworm-associated phyla described in other

earthworm microbiome studies (Knapp *et al.*, 2009; Singh *et al.*, 2015; Sun *et al.*, 2020). In particular, members of the genus *Verminephrobacter* are known symbionts found in Lumbricid earthworms and have a known nephridial association (Davidson, Powell and James, 2013; Lund, Kjeldsen and Schramm, 2014; Viana *et al.*, 2018). *Aeromonas*, a genus consisting of facultative anaerobic species, are a further taxa that are frequently earthworm-associated including with *L. terrestris* (Meier, Hunger and Drake, 2018; Zeibich, Schmidt and Drake, 2019).

Among taxa indicating potential resistance, the near ubiquitous detection of *Aeromonas* in the culturable microbiome of both control and antibiotic cocktail treated individuals suggests that representatives of this genus were resistant to antibiotic treatment. *Aeromonas* are considered to be naturally resistant to β-lactam antibiotics, such as ampicillin (Saavedra *et al.*, 2004; Zdanowicz, Mudryk and Perliński, 2020) and resistance to ciprofloxacin and nalidixic acid has also been reported for environmental strains, including multi-antibiotic resistance (Zdanowicz, Mudryk and Perliński, 2020). In contrast, resistance of this genera to gentamicin appears to be rare (Skwor *et al.*, 2020; Zdanowicz, Mudryk and Perliński, 2020). Further characterization of the antibiotic resistance profile of our *Aeromonas* isolates would be required to discern if these strains were indeed gentamicin resistant as may be suggested by their presence in the cocktail exposure or, alternatively, evaded exposure. *Aeromonas hydrophila* has been isolated from the coelomic cavity of *L. terrestris* (Marks and Cooper, 1977). If *Aeromonas* were in this compartment, their exposure may be more limited than for bacteria in the gut. The organ-specific location of *Verminephrobacter* may similarly result in a lower exposure for members of this genus.

In contrast to the apparent tolerance of *Aeromonas* species to the antibiotic exposure, bacteria belonging to the genera *Comomonas*, *Kosakonia* and *Sphingobacterium* that were part of the core in control *L. terrestris* were not detected in cocktail-treated individuals. This absence

suggests a possible antibiotic sensitivity of these strains. No antibiotic resistance genes have been annotated in environmental isolates of *Comamonas* (Jiang *et al.*, 2018) and we could not find reports of resistance traits in environmental *Kosakonia* and *Sphingobacterium* strains. The genus *Pseudochrobactrum*, however, was not detected in control individuals but was present in all cocktail-treated individuals (NA) suggesting that antibiotic treatment potentially promoted the growth of this putatively multi-antibiotic resistant group to densities above the limit of detection of the spread plate. We could not find any previous descriptions of the resistance of *Pseudochrobactrum* to the antibiotics used here. Further characterization is required to verify the antibiotic resistance profile of this group and to explore the earthworm as a bacterial environment conducive to acquisition of antibiotic resistance genes, particularly under the pressure of antibiotic selection (Van Hoek *et al.*, 2011).

In conclusion, we have shown that is it possible, across three ecologically-contrasting earthworm species (*E. fetida*, *L. terrestris, A. chlorotica*), to significantly reduce the abundance of earthworm-associated culturable microorganisms through a 96 h exposure of earthworm individuals to a cocktail of antibiotics containing cycloheximide (150 μg ml^{-1}), ampicillin (100 μg ml^{-1}), ciprofloxacin (50 μg ml^{-1}), nalidixic acid (50 μg ml^{-1}), and gentamicin (50 μg ml^{-1})) in a semi-solid agar carrier. Abundance was reduced to below detection limits (50 CFU $individual^{-1}$) in *E. fetida* and *A. chlorotica* and by >100-fold for *L. terrestris* with accompanying shifts in *L. terrestris* bacterial community composition. The culturable bacterial microbiome of control (non-exposed) and antibiotic cocktail-exposed *L. terrestris* individuals revealed between-individual variability in richness and diversity but also 'core' genera that were putatively sensitive (*Comomonas*, *Kosakonia* and *Sphingobacterium)* or resisted *(Aeromonas, Pseudochrobactrum)* antibiotic exposure. This characterization of the efficacy of antibiotic treatment in suppressing the microbiome of *E. fetida*, *A. chlorotica* and *L. terrestris* individuals provides the foundation for future

experiments aimed at understanding the importance of earthworm-associated microorganisms, be they transient gut inhabitants or more permanently-associated, for host health and ecosystem functioning.

Chapter 4 Assessing the role of the earthworm's tightly-associated microbiome and soil microbiome in influencing earthworm's feeding activity and choice.

4.1 Introduction

In terrestrial ecosystems, leaf litter is a significant source of soil organic matter (SOM), nutrients and acts as an interface between the aboveground and belowground ecosystems (dos Reis Martins and Angers, 2015; Wardle *et al.*, 2004; Yan *et al.*, 2018). Soil macroinvertebrates, play a crucial role in the fate and decomposition of leaf litter inputs (Hättenschwiler, Tiunov and Scheu, 2005; Liu *et al.*, 2019; Ohgushi, Wurst and Johnson, 2018) through their stimulation of microbial primary decomposer activities (Hättenschwiler, Tiunov and Scheu, 2005; Lavelle, 1997).

Among the soil macroinvertebrates, earthworms are widely recognised and abundant key organisms (Hendrix *et al.*, 2008; Lavelle, 1997; Lavelle *et al.*, 2016). Through their litter bioturbation mechanisms, earthworms fragment and mix litter with soil initially increasing microbial access for decomposition and also expose ingested material to ingested soil, microbes, and mucus present in the gut. The mucus exposure activates dormant soil microbes (through a priming effect) necessary for OM decomposition and further exposure for microbial activity (Guhra *et al.*, 2020; Zhang *et al.*, 2016). Hence, the earthworm plays a significant role in stimulating the microbial decomposition of plant litter (Blouin *et al.*, 2013; Cesarz *et al.*, 2016; Edwards, 2004; van Groenigen *et al.*, 2015; Lavelle *et al.*, 2016; Melman *et al.*, 2019).

Earthworms can be typically categorised into three functional groups: epigeic species, endogeic species and anecic species, based on ecological and morphological characteristics and their vertical distribution (Bouché, 1977; Thakuria *et al.*, 2010). Recently, Bottinelli et al. (2020) applied a numerical approach to the classification of earthworms to ecological

categories. This approach has enabled each species to be defined by three percentages of membership to the three main categories: their projection to zones in a ternary plot with zones for the three main categories at the poles and allowing for other zones (e.g., epi-anecic or epi-endo-anecic) in between these poles. Going by the general classification described by Bouché, epigeic species, for instance, are surface-dwelling, non-burrowing and consume decaying plant residues on the soil surface. Anecic worms build permanent vertical burrows but feed on plant litter at the surface or dragged into their vertical burrows to be pre-decomposed by microorganisms. Endogeic species live in the upper mineral soil layer and feed on mineral soil material and decaying soil organic matter (Bouché, 1977; Eisenhauer, 2010; Lee, 1985; Wu *et al.*, 2017). It is likely that earthworms from different ecological groups act synergistically to stimulate organic matter decomposition but out of the ecological groups, anecic earthworms might be the most influential in determining the fate of fresh litter through their role in dragging litter into their burrows, hence the focus of this experiment.

Although the feeding ecology of earthworms and the general importance of litter quality to decomposers has been widely acknowledged (Ashwood *et al.*, 2017; Hendriksen, 1990; Neilson and Boag, 2003; Rief, Knapp and Seeber, 2012; Seeber *et al.*, 2009), with several studies revealing earthworm feeding choice through gut content analyses (Hendrix *et al.*, 1999), palatability tests (Hendriksen, 1990), recording of ingestion and consumption rates (Daniel, 1991; Holdsworth, Frelich and Reich, 2012), isotopic measures (Briones *et al.*, 2001; Dungait *et al.*, 2008), and measuring growth rates of worms fed on different substrates (Satchell and Lowe, 1967), the outcomes of these feeding ecology studies suggest that earthworms might be selective in their feeding activity and hence show preferences for the consumption of different types of litter (Seeber *et al.*, 2009). According to these studies, earthworms, when given a choice, express a preference for high-quality food sources with low C: N ratios (Hendriksen, 1990), avoiding foods rich in lignin (due to its physical

toughness) (Edwards, 1974; Rief, Knapp and Seeber, 2012) or tannins and polyphenols, a defence chemical to reduce herbivory (Cornelissen and Thompson, 1997; Duffey and Stout, 1996; Endara and Coley, 2011; Hättenschwiler, Tiunov and Scheu, 2005; Liebeke *et al.*, 2015) and they prefer senescent litters as opposed to fresh litters possibly due to an undesirable higher content of total phenolic compounds in the latter (Ashwini and Sridhar, 2005; Rief, Knapp and Seeber, 2012).

Some studies have looked at the role of soil microbial composition in the feeding ecology of earthworms by looking at soil microbes' role in making plant litter more desirable for consumption. These studies focus on the importance of microbes to litter polyphenols and their preferences. Microbes break down the complex structures of litter by altering their chemical and physical nature through exoenzyme activity to make them fragile and more desirable for earthworm consumption (Šlapokas and Granhall, 1991; Suberkropp, Arsuffi and Anderson, 1983; Veen *et al.*, 2019).

Role of the soil and earthworm microbiome in litter decomposition

When the litter is consumed by the earthworm, the activities of the co-ingested soil microbiome and the potentially less transient, more tightly earthworm-associated microbiome contribute to litter (and also other SOM) decomposition in the earthworm gut and confer a nutritional benefit to the host (Fujii, Ikeda and Yoshida, 2012). However, it is not clear if the soil and earthworm microbiome also perform other beneficial function (Sansone *et al.*, 2015; Zhang *et al.*, 2019).

Whilst previous feeding ecology studies suggest that earthworm food preference is influenced by the activity of the leaf litter microbiome, it is not clear if the presence and activity of the soil and earthworm microbiome also feedback to influence earthworm behaviour in this respect. If earthworm health is compromised by lack of microbiomes that provide general

nutritional and/or other benefits that are independent of food quality, then behaviour may alter non-specifically through reduced or increased feeding. If the microbiome benefits relate to a detoxification or nutritional function that depends on the food source quality and the earthworm can sense this function is absent (and the quality of the food) it might then change food preferences.

In this present study, we hypothesised that (1) L. terrestris would show a feeding preference related to litter quality determined by the carbon: nitrogen ratio (C: N) and phenol concentration. (2) Litter feeding behaviour (both quantity of litter consumed and preference for litter of differing quality) would depend on the soil and earthworm's microbial status.

To test these hypotheses, we examined the feeding preference of *L. terrestris* in feeding choice chambers on three different plant litter residues, namely, *Q. robur* (oak), *F. excelsior* (ash) and *L. multiflorum* (ryegrass), using the earthworm's tightly-associated microbiome and the soil microbiome as factors.

4.2 Materials and methods

4.2.1 Earthworm, soil and plant material

4.2.1.1 *Earthworm*

Earthworm individuals (*L. terrestris*) were purchased from Worms Direct (Essex, UK). The worms were acclimated to the laboratory conditions for two weeks before the start of the experiment in a culture made from Kettering loam, and Irish moss peat (2:1 ratio) in the dark at 20 ± 2ºC (Hendriksen, 1990) and the earthworms were fed Irish moss peat at approximately 1 g earthworm-1 week-1 after the two-week acclimation period (Satchell and Lowe, 1967; Hendriksen, 1990; Enríquez, Duarte and Sand-Jensen, 1993). The chosen

earthworms (responsive to stimuli, well-hydrated, healthy, and similar sizes) were cleaned with autoclaved deionised water, dried, and weighed before use.

Near-axenic and intact (control) earthworms were produced following the method described in (**Chapter three**). Briefly, *L. terrestris* individuals were incubated in Duran bottles containing technical agar (0.65 % (m/v)); 150 ml) supplemented with antibiotics for near-axenic and no antibiotic for 'intact'/control as shown in **Table 4-1** for 96 hours. This procedure was previously shown to significantly ($P<0.05$) reduce the abundance of associated microbial communities to produce earthworm individuals deemed near-axenic for use in ecological studies.

Table 4-1. Antibiotic types and concentrations

Antibiotic	**Antibiotic concentration (µg/ml agar)**
Cycloheximide	150[a]
Ampicillin	100 [b]
Ciprofloxacin	50
Nalidixic acid	50 [a]
Gentamycin	50 [a]

[a] Antibiotic concentration as in Whiston and Seal, 1988

[b] Antibiotic concentration as suggested in Sigma Aldrich, 2017

4.2.1.2 *Soil*

The soil used for this study is of a loamy texture and was collected from a depth of around 30 cm from The University of Reading Hall's farm (grid reference 51.408580, -0.927353). The was sieved (2 mm) and homogenised before dividing into two batches. The soil texture was analysed with Coulter's LS Particle Size Analyser, and the soil characterised in the lab using the laboratory standard method as detailed below. The soil's nitrate content (42.5 ± 0.61

mg.kg-1; n = 3), ammonium (1.07 ± 0.11 mg.kg-1; n = 3), soil pH (5.29 ± 0.66; n = 5), organic matter % (6.02 ± 0.31; n = 3) and calcium carbonate % (3.77 ± 0.55; n = 3) were recorded

The first batch was stored at 4 ºC before being used as non-autoclaved intact soil for the experiment. Aliquots of 800 g of the second batch were stored in polypropylene autoclave bags (Fisher Scientific, U.K.). The bags containing the soil were left open and autoclaved at 121 ºC for 1 hour and then sealed immediately. The bags were then allowed to equilibrate (at 20 ºC) for three days before being autoclaved again on three separate occasions, each time leaving three days between autoclaving. Sterility was checked in a subsample of the autoclaved soil by suspending 5 g of soil in 10 ml of sterile distilled water; the suspended autoclaved soil was then spread onto both nutrient agar (NA) and potato dextrose agar (PDA) media. The soil samples were considered sterile when there were no visible growths after an incubation period of 14 days at 30 ºC. Intact soil samples were also analysed as a positive control.

Subsamples of the intact and autoclaved soils were taken to determine gravimetric moisture content (using weight loss at 105° C). The water holding capacity was determined using saturation and drain method, by submerging a 30 g air-dried sample in a plastic cylinder that has a mesh bottom in water for 12 hours. This is to ensure complete saturation. The water was allowed to drain for another 12 hours. The drained soil was then oven-dried at 105° C for 24 hours and the dried weight recorded). The soil pH was determined by shaking soil samples with deionised water at 1: 2.5 m/v ratio for 10 min and allowed to stand approximately 10 minutes before measuring with digital pH meter (Thermo Orion). The mineral- (nitrate- and ammonium-) N concentration determined by extraction (1 h, 20 ºC) of 40g soil with 1M KCl (soil: KCl], 1: 5 (m/v)) and subsequent colorimetric analysis of filtered (GF/A) extracts

(Skalar SAN++series continuous flow analyser)). The nitrate-N and ammonium-N concentrations were expressed as mg per kg of dry soil weight.

4.2.1.3 *Plant litter*

Three plant species, previously used in earthworm feeding preference studies (Hendriksen, 1990), were chosen: *L. multiflorum* (ryegrass), *Q. robur* (oak) and *F. excelsior* (ash).

Leaf litter samples of *Q. robur* and *F. excelsior* were collected from the forest floor in the autumn of 2018, on the grounds of The University of Reading, Whiteknights campus, Reading. Seeds of *L. multiflorum* were purchased commercially (Emorsgate Seeds, Norfolk) and sown in potting compost in 20 cm by 30 cm seed trays. Three weeks after emergence, above-ground biomass was harvested. All litters collected from the forest floor and grown in the lab were stored in plastic bags at -20 ^{o}C.

Before starting the experiment, the litters were oven-dried at 80 °C until a stable weight was reached, and sub-samples milled to a fine powder for determination of total carbon and nitrogen content (n = 3; FLASH CN elemental analyser; Thermo Fisher Scientific) and total phenol content.

The total phenolic content was determined using the Folin-Denis method (Hendriksen, 1990). Briefly, samples (0.75 g ± 0.001g; n=3) were extracted with 50% (v/v) methanol in a static water bath (80 ° C, 1 h). Extracts were filtered (Whatman No. 1) and 1 ml of filtrate added to distilled water (20 ml), Folin-Denis reagent (2.5 ml; purchased ready-made; Sigma-Aldrich), and sodium carbonate (17 % (m/v); 10 ml). Following incubation in the dark (1 h), the absorbance at 760 nm was determined using a spectrophotometer against a calibration curve constructed from tannic acid (Sigma-Aldrich) (concentration range (0.002-0.012 mg ml-1) with results expressed as mg of tannic acid equivalents per g dry weight.

4.2.2 Feeding choice chamber experiment

4.2.2.1 *Experimental design*

The experiment consisted of a 2 x 2 factorial design with soil sterility (intact or autoclaved) and earthworm microbiome (intact or near-axenic) as factors resulting in four treatments:

1. Autoclaved soil + near-axenic earthworm
2. Autoclaved soil + intact earthworm
3. Intact soil + near-axenic earthworm
4. Intact soil + intact earthworm

Based on previous trials, there was a high degree of biological variability, and hence six replicates were needed for this experiment. However, with just (12) chambers available, the experiment was performed in two batches, with each of the four treatments in triplicate per batch.

4.2.2.2 *Choice chamber design*

The design of the food choice chambers used in this experiment was adapted from (Hendriksen, 1990). The feeding chambers (15 cm in diameter, 30 cm in height) were constructed from Perspex, and the bottom of the chamber was sealed with mesh and secured with tape. There were three equally-spaced tubes for food source delivery (7 cm in diameter) attached 15 cm from the bottom **Figure 4-1**. Each chamber contained approximately 1.5 kg of soil (intact or autoclaved), and one gram of each of the three plant litters was added to the feeding tubes. Prior to use, the plant litters that were dried to constant weight were cut up to approximately 1 mm in size. This was to ensure consistency in the size and to prevent the introduction of size bias by the worms.

The experiment was initiated when a single earthworm individual per chamber (intact or near-axenic as appropriate to the treatment) was placed in a central position on the soil surface aided by an earthworm guide, designed using round cardboard with a centralised hole. The control chamber contained plant litters (1 g per litter type) only (n = 3, batches = 2) to monitor litter decomposed throughout the experiment. Chambers were checked daily for moisture loss and moisture content and adjusted using sterile water as required. Any casts visible on the surface during daily checks were removed to prevent coprophagy. The casts were weighed and stored at -20 ºC until DNA analysis. In chosen chambers, one at a time, earthworm activity was monitored using the time-lapse camera and time-lapse videos created. At the end of the experiment, the earthworms were removed, washed with sterile water, dried, and weighed. They were then depurated to empty their guts. The depurate soil was air-dried, weighed, and stored until analysis. The remaining plant litters from the tube for food source were removed, weighed, dried to a constant weight. Soil subsamples (25 g) from the chambers were collected and stored.

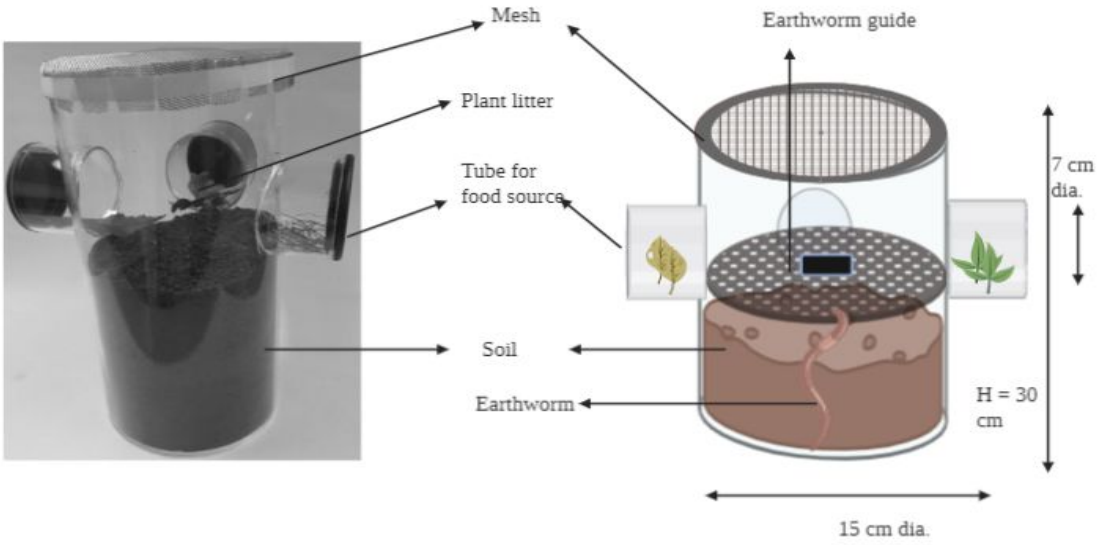

Figure 4-1. A choice chamber with mesh covering, seen from the side. The main chamber is 15 cm in diameter, 30 cm in height; the tube for food source (7 cm in diameter) attached 15 cm from the bottom.

4.2.3 DNA extraction, sequencing and bioinformatics

Total earthworm casts over two weeks, and depurate soil collected at the end of the experiment from each chamber were added together. The combined samples were used for DNA extraction, PCR and 16S rRNA gene amplicon sequencing.

DNA was extracted from the pooled cast and depurate soil samples using the Qiagen Powersoil Pro-Kit (Qiagen, Valencia, CA), following the manufacturer's instructions. The extracted DNA samples were stored at -20 ºC until further use.

16S rRNA gene amplification and sequencing of the V4 variable region was performed at Next-Generation Sequencing provider Molecular Research Laboratory (MRDNA) (www.mrdnalab.com, Shallowater, TX, USA). Briefly, in-house primers 515F/806R were utilised to analyse the microbial communities of combined casts and depurate samples on the

Illumina Miseq platform with the method based on the bTEFAP® process (Dowd *et al.*, 2008). Briefly, a single-step 30-cycle PCR with HotStarTaq Plus Master Mix Kit (Qiagen, USA) was used under the following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, with a final elongation step at 72°C for 5 minutes. After amplification, PCR products were checked in 2% agarose gel to determine the amplification's success and bands' relative intensity. The PCR product was used to prepare the Illumina DNA library according to the Illumina TruSeq DNA library preparation protocol. The data sequences were processed using the MRDNA analysis pipeline (MRDNA, Shallowater, TX, USA). In summary, the sequences were joined, depleted of barcodes, sequences <150bp removed, and sequences with ambiguous base call removed. The sequences were denoised, OTUs generated, and chimaeras removed. Operational taxonomic units (OTUs) were defined by clustering at 3% divergence (97% similarity). The final OTUs were taxonomically classified using BLASTn against a curated derived database based upon RDPII and NCBI (www.ncbi.nlm.nih.gov, http://rdp.cme.msu.edu).

4.2.4 Statistical analysis

The effect of autoclaving sterilisation on the soil properties (pH, WHC, ammonium and nitrate-N) were assessed using a t-test analysis on Minitab 19.1.1. The relationship between the diverse types of litters species and their chemical properties, such as C: N ratio and phenol concentration, were assessed using ANOVA analysis on Minitab 19.1.1.

The initial statistical analysis to investigate the effect of the experimental batch as a factor was found not to be significant ($P>0.05$) and therefore not carried forward as a factor for subsequent analyses. The effect of soil and earthworm microbiome, as well as earthworm and soil microbiome interaction on the percentage total litter removed, as well as percentage litter preference against the treatments, was determined using Two-way analysis of variance (ANOVA) followed by post hoc Tukey comparison, where appropriate ($P>0.05$), on Minitab

19.1.1. The normality of residuals (Anderson-Darling test) and equal variance (Levine's test) assumptions of the data were verified. Data passed verification.

The resulting OTU table from the 16S rRNA analysis of the cast and depurate soil was used for analyses of alpha diversity that included Chao1 richness, Simpson and Shannon indices using the 'vegan' package in R programming (R package vegan v2. 5-6). The normality of the dataset was checked using the Shapiro-Wilk normality test. The significance of differences between alpha diversity and relative abundance of taxa was evaluated using variance analysis (ANOVA). Tukey analysis was also performed to check for significant differences between each treatment in each diversity index. The beta diversity was estimated by calculating weighted UniFrac distances. This was visualised using principal coordinate analysis (PCoA), The effect of sterilisation treatments on bacterial community of the soil and depurate soil patterns were further analysed by permutation analysis of variance (PERMANOVA) with the Adonis function (999 permutations) of the 'vegan' package. The effect of treatment on the soil bacterial community patterns was also examined using ANOSIM.

4.3 Results

4.3.1 Characterisation of treatment effect on soil properties

4.3.1.1 *Soil chemical properties*

The chemical properties of the soil are summarised in **Table 4-2**.Results demonstrated that autoclaved soil was slightly less acidic than intact soil, t-test analysis showed a significant difference p value of (P = 0.027). Ammonium-N in autoclaved soil increased by 894.69 % from 1.13 to 11.24, and nitrate-N increased by 62.91 % from 40.17 to 65.44, both showing a significant difference between the intact soil and autoclaved soil (P<0.05).

Table 4-2. Mean nitrate, ammonium, and pH of soil (autoclaved / intact). Values are means (±SD) of measure values (n = 3) for nitrate and ammonium; (n = 5) for pH.

Chemical property	Autoclaved soil ± S.E.	Intact soil ± S.E.
NH_4-N*	11.24±0.60	1.03±0.09
NO_3-N*	65.44±1.67	40.17±0.66
pH	5.80±0.01	5.29±0.22

*$mg.kg^{-1}$

4.3.1.2 *Diversity and composition of earthworm associated bacterial communities*

Illumina 16S rDNA amplicon sequencing of DNA extracted from the collected cast and depurate soil from *L. terrestris* identified 2382 OTUs in total. Taxonomy was assigned using Silva v132 database which resulted in the detected OTUs being classed into 19 phyla, 45 classes, 198 families and 503 genera.

Two-way ANOVA **Figure 4-2** revealed an overall highly significant effect of soil sterility on alpha diversity as shown by Shannon (P = 6.92 e-13), Simpson (P = 3.8 e-08) and Chao1

richness (P = 1.44 e-09); intact soil had an overall three-fold higher number of OTUs detected compared to autoclaved soil. In contrast, earthworm microbiomes had no effect (P>0.05) on any of the alpha diversity measures and the effect of the soil sterility did not depend on earthworm microbiome as evidenced by an insignificant (P>0.05) interaction between the two factors.

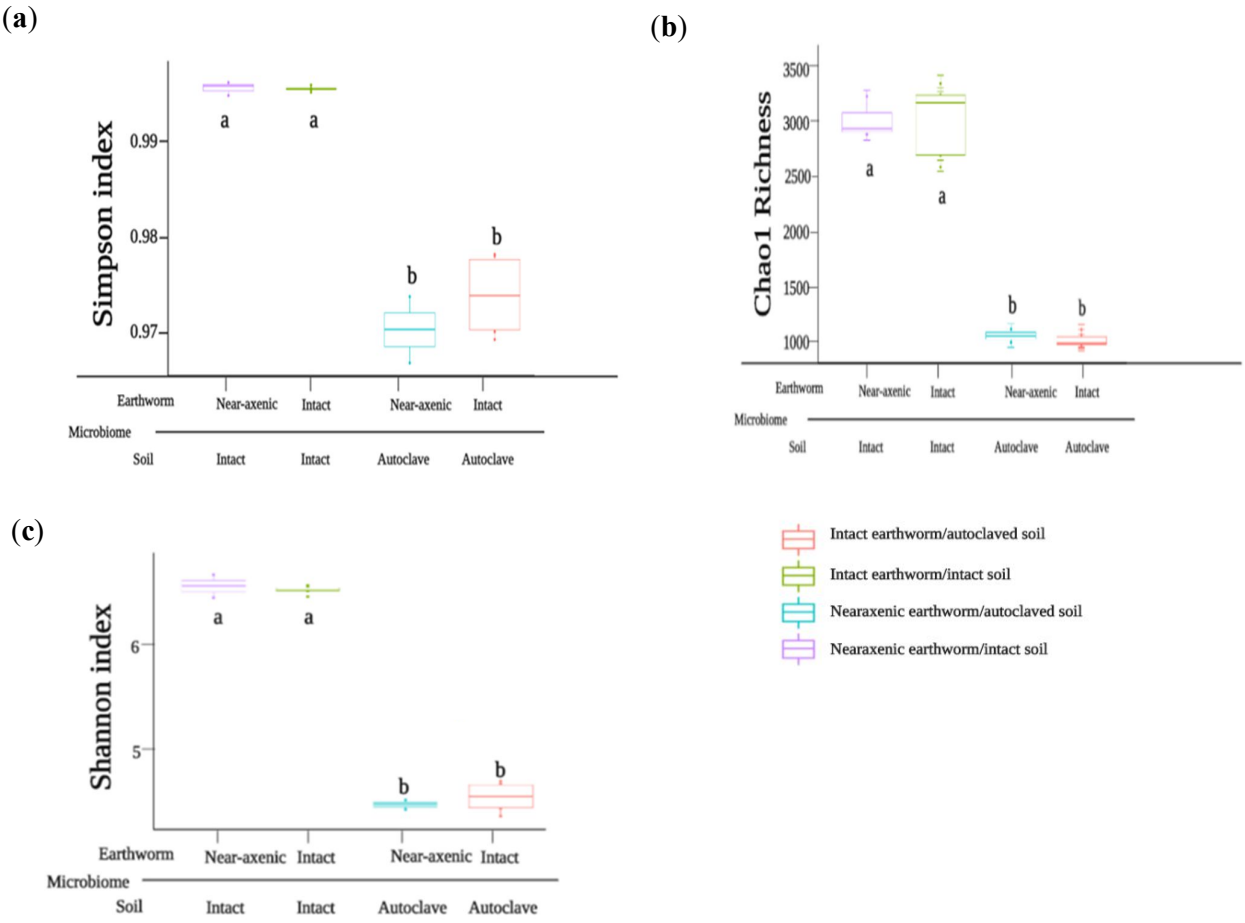

Figure 4-2. Effect of manipulation of soil (autoclaving or not) and earthworm (antibiotic treatment or not) microbiomes on alpha-diversity of earthworm-associated (cast/depurate) soil as assessed by (a) Simpson index, (b) Chao1 richness, and (c) Shannon index. Samples with different letters are statistically different ($P<0.05$).

PCoA plot using weighted UniFrac distance matrix was used to visualise the similarity in the bacterial composition for cast and depurate soil samples from earthworms in various treatments **Figure 4-3**. There was a reduction in the number replicates in treatment containing near-axenic earthworm and autoclaved soil due to earthworm's death and no visible cast and depurate recovered during the experiment. Overall, three replicates were lost (2 in 2nd batch both occurring with chambers in near-axenic earthworm). The third loss was in the first batch with chamber containing earthworm with intact earthworm. Both PERMANOVA and ANOSIM analyses showed that there were significant differences between the soil samples based on their treatments (P>0.05). These analyses showed a clear distinction in the microbial structure before and after the autoclaving process. It also showed the huge influence of the soil microbiome on earthworm casts and depurates samples.

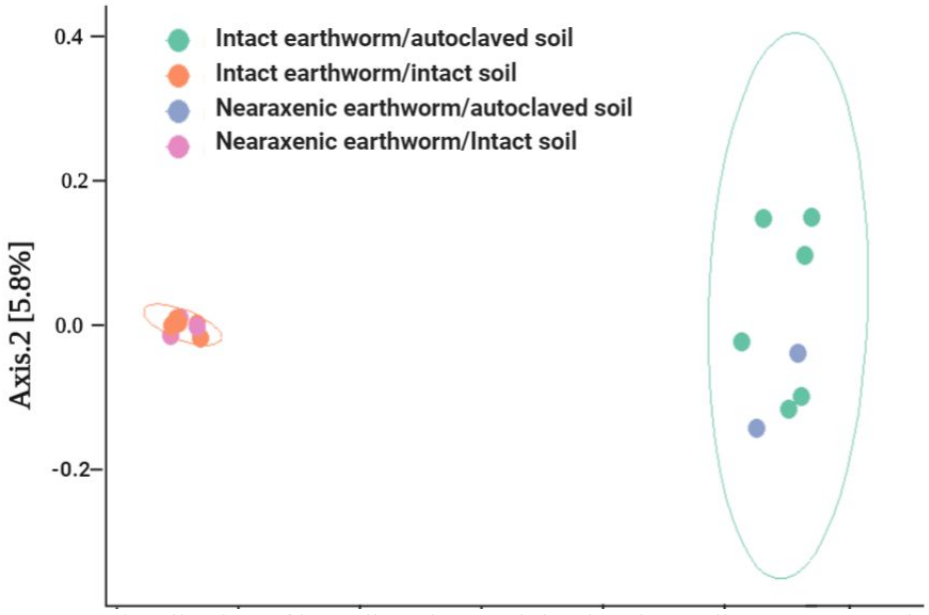

Figure 4-3. Visualisation of beta diversity. Weighted UniFrac distances were plotted using Principal Coordinate analysis (PCoA) of OTUs. Each point represents each sample.

4.3.2 Effect of disruption of earthworm and soil microbiome on earthworm feeding behaviour on litters of different quality

4.3.2.1 *Litter quality*

The litter quality chemical properties indicating potential desirability of the litter residues are represented in

Table 4-3. Using the ANOVA analysis, the mean carbon: nitrogen ratio and phenol concentration of each litter residue was significantly different ($P<0.05$) from one another. The mean carbon: nitrogen ratio of ryegrass residue was also distinctively much lower than the other two residue.

Plant species litters	**Mean %Nitrogen ± S.E.**	**Mean %Carbon ± S.E.**	**C: N ratio ± S.E.**	**phenol concentration ± S.E.**
Quercus robur (oak)	2.21±0.01	45.60±0.09	20.7±0.06[a]	17.14 ±0.03*a
Fraxinus excelsior (ash)	2.28±0.02	42.49±0.15	18.61±0.13[b]	16.26 ±0.11*b
Lolium multiflorum (ryegrass)	5.98±0.04	40.5±0.22	6.78±0.02[c]	12.39 ±0.73*c

Table 4-3. The mean nitrogen and carbon, carbon: nitrogen ratio and the phenol concentration of each food type presented to the earthworm in the chamber. Values are means (n=3) with standard error.

*mg of tannic acid equivalent of g dry weight, $^{a\,b\,c}$ letters indicate statistical significance group at $\alpha = 0.05$.

4.3.2.2 *Feeding activity and food preference*

Analysis of the effect of manipulation of earthworm microbiome, soil microbiome and the earthworm x soil microbiome interaction using ANOVA revealed an overall significant effect of both earthworm ($p= 0.019$) and soil ($p=0.024$) microbiome and earthworm x soil microbiome ($p=0.013$) on the overall amount of litter consumed per chamber (i.e. the sum of oak, ash and ryegrass) (**Table 4-4; Figure 4-4**). The **Figure 4-4** showed that the chamber with both intact soil and intact earthworms has significantly ($P<0.05$) more overall % plant litter residue removed when compared to the other chambers with either manipulated soil microbiome, earthworm microbiome, or both.

Table 4-4. ANOVA analysis result for the total litter removed with factors earthworm microbiome, soil microbiome and earthworm microbiome and soil microbiome interaction.

Variation	**Sum of squares**	**df**	**sig**
Earthworm microbiome (E)	0.059	1	0.019
Soil microbiome (S)	0.055	1	0.024
E X S	0.015	1	0.013

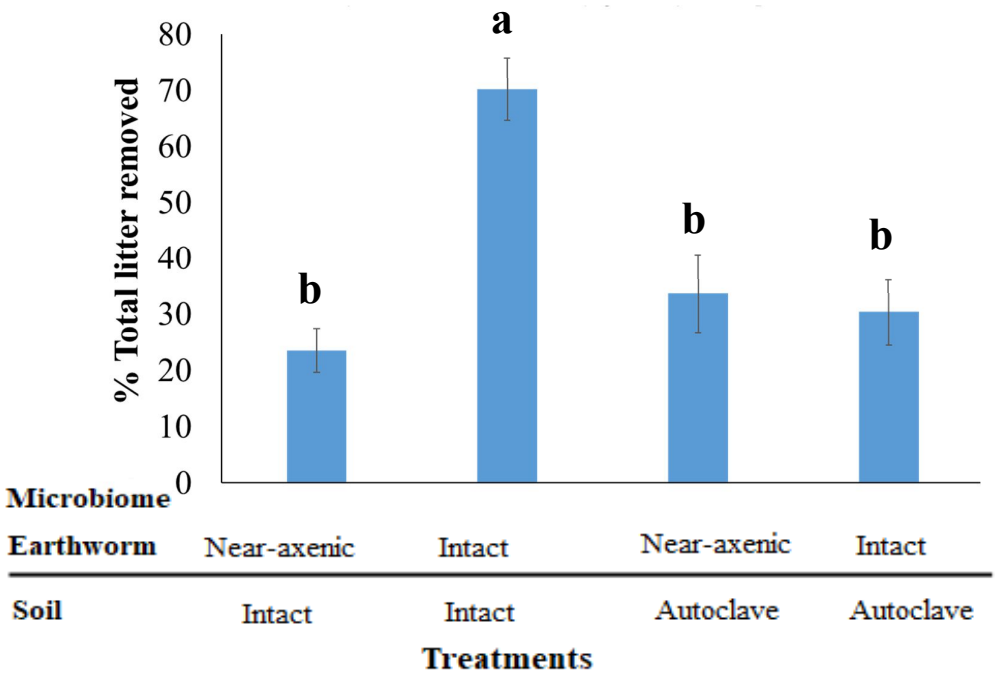

Figure 4-4. Effect of earthworm and soil microbiome treatments on total litter removed of oak, ash and ryegrass combined calculated as: [(initial mass of litter (oak, ash and ryegrass) added – final mass of litter (oak, ash and ryegrass) recovered)/ initial mass of litter (oak, ash and ryegrass) added] * 100%. Data are mean ± SE (n =6). Bars that do not have a letter in common are significantly different ($p < 0.05$; Tukey test).

Calculation of a litter preference for each of the litter types (oak, ash and ryegrass; **Figure 4-5)** showed that the ryegrass litter with the lowest C:N ratio and polyphenol concentration (**Table 4-4. ANOVA analysis result for the total litter removed with factors** earthworm microbiome, soil microbiome and earthworm microbiome and soil microbiome interaction.) was generally the least preferred out of the three. While the second highest C:N ratio litter residue (ash) was consistently preferred irrespective of the soil and earthworm microbiome.

In the choice chambers with intact earthworm and soil microbiomes, ryegrass litter with the lowest C:N ratio and polyphenol concentration (

Table 4-3) was the least preferred out of the three litters with the preference for ash and oak approximately equal. This preference pattern of ash » oak > ryegrass was also recorded for treatments with either soil or earthworm microbiome disrupted. However, the preference of *L. terrestris* for the three litters changed when both soil and earthworm microbiome were disrupted with oak litter being preferred significantly ($P<0.05$) less, making up only ~20% of the total mass of litter removed when compared to ~40% in the fully intact (soil and earthworm) microbiome treatment.

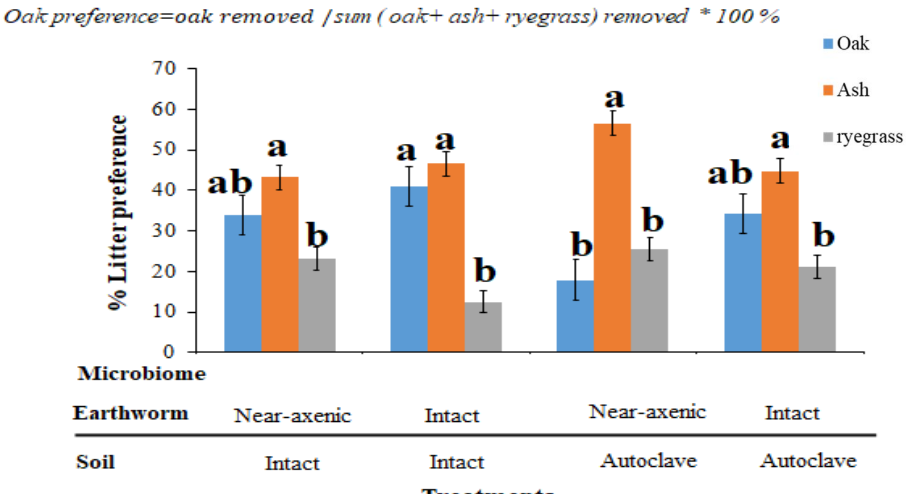

Figure 4-5. Effect of earthworm and soil microbiome treatments on litter preference of oak, ash, and ryegrass. Preference for a given litter (i) from a choice of litters i, j and k were calculated as: [mass of litter (i) removed / (mass of litter i removed + mass of litter j removed + mass of litter k removed)] * 100%. Bars of the same colour that do not have a letter in common are significantly different ($p < 0.05$; Tukey test).

4.4 Discussion

Feeding choice chambers were set up with three diverse sources of litter residues. Each chamber contained soil (intact microbiome or disrupted microbiome through autoclave sterilisation), and earthworms (intact microbiome or disrupted microbiome using a cocktail of antibiotics). This experiment was set up to establish whether feeding activity and preferences for the earthworms were influenced by interactions between litter quality (C: N ratio, phenol concentration) and soil and earthworm microbial communities. The success of creating near-axenic earthworms with disrupted microbiomes could not be determined due to the destructive nature of the method for quantifying the response of earthworm-associated microbial abundance to antibiotic treatment. However, assessment of the antibiotic treatment method in Chapter 3 showed that, for *L. terrestris*, earthworm-associated culturable microbial abundance was reduced by >100-fold with accompanying shifts in bacterial community composition. We assume that the same level of suppression to the *L. terrestris* microbiome was achieved by antibiotic treatment in the current experiment.

The creation of sterile soil through autoclaving was tested by plating the soil on agar plates and examining the plates for microbial growth. In this case, the soil was considered 'sterile' if there was no observable growth, although it is accepted that this test would not identify any viable but non-culturable microorganisms that survived the autoclaving process. However, because of the introduction of, and re-colonisation of soil by, the microbial community as a result of the addition of earthworms to the soil on initiation of the experiment, the initially sterile soil would likely not remain sterile throughout the experimental duration. This earthworm-mediated re-introduction of microorganisms would almost certainly be the case

for the chambers receiving microbially-intact earthworms (shown in Chapter 3), but also for the 'near-axenic' earthworms which might also still host viable microbial cells (Chapter 3).

The cast and depurate soils used for the high-throughput sequencing were the soils that had been in direct contact with the earthworms instead of the large body of the soil in the chamber. *L. terrestris* with permanent burrows may not come in to contact with all of the soil in the chamber and therefore we wanted to characterize the soil that had definitely been in contact with the worm, since we are interested in microbiome effects on earthworm behaviour. Autoclaving, the soil sterilisation method used in this study, is a process used to control significant classes of fungi and bacteria. It can effectively kill and inactivate microbes and eventually be used to quantify microbial contribution such as in soil enzyme assays, soil respiration and biodegradation of chemicals (Berns *et al.*, 2008; Otte *et al.*, 2018; Trevors, 1996; Trevors and van Elsas, 1995). However, some resilient DNA, RNA and microbes could persist, with some studies showing evidence of microorganisms that often survive the autoclaving process, including spore-forming bacteria (Sahlström *et al.*, 2008). We also cannot be sure that dead microorganisms were not amplified during the sequencing process in our use of sterilisation method. Still, it is also likely that the re-colonising microorganisms were introduced by the added earthworms, which decomposed most of the DNA associated with dead microbial cells and are not detectable in the OTUs detected. Therefore, the detectable OTUs were from the introduced microbial cells. Overall, from the result, the disrupted soil microbiome showed a considerable difference in the casts and depurated soil diversity and richness compared with intact soil. The sterilisation of earthworms through antibiotics had no significant effect on the soil sample from already autoclaved soil, but a significant difference was observed on antibiotic-treated earthworms in intact soil.

Apart from the desired effect of autoclaving on microbial inactivation, studies have shown that autoclaving influences the soil's physio geochemical properties (Berns *et al.*, 2008; Lotrario *et al.*, 1995; Razavi darbar and Lakzian, 2007). The result in this study showed an increase in pH which agrees with (Berns *et al.*, 2008; Lotrario *et al.*, 1995; Razavi darbar and Lakzian, 2007; Shaw *et al.*, 1999) which could be due to autoclave-induced solubilisation of organic acids from soil organic matter. In contrast, a study by (Wolf *et al.*, 1989), has recorded no changes in soil pH after autoclaving. The magnitude of the decrease in the pH value depends on the acidic buffering capacity of the soil (Razavi darbar and Lakzian, 2007) might be an explanation for the discrepancy between findings. Autoclaving also increased soil ammonium-N concentration which agrees with the findings of Jager, Van der Boon and Rauw, (1970) and Trevors, (1996). The increase in ammonium-N could be caused by the autoclaving process release of N that was mineral associated or abiotic mineralisation of N. There is not a lot of literature that addresses what the implication was of the autoclaving process increasing ammonium-N on the earthworm health but Berns *et al*., (2008) observed that autoclave process increases extractable ammonium and organic nitrogen and Yeardley, Lazorchak and Pence, (1995) suggests that it might be toxic. Also Iordache and Borza, (2010) and Reinecke and Reinecke, (2004), suggested that prolonged exposure of excessive inorganic compounds in soil such as nitrogen led to the acidification of the soil and negatively affected earthworms. We can therefore infer that prolonged exposure of earthworms to high inorganic concentration could be toxic to the earthworms. However, during the designing of this experiment, caution was taken to check for the implication of autoclaving the soil to the health of the earthworms, the result showed that the autoclaving method did not result in any significant increase ($P<0.05$) in inorganic compounds such ammonium and nitrate that was toxic to the earthworms, as all test earthworms in the preliminary work were alive after the incubation period and were responsive to

stimuli. However, autoclaving influenced both microbial and abiotic properties of the soil and therefore cannot be certain that all the effects of the autoclaving treatment on earthworm behaviour were due to the manipulation of the soil microbiome. There may also be influences of the changed pH and nitrogen availability.

In preliminary trials using the choice chamber experimental design, *L. terrestris* individuals, when placed on the soil surface, burrowed at variable positions on the surface that were not necessarily central in the chamber. It was noted that the position of this initial burrow tended to influence food preference with earthworms preferring the litter residues in the feeding tube closest to the burrow. Previous studies (Doube *et al.*, 1997; Neilson and Boag, 2003; Valckx *et al.*, 2011) adopting choice chamber designs resembling the design here have not reported a potential influence on results of this initial borrow position. However, our observations led to the modification of the design to introduce a guide to encourage the earthworm to burrow in the centre of the chamber and therefore to choose between the different food sources without the bias of proximity to a given food source.

When looking at the restrictive nature of replicating a field experiment in a small chamber of 30 cm by 15 cm, we can expect the exploration of all the feeding tube. Therefore, it was reasonable not to discount the effect of earthworms entering these tubes, not eating the litters, or intentionally dragging the litters, but the litters adhering to the surface and therefore being removed from the feeding tube this way. However, there was little to no evidence of this happening. Instead, we observed that earlier on in some chambers, the earthworms repetitively visited specific feeding tubes to drag the litters to their burrow entrance in readiness to be dragged into the burrow.

This study's primary goal was to obtain an insight into the role of the soil and the earthworm microbiome in influencing the feeding preference of *L. terrestris*. It was hypothesised that

(1) *L. terrestris* will show feeding preference related to litter quality determined by C: N ratio and phenol concentration. (2) Litter feeding behaviour (both quantity of litter consumed and preference for litter of differing quality) will depend on soil and earthworm's microbial status.

The litter quality test in

Table 4-3 , showed that oak and ash litter C: N ratio and polyphenol compound values were observably close. Although statistically different, the magnitude of the difference in C: N and phenol values between oak and ash was very small (e.g., values for oak were 5-10% greater than for ash) and therefore might not be biologically relevant from a nutritional point of view. It is not surprising that this similarity in composition did not lead to substantial preference for one litter over the other in *L. terrestris* individuals with the intact soil and earthworm microbiomes microcosm **Figure 4-5**. The results obtained in this study in relation to litter quality is different to results from studies such as Satchell and Lowe, (1967) and Hendriksen, (1990) where the litter quality especially between the oak and ash litter C:N ratio and the polyphenol concentration are observably different and also significantly different in litter preferences.

Although the ryegrass has the lowest C: N and phenol, the earthworms did not prefer it. This is in contrast to Rajapaksha *et al.*, (2013), that suggested that earthworms particularly preferred litters with low C:N ratios. This indicates that other plant litter qualities such as the texture and physical form may have influenced the choices. The state of decomposition/weathering of the litters (oak and ash) could have also influenced the litter selections. This is because both the oak and ash were picked when they had freshly fallen from trees while the ryegrass was grown and harvested in the lab so it is unlikely that any senescence would have started.

The preference results also showed that although *L. terrestris did* not avoid other litters in the feeding chamber, *L. terrestris* earthworms were still selective in their feeding. They distinctively choose certain litter types in all treatments.

The soil and earthworm microbiome results indicated that earthworm feeding behaviour was influence by the soil and earthworm microbiome and by soil x earthworm interaction **Table 4-4.** This was also observable in **Figure 4-4** showing the overall litter consumed.

The disruption of either earthworm or soil microbiome resulted in reduced feeding activity but did not result in a significant impact on food preference when compared to *L. terrestris* individuals with both earthworm and soil microbiome intact. Only when both soil and earthworm microbiomes were disrupted did this lead to a change in food preferences, specifically the avoidance of oak, although the overall level of feeding activity stayed the same as for worms with either soil or earthworm microbiome intact.

Knowing that both litters significantly differ in their litter quality, measurable by the C: N and phenol concentration, there is no clear suggestion as to exactly how the changes in soil community mediate the earthworm function, as suggested by Medina-Sauza *et al.*, (2019). It was possible that the reduction in soil and earthworm microbiome, facilitated by the various sterilising process, affected the health of earthworm, which was observable in the weight loss seen in earthworms with the disrupted microbiome and soil microbiome ***Supp table 4-1***, compared to weight gain in the control sample. This weight loss can be assumed to result from poor health, and earthworms becoming uninterested in carrying out essential function like feeding or making the appropriate feeding choice.

In conclusion, our study observed that the earthworms did show preference between the offered food sources. These preferences were not dependent on litter quality based on C:N ratio and polyphenol content since the litter with the lowest C: N and polyphenol content and

interpreted to be highest quality was least favoured as a food choice. However, other litter quality factors not analysed in this study could have influenced the choice. Disruption to either earthworm or soil microbiome (or both) is influential for feeding activity, but an intact soil microbiome can compensate for a lack of earthworm microbiome and vice-versa when it comes to food preference. The results observed in this study make one of the first contributions to understanding the role of the soil and earthworm microbiome (and their interactions) on earthworm health and potential feedbacks to feeding behaviour. When it comes to activities such as their feeding habit, it can alter how organic matter cycles from plant litters into the soil as an essential part of the carbon cycle (Mohammed *et al.*, 2019). The next step is to qualify that that this study microbiome treatments were rather extreme, and these levels of microbiome disruption will not be found in nature. The earthworm microbiome might be vertically transmitted and therefore have constant presence, but the soil microbiome is variable depending on soil type, management other external factors (pollution, drought), it is not clear if more subtle variation in soil microbiomes might influence earthworm feeding activity. The next chapter will address this question through using a dilution to extinction approach to vary soil microbial composition within the same soil type.

Chapter 5 Creating a gradient of soil microbial diversity using dilution to extinction process

5.1 Introduction

Soil is a critical part of terrestrial life and contributes significantly to ecosystem processes such as organic matter decomposition and nutrient cycling (Fernández-Luqueño *et al.*, 2011; Nazaries *et al.*, 2021; Philippot, Raaijmakers, *et al.*, 2013; Trivedi *et al.*, 2016). It is one of the most complex habitats on earth with a large community of diverse microorganisms (Martiny, Treseder and Pusch, 2013; Roesch *et al.*, 2007; Wagg *et al.*, 2014; Wall, Nielsen and Six, 2015) whose functions underpin soil ecosystem services (Tiedje *et al.*, 1999; Torsvik, Goksoyr and Daae, 1990; Wall, Nielsen and Six, 2015).

With the current global challenges of climate change and food security, and the unprecedented increase in worldwide depletion to ecosystem services, due to threats from human-made pressures (European Commission, 2015), the shift in communities, as well as extinction of some species, raises important questions on the effect of biodiversity loss for ecosystem functioning (Cardinale *et al.*, 2012). Traditionally, there have been many studies on understanding biodiversity effect on ecosystem functioning. Many of these studies have shown us clear evidence of a significant positive effect of biodiversity on ecosystem function (Lavorel *et al.*, 2013; Mouillot *et al.*, 2011; Powell *et al.*, 2015; Trivedi *et al.*, 2016). Some studies have hypothesised that due to the overwhelming biodiversity present in the soil, most soil species are redundant, which meant that the loss of a few species would generally not influence the rate of the ecosystem services provided due to the replacement of functions by other species present (Banerjee *et al.*, 2016; Loreau, 2004; Mendes *et al.*, 2015; Wall, Nielsen and Six, 2015). However, in a community with a poor level of species richness, it is hypothesised that the loss of certain species could result in an adverse effect on the services provided, which alludes to the idiosyncratic relationship (Birkhofer *et al.*, 2018; Liu *et al.*, 2021; Nielsen *et al.*, 2011; Philippot, Spor, *et al.*, 2013). Some studies also hypothesised a linear relationship, whereby each species contributes uniquely to ecosystem functioning and

that the addition of each new species would cause an increase in the ecosystem functioning (Lawton and Brown, 1994).

The overall aim of this PhD thesis is to improve our understanding of how earthworms and soil microbial diversity interact to determine the fate of organic matter (OM) amendment to soil, with an overall hypothesis that earthworm health and function with respect to the promotion of OM mineralisation depends on soil microbial biodiversity. So far, the hypothesis has been tested by assessing the importance of both soil and earthworm microbiomes, using near- axenic *L. terrestris* created in chapter 3, for feeding activity and the food preference of *Lumbricus terrestris* (chapter 4). These experiments revealed that initial elimination via autoclaving of the soil microbiome resulted in reduced feeding activity. However, an initially sterile soil would not be a situation that earthowrms would encounter in the natural envionment. Rather, earthworms inhabit soils with variable microbial diversity depending on soil type, management and environmental factors. Therefore, the next experiment aimed to examine if earthworm health and functioning are sensitive to more subtle reductions in microbial richness. To test the role of microbial richness, it is necessary to create, through manipulation, a gradient of microbial diversity within a specific soil type so that the role of diversity can be isolated.

The dilution-to-extinction method is a proactive approach that has been used in soil ecology. It can manipulate microbial diversity in a controlled environment (Van Elsas *et al.*, 2012; Philippot, Spor, *et al.*, 2013; Wertz *et al.*, 2006; Yan *et al.*, 2017).This method, initially described by Salonius, (1981), involves the inoculation of sterilised soil with diluted inoculants derived from the suspension of the same non-sterile soil in sterile water to create soil that contains a gradient of varying species diversity, depending on the dilution factor. Based on an assumed uneven species abundance distribution in the starting non-sterile soil (Castro *et al.*, 2010; Fierer *et al.*, 2013; Ladau *et al.*, 2018; Mod *et al.*, 2021; Singh *et al.*,

2010), it follows that this manipulation approach will create a gradient of species richness. There is likely to be a reduction in species as they are lost as dilution progresses due to their initial rarity in the initial soil sample.

Other methods that can be used to study the relationship between microbial diversity and ecosystem function includes (1) Inoculations of microcosms with known species (Van Elsas *et al.*, 2012; Naeem and Li, 1997; Salles *et al.*, 2009). This methodology is an alternative approach that allows the assertion of certain desired properties like specific species identity. It also allows the effective use of data, mainly where the functional contribution of the species is unknown. However, this approach is limited, and only a few soil microbial groups can be used at a time. There is also the issue of underrepresentation of indigenous soil microbial communities.

(2) Fumigation of soil with chloroform (Van Elsas *et al.*, 2007; Griffiths *et al.*, 2000). This is a destructive progressive approach that results cell lysis and in halting all microbial growth as time passes. It is difficult to determine if this method results in a reduction in species that is random, which would be the preferred approach compared to being selective. Although, Griffith et al., (2000) in their genetic fingerprinting and physiological tests suggested that changes in the various dominant bacterial groups after the fumigation process appeared to be random, the evidence from the functional assays showed that the microbes colonising the fumigated soils were physiologically different from the unfumigated soil, suggesting that the chloroform did target specific microbes and may not have been random as previously thought.

To obtain a series of soils with a gradient of microbial richness in order to examine if earthworm health and functioning are sensitive to reductions in microbial biodiversity (chapter 6), the dilution to extinction method was chosen. The specific objectives for this

study were: (1) to use the dilution-to-extinction process to create soil with a gradient of bacterial richness **Figure 5-1** and to describe the gradient achieved using high throughput amplicon sequencing. This characterization of bacterial community richness and composition of the resultant soil series was necessary since the communities that are established cannot be predicted from the composition of the initial inoculum due to biotic and abiotic selection that will determine which components of the inoculum will survive and proliferate and which will not survive during post-inoculation equilibration; (2) to examine whether, following the inoculation with soil dilutions, soil microbial biomass and activity recovered during the equilibration period to levels that were comparable between dilution treatments so that effects of differing bacterial richness could be isolated from any potential differences in biomass and activity between dilution to extinction treatments.

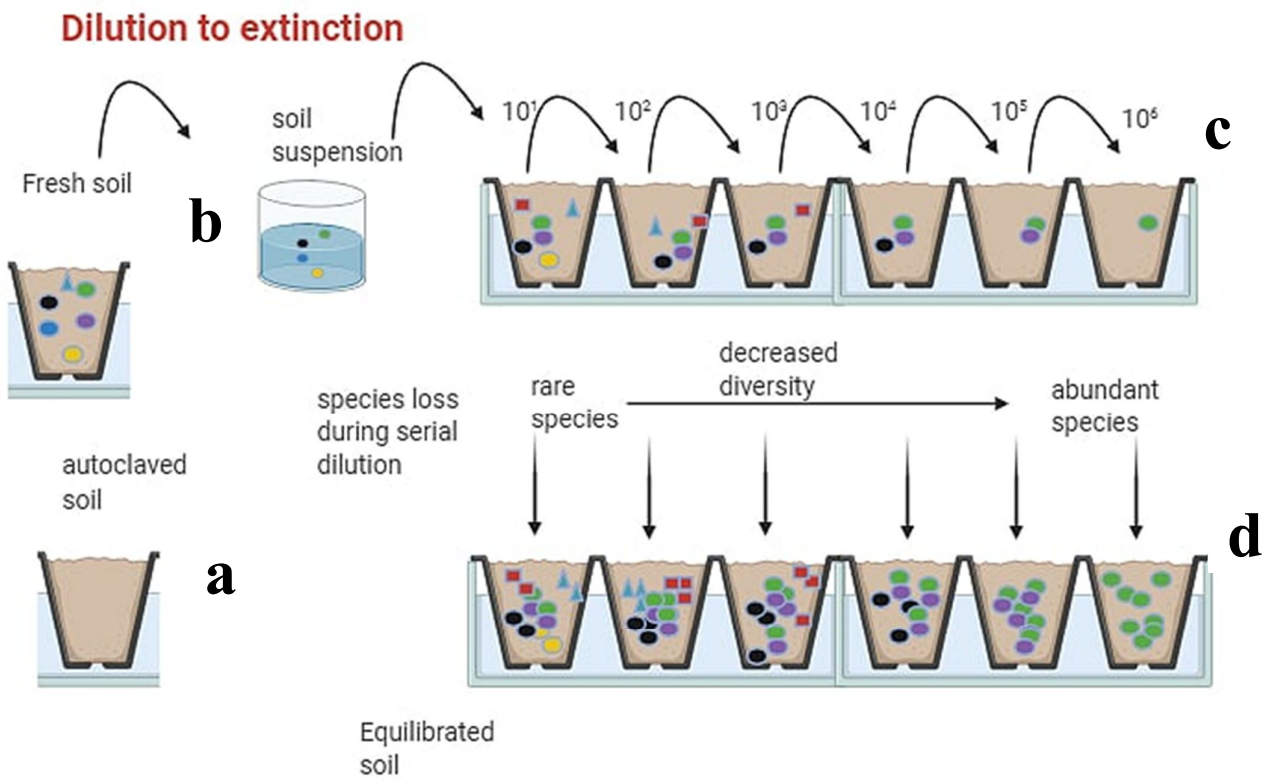

Figure 5-1. An illustration of the dilution-to-extinction approach. (a) Sterilisation of soil by autoclaving. (b) create microbial soil suspension using subsamples of source soil and sterile water (c) Create microbial dilutions from the soil microbial suspension and add varying dilution to autoclaved sterile soil. Incubate for 27 weeks at 20º C to allow microbial re-colonisation of the soil (d) Equilibrated soil/re-colonised soil, after the incubation period, should have varying microbial community.

This is based on the notion that the less diluted inoculum would contain greater diversity, while the richness reduces as the dilution increases. It is also assumed that the initial soil contains an uneven microbial community structure that will result in the most diluted inoculum containing the most initially abundant species present and none of the rarest species that are not carried forward to the higher dilution

5.2 Material and methods

5.2.1 Soil sampling and treatments

Sixty-five litres of soil of a loamy texture (sand 52%, silt 32%, clay 16 %) were collected at a depth of around 0 - 30 cm from the University of Reading's Hall farm (51.408580, -0.927353). The soil was sieved to < 4 mm and homogenised by thoroughly mixing the components using a spatula before use. The soil texture was analysed with Coulter's LS Particle Size Analyser, and the soil characterised using the laboratory standard method. The soil's nitrate content (40.2± 0.67 mg.kg-1; n = 3), ammonium (1.03± 0.09 mg.kg-1; n = 3), soil pH (5.29 ± 0.22; n = 5), organic matter % (5.88 ± 0.20; n = 3) and calcium carbonate % (3.47 ± 0.46; n = 3) were recorded. Aliquots of 800 g * 13 of the soil were stored in sealed clear bags at 4º C to be used as 'intact' soil, and further aliquots of 800 g * 60 were stored in autoclaved plastic bags (Polypropylene clear autoclave bags, Fisher Scientific, UK). One bag of the stored 'intact' soil was used as the inoculum. The autoclave bags containing the soil intended for autoclaving were left open and autoclaved at 121 ºC for 1 hour and then immediately sealed. The bags were then incubated at room temperature for three days before being autoclaved again (121 ºC, 1 hour) on three separate occasions, each time leaving three days between autoclaving according to the method used by Shaw *et al.*, (1999). Sterility was checked in subsamples of the autoclaved soil by suspending 5 g of soil in 10 ml of sterile distilled water and spreading the suspended sterilised soil onto both nutrient agar (NA) and potato dextrose agar (PDA) media (Sigma Aldrich, Gillingham, Dorset, UK). The previously autoclaved soil samples were considered sterile when there was no visible growth after an incubation period of 14 days at 30 ºC. Subsamples of the intact soil and the sterilised soil were then taken to determine the soil moisture content and water holding capacity.

The soil dilution was achieved using the following process:

The final outcome was that the 10^{-2} soil microcosm was made by adding 5.56g dry weight equivalent of fresh soil to 556 g (dry weight equivalent) autoclaved soil in 0.25 ml/g (calculated moisture content required to adjust soil to 70% moisture content capacity)

Which is 0.25 ml/g* 556 g soil = 139 ml water added in to 556 g (dry weight equivalent) autoclaved soil.

The fresh weight of soil and fresh weight per ml water were calculated as follows:

5.56 g dry weight soil =5.56*(1 + 0.182(moisture content of fresh soil) = 6.57 g of fresh soil.

6.57 g fresh soil in 139 ml water = 6.57 g/139 ml = 0.0473 g fresh soil per ml water.

200 ml was prepared, and the amount of fresh soil used was calculated as follows:

0.0473 * 200 ml = 9.45 g fresh soil in 200 ml sterile water. Therefore, 9.45 g of fresh soil was added to 200 ml of sterile water, 139 ml of which was added to the autoclaved soil to achieve the 10^{-2} dilution.

A hundred-fold dilution of the initial sample was made (2 ml in 200 ml) and 139 ml of the dilution was added to 556 g autoclaved soil to make a 10^{-4} soil microcosm. The 100-fold dilution was repeated to achieve a 10^{-4} and 10^{-8} soil microcosm.

The schematic representation of the dilution series process is represented in **Figure 5-2**.

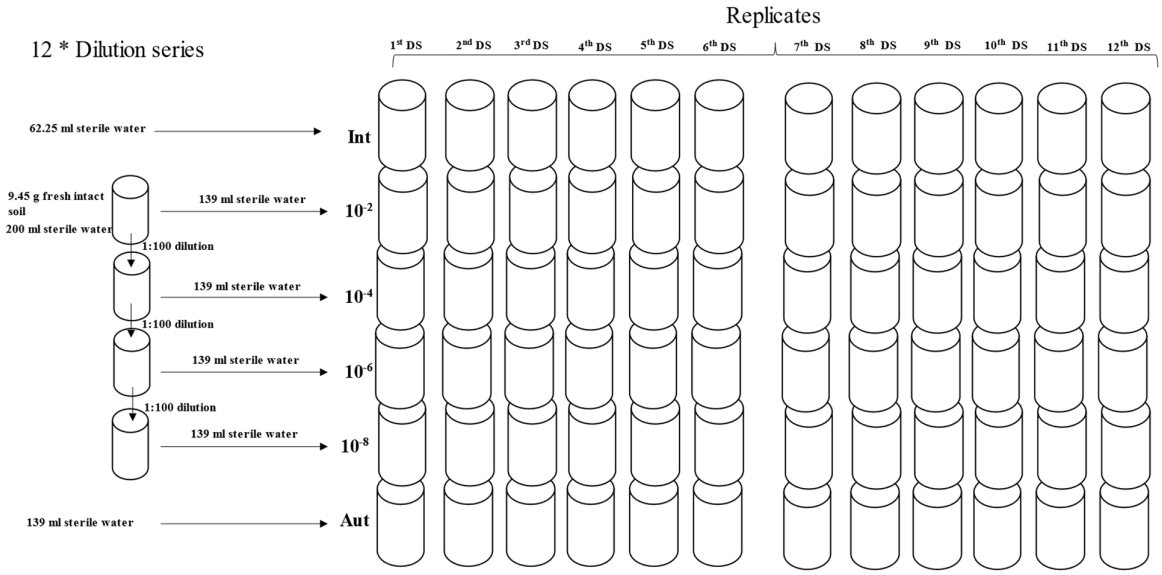

Figure 5-2. schematic representation of the dilution series process

In total, 72 microcosms of soil resulting from 12 dilution series (DS) were made.

The microcosms were incubated in the dark at 20 ºC and loosely covered with a lid to allow gas exchange while preventing microbial contamination. The soil moisture was monitored weekly for the first four weeks and biweekly afterward and adjusted to 70% of the water holding capacity when necessary. After 27 weeks of incubation, some sub-samples of soil were taken from randomly selected microcosms to determine the microbial biomass carbon (fumigation extraction; n = 4), microbial activity (dehydrogenase activity (DHA; n = 12), fluorescein diacetate hydrolysis (FDA; n = 12) and microbial diversity (DNA analysis; n = 6) in all treatments.

5.2.2 Microbial biomass, abundance, and activity

5.2.2.1 *Fumigation extraction*

The fumigation extraction method is a method based on the basic principle that soil microbial biomass can be estimated because microbial cells lyse after exposure to chloroform, and the microbial component is transformed to extractable components (Alessi, Walsh and Fein, 2011; Diaz-Raviña *et al.*, 1992; Joergensen, 1996; Powlson and Jenkinson, 1976).

Four out of the twelve replicates for each soil sample treatment were randomly chosen. For each of the chosen moist soil samples, the samples were divided into two portions of 30 g (on an oven-dry basis). The samples were weighed into 100 ml borosilicate glass beakers. The beakers were marked with pencil on paper-based stickers, and a map of the beakers was drawn. The beakers were then placed in a vacuum desiccator lined with moist filter paper to maintain high humidity. One 30 g portion from each sample was fumigated, while the other remaining sample portions were not. For fumigation, approximately 50 ml of ethanol-free chloroform and a few anti-bumping granules were placed in the vacuum desiccator. The desiccator was evacuated until the chloroform had boiled for 2 min, the valve was closed, and the desiccator kept in the dark for 24 hours in a fume cupboard. After 24 hours, the chloroform was removed from the desiccator. The desiccator was evacuated three times, and the samples left to vent in a fume cupboard for 1 hour to ensure that no chloroform was trapped in the soil samples. Both the fumigated and the non-fumigated soil samples were extracted using 0.5 M potassium sulphate (K_2SO_4) made by dissolving 871.35 g of (K_2SO_4) in 10 L deionised water. The soil samples were placed in 350 ml polypropylene bottles, and 120 ml of the 0.5 M K_2SO_4 added. The bottles were shaken on an oscillating shaker for 30 min and the suspensions filtered with Whatman No. 42 filter papers. The filtrate was analysed for total dissolved carbon using a Shimadzu TOC 5000 (Shimadzu TOC-L analyser, Shimadzu Corporation, Columbia, Maryland, USA) after diluting the filtrate by a factor of 10

and then filtering to remove a white precipitate (calcium sulphate) that is sometimes observed. Total organic carbon (TOC) is determined by subtracting inorganic carbon (IC) from total carbon (TC). Microbial biomass C was calculated as the difference between the C extracted from the chloroform fumigated and the non-fumigated sample.

5.2.2.2 *Fluorescein Diacetate Hydrolysis*

Fluorescein diacetate (FDA) is an esterase substrate that can diffuse quickly through the cell membrane, therefore serving as a viability probe that measures cell membrane integrity and enzymatic activity. The presence of enzymes such as protease, lipase, and esterase hydrolyse the fluorescein diacetate compound to yield fluorescein. Because of the non-specificity of the enzymes that can hydrolyse this compound, FDA analysis has been used as a broad indicator of soil microbial activity (Adam and Duncan, 2001; Bandick and Dick, 1999; Perucci, Vischetti and Battistoni, 1999; Sánchez-Monedero *et al.*, 2008; Shaw and Burns, 2006).

One-gram sub-samples of 2 mm sieved soil * 72 microcosms (all 12 replicates of each treatment) soil samples were weighed into sterile McCartney bottles and 7.5 ml of potassium phosphate buffer (pH 7.6, 60 mM), made by dissolving 8.7 g of K_2HPO_4 and 1.3 g of KH_2PO_4 in 1 L of distilled water, sterilised by autoclaving (121 ºC, 20 min), was added and allowed to equilibrate at 25 ºC on a counter rotator for 30 min, the incubation time was based on in-lab protocol. The reaction was then started by adding 0.1 ml FDA solution (1000 µg/ml) solution made by dissolving 25 mg of fluorescein diacetate (3'6' diacetyl-fluorescein) in 25 ml acetone to the samples and returned to the rotator for another 30 min based on set laboratory protocol. After 30 min had elapsed, 7.5 ml of extractant (2:1, chloroform: methanol) was added to stop the reaction. The contents were mixed by vortex for 10 s and centrifuged (RCF = 300 x g, 5 min) to clarify the phases. The transferred top phase was then centrifuged (RCF = 16, 500 x g, 5 min) to remove suspended fines. The supernatant was analysed using a spectrophotometer at 490 nm against an appropriate blank consisting of the

soil and buffer mixture with the FDA solution replaced by 0.1 ml acetone and incubated simultaneously as the samples. The calibration curve for FDA analysis was constructed using fluorescein working standards (0 -5 µg/ml) prepared from the fluorescein master solution (2000 µg/ml), made by dissolving 113.2 mg fluorescein disodium salt in 50 ml of potassium phosphate buffer (60 mM, pH 7.6) and extracted with chloroform: methanol extractant, as for the samples.

The mass of fluorescein produced per mass of soil was determined using the calibration curve by dividing the corresponding OD490nm values by the equivalent dry weight of soil.

Fluorescein diacetate hydrolysis activity was expressed as µg fluorescein/g dry soil/ 0.5 h.

5.2.2.3 *Dehydrogenase activity*

The dehydrogenase activity method is used to determine the intracellular activity of dehydrogenase enzymes that can be used as an indicator of microbial activity (Goel *et al.*, 1998; Hongwei *et al.*, 2002). Dehydrogenases only function in living cells and are thought to indicate the overall activities of microorganisms in respiring soil organic matter. Dehydrogenase activity is measured by adding tetrazolium salts such as 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to the biological system. This salt is used as a terminal hydrogen and electron acceptor. The salt is reduced after the addition of two hydrogen atoms and electrons. The colour changes from colourless to red insoluble p-iodonitrotetrazolium formazan (INTF) crystal that dissolves in organic solvent and thus can be extracted from microbial cells. The extracted solution concentration of INTF is measurable by spectrophotometer at 464 nm (Hongwei *et al.*, 2002; Kim, Koopman and Bitton, 1994). The method used in this study is a modified method based on the method used by (Shaw and Burns, 2006).

One-gram sub-samples of soil * 72 microcosms (all 12 replicates of each treatment) soil sample (2 mm sieved) were weighed into sterile McCartney bottles and 4 ml of filter (0.2 µm)-sterile aqueous INT solution (3%, m/v) added to the sample. The lids of the bottles were closed, and the samples were incubated in the dark at 25 ºC for 48 hrs. After the incubation period, 10 ml of the extractant (N, N-dimethyl formamide: ethanol (1:1, v/v)) was added and incubated in the dark with agitation for one hour. Approximately 1.2 ml of the extractant/soil mixtures were transferred to microcentrifuge tubes and centrifuged (relative centrifugal force (RCF) = 11,600 x g, 5 mins). The absorbance of the supernatant was determined using a spectrophotometer at 464 nm against an appropriate blank (sterile water instead of INT solution) and INTF calibration standard solutions (0 -25 µg/ml) made in a mixture of extractant: distilled water (5:2, v/v) which was prepared from the INTF master standard solution (500 µg/ml in extractant).

The concentration of INTF in the samples were estimated using the INTF calibration curve from corresponding OD464nm values and Equation 1.

Equation 1. Dehydrogenase activity calculations.

Dehydrogenase activity (µg INTF/g dry soil/48 h) = ([INTFs] – [INTFc]) * 14/Edw

where: [INTFs] is the INTF concentration (µg/ml) in the sample; [INTFc] is the INTF concentration in the control; Edw is the equivalent dry weight of 1 g of soil (determined by loss of weight of field-moist subsamples after heating at 105 ºC until constant weight), and 14 is the volume (ml) of solution added in the assay (INT + extractant).

5.2.3 DNA extraction and 16S rDNA amplicon-based sequence analysis of soil samples

Soil DNA was extracted using Qiagen Powersoil Pro-Kit (Qiagen, Valencia, CA), following the manufacturer's instruction. The extracted DNA samples were stored at -20 ºC until further use. The 16S rRNA gene amplification and sequencing of the V4 variable region were performed at Next-Generation Sequencing provider Molecular Research Laboratory (MRDNA) (www.mrdnalab.com, Shallowater, TX, USA) (Caporaso *et al.*, 2012). The samples were barcoded and in-house primers 515F/806R were utilised to analyse the microbial communities of soil samples on the Illumina Miseq platform with the method based on the bTEFAP® process (Dowd et al., 2008). A single-step 30-cycle PCR with HotStarTaq Plus Master Mix Kit (Qiagen, USA) was used under the following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, with a final elongation step at 72°C for 5 minutes. After the amplification process, the PCR products were checked in 2% agarose gel to determine the success of the amplification and the relative intensity of bands. The PCR products were used to prepare an Illumina DNA library according to the Illumina TruSeq DNA library preparation protocol. The data sequences were processed using the MRDNA analysis pipeline (MRDNA, Shallowater, TX, USA). In summary, sequences were joined, sequences <150bp removed, and sequences with ambiguous base calls removed. Sequences were quality filtered using a maximum expected error threshold of 1.0 and dereplicated. The dereplicated or unique sequences were denoised; unique sequences were identified with sequencing and/or PCR point errors and removed, followed by chimera removal, thereby providing a denoised sequence or zOTU. The final OTUs were taxonomically classified using BLASTn against a curated derived database based on NCBI (www.ncbi.nlm.nih.gov).

5.2.4 Data analysis

All graphs showing the effect of dilution to extinction and the equilibration over the 27 weeks on the microbial biomass C/ bioactivity using fluorescein diacetate hydrolysis, dehydrogenase activity analysis and fumigation extraction were presented using excel. The effect of the treatment on the biomass/bioactivity was analysed using one-way analysis of variance (ANOVA) and the post hoc test based on the Tukey comparative model to test for statistical analysis. The data was checked for normality with Shapiro-Wilk's test, and homogeneity of variance checked with Levene's test before using ANOVA.

The resulting OTU table from the 16S rRNA analysis of the soil was used for analysis. Chao1 richness, Simpson and Shannon indices were determined using the 'vegan' package in R programming (R package vegan v2. 5-6). The sequencing generated 29655040 high quality forward reads with an average of 30476 reads per sample. The dataset was rarefied to 20246 reads per samples. Taxonomy assigned using BLAST database resulted in bacteria being classed into 17 phyla, 46 classes, 107 orders, 191 family, 430 genera and 830 species. Differences between diversity treatments were tested using one-way analysis of variance to test the difference in the alpha diversity index between the groups ($p < 0.05$). Tukey analysis was also performed to check for significant differences between each treatment in each diversity index. The beta diversity was estimated by calculating Bray-Curtis distances. This was visualised using principal coordinate analysis (PCoA). The PERMANOVA test was used to determine the matrix distance (permutations = 999).

5.3 Results

5.3.1 Assessment of microbial biomass and bioactivity of the dilution-to-extinction soil

The microbial biomass and bioactivity of the soil inoculated with series of microbial suspensions produced through the dilution-to-extinction approach and control (10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}, autoclaved and intact) were assessed at the end of a 27-week incubation period **Figure *5-3***a-c. After the equilibration period, ANOVA revealed an overall significant ($p<0.05$) effect of treatment on the level of biomass and bioactivity (FDA and DHA). Post-hoc analysis revealed in each case that this treatment effect was due to significantly reduced biomass/bioactivity in the autoclaved soil but with no statistical difference ($p>0.05$) between measurements for intact soil and all autoclaved but re-inoculated treatments.

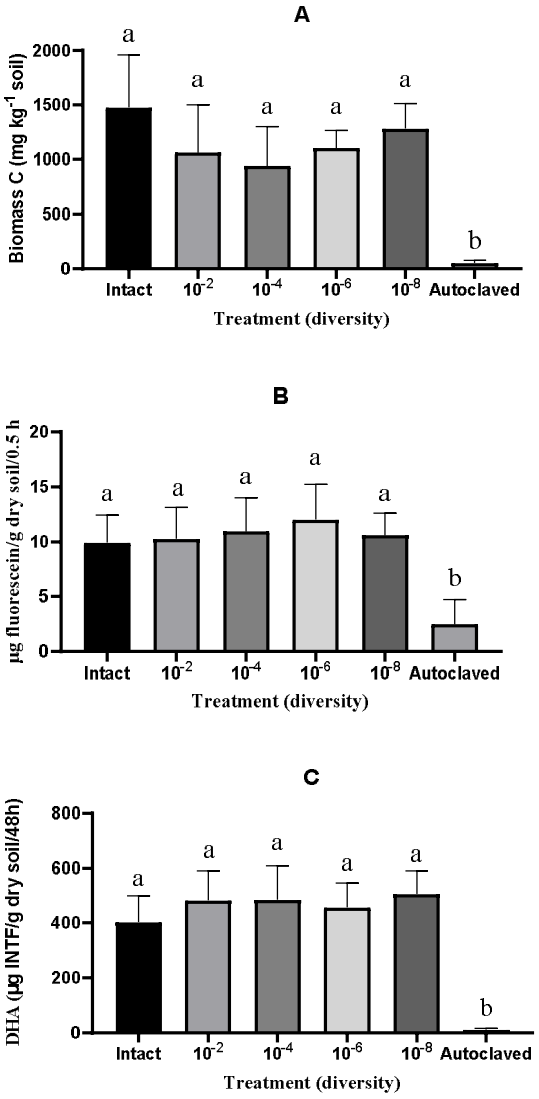

Figure 5-3. (A) Microbial biomass using fumigation analysis, (B) Fluorescein diacetate analysis (FDA), and (C) dehydrogenase activity (DHA) in soil inoculated with 10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8} microbial suspensions. Also included is the fresh intact soil and autoclaved soil, all after 27-week equilibration period. Bars marked with the same letters indicate that they are not significantly different at $p > 0.05$. Values are mean (biomass; n = 4; FDA; n = 12; DHA; n = 12) and error bars indicate standard error.

5.3.2 Effect of dilution on the diversity of the bacterial community

5.3.2.1 *16S rRNA amplicon sequencing*

Illumina 16S rRNA amplicon analysis was performed to examine the effect of the microbial suspension dilution method on the bacterial communities present in soil after the equilibration period. Taxonomy was assigned which resulted in bacteria being classed into 17 phyla, 46 classes, 107 orders, 191 family, 430 genera and 830 species.

The α diversity **Figure 5-4**, showed a significant difference between the treatments (Chao1 richness ($p < 0.05$); Shannon index ($p< 0.05$) and Simpson index ($p < 0.05$)). For β diversity, a principal coordinate analysis (PCoA) plot using Bray-Curtis similarity matrix **Figure 5-5** was used to visualise the similarity in the bacterial composition for soil samples after the equilibration period. The pattern of sample clustering in the PCoA showed the intact soil was distinct from other communities. Bacterial communities from Microcosms containing 10^{-2} and 10^{-4} were grouped together and autoclaved, 10^{-6} and 10^{-8} grouped together. Permutational multivariate analysis of variance (PERMANOVA) showed a significant difference ($P = 0.001$).

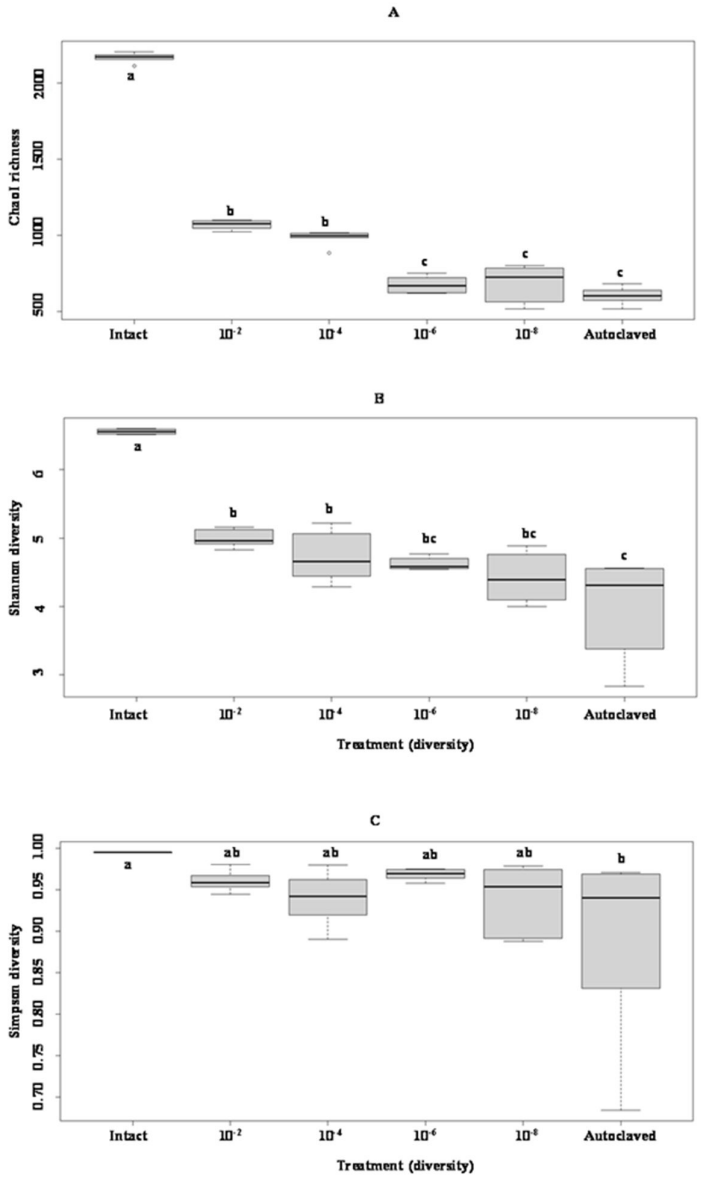

Figure 5-4. Box plot showing the alpha diversities estimated using the Chao1 estimator, Shannon index and Simpson index in autoclaved soil reinoculated with diluted suspensions of the initial non-autoclaved soil at four dilutions ($10^{-2,}$ 10^{-4}, 10^{-6}, and 10^{-8}), intact soil and autoclaved soil after 27 days of incubation in soil. Treatments marked with the same letters indicate that they are not significantly different at $p < 0.05$. $n = 6$.

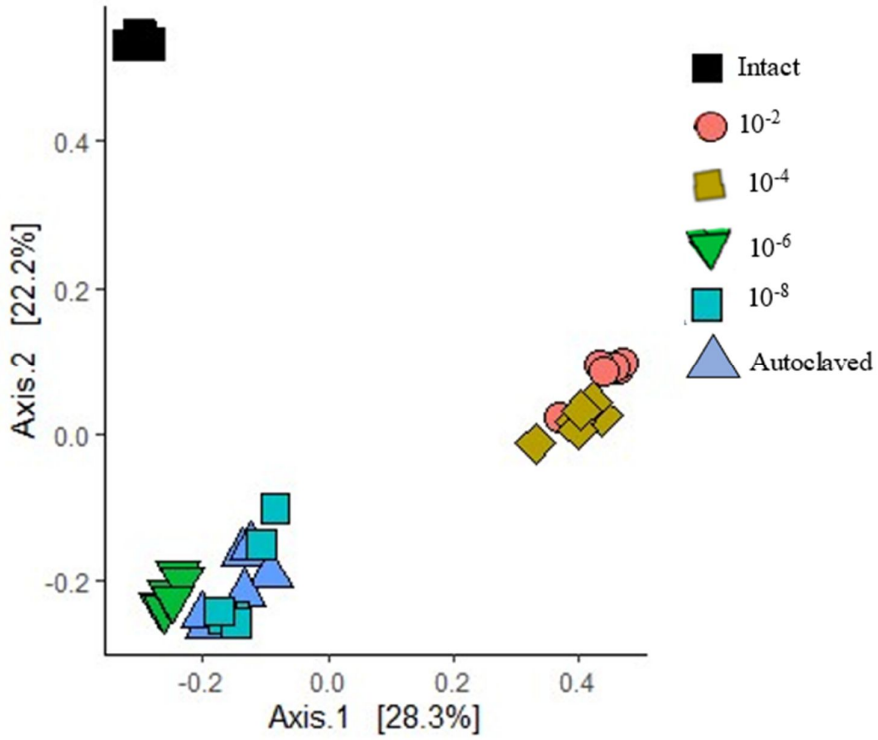

Figure 5-5. Principal coordinate analysis (PCoA) plot of Bray-Curtis similarity matrix among six replicate samples of autoclaved soil reinoculated with diluted suspensions of the initial non-autoclaved soil at four dilutions (10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8}), intact (non-autoclaved) soil and autoclaved (non-inoculated) soil after 27 days of incubation. Points that are closer together on the ordination reflect communities that are similar. Permutational multivariate analysis of variance (PERMANOVA) indicated a significant ($p < 0.05$) difference between communities.

5.3.2.2 *Effect of dilution on bacterial communities*

The 16S rRNA gene sequencing was used to assess the bacterial community composition of the soil to show the effect of the microbial suspension dilution method after the equilibration period. The relative abundance of bacteria community composition at the phylum level in soil with 10^{-2} and 10^{-4} were similar to each other and dominated by Acidobacteria (4.3% -2.6%), Actinobacteria (8.5% -3.2%), Bacteroidetes (26.8%-19.6%), and Proteobacteria (60.9%-45.9%). The 10^{-6}, 10^{-8} and autoclaved soil were similar and dominated by Acidobacteria (5.1%-0.1%), Actinobacteria (8.8%-7.0%), Bacteroidetes (23.8%-22.5%), and Proteobacteria (58.4%-45.8%). The Intact soil was dominated by Acidobacteria (5.8%), Actinobacteria (14.2%), Bacteroidetes (7.2%), Chlorofexi (2.7%), Proteobacteria (41.9%). When compared to the intact soil, the effect of dilution method resulted in the loss of Armatimonadetes, Nitrospirae and Ignavibacteria.

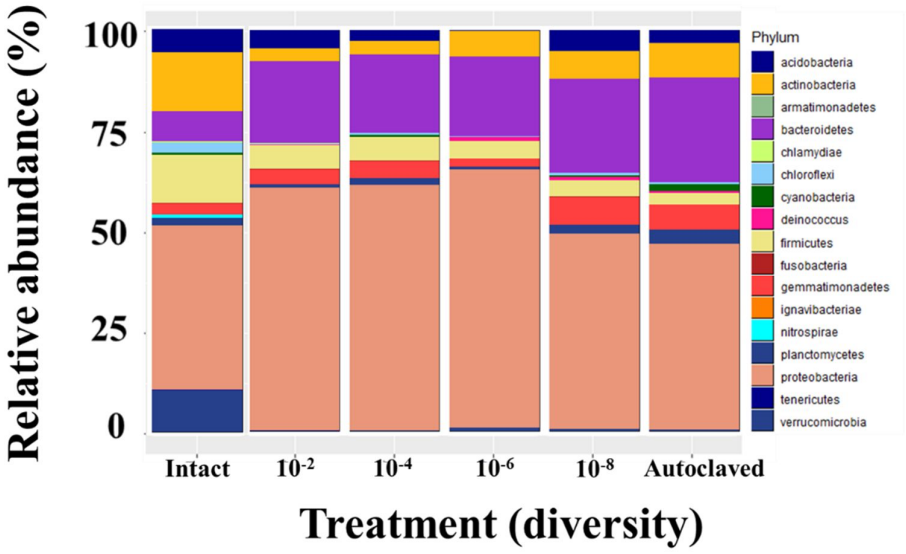

Figure 5-6. Bar chart showing the bacterial community composition at the phylum level for the different treatments.

5.4 Discussion

The objectives of this experiment were to: (1) use a dilution-to-extinction process to create soil with a gradient of microbial richness and to describe the gradient achieved using high throughput amplicon sequencing; (2) examine whether, following the inoculation with soil dilutions, soil microbial biomass and activity recovered during the equilibration period to levels that were comparable between dilution treatments

The dilution to extinction approach has been used in several studies to manipulate soil microbial diversity (Van Elsas *et al.*, 2012; Hol *et al.*, 2015; Maron *et al.*, 2018; Matos, Kerkhof and Garland, 2005; Tardy *et al.*, 2014; Wertz *et al.*, 2006; Zhang and Zhang, 2015). Our approach here is one of the available methods that can produce series of soils with a gradient of microbial diversity. Based on probability, this method should result in the loss of the rare members of the soil microbial community at the higher dilution factors while the initially abundant members remain prominent, depending on their ability to re-colonise the soil following inoculation. It is necessary to allow the re-colonised soil a period of incubation to allowed re-inoculated cells, initially at low and unequal (depending on the dilution factor of the inoculum) abundance, to recolonise soil and for populations to stabilise and reach a new equilibrium and carrying capacity of the soil (Vivant *et al.*, 2013). To test hypotheses about the significance of microbial diversity for ecosystem function (chapter 6), the soils used should only differ in diversity and not have unequal biomass which would confound the interpretation of the data with respect to biodiversity differences.

The pattern observed in the alpha diversity **Figure 5-4** showed that the dilution-to-extinction treatment was able to create soil with varying microbial diversity. This agrees with (Maron *et al.*, 2018; Wertz *et al.*, 2006; Zhang and Zhang, 2015), where the action of the dilution process resulted in the progressive decline in the species richness depending on the dilution

treatments when compared to the source (intact) soil. This varying microbial diversity evident in the diversity measurement **Figure 5-4** could be attributed to the loss of rare species and persistence of abundant species.

Zhang and Zhang, (2015), in their work, used the dilution - to - extinction approach to look at the patterns of biomass production in two diverse types of soil (sandy soil microcosms and grassland soil microcosms). Their work agrees with this current study methodology in terms of the dilution factors used and the use of gene sequencing. Still, it differs in their biomass analysis methodology, which involved the survey of enumerating bacteria culturable on agar plates. Overall, their biomass survey showed that the bacterial biomass in microcosms varied among the treatments and some significant departure from their source soil, but not in a consistent manner. This disagrees with the findings in this current study, the use of fumigation analysis to determine microbial biomass did not show any significant difference between the control (intact soil) and soil with microbial dilution. But it agrees with (Tardy *et al.*, 2014), where their bacterial densities and bacterial biomass in three diversity treatments did not significantly differ. However, with no significant differences observed, what can be seen in our study was the differences in the magnitude of biomass between the control (intact soil) and the soil with microbial dilution.

Another way to measure the success of the recolonisation of soil after the dilution process would be to check the microbial activity of the re-colonised microcosm. Soil dehydrogenase activity, as well as fluorescein diacetate analysis, are good indicators of total soil microbial activity (Goel *et al.*, 1998; Hongwei *et al.*, 2002; Salazar *et al.*, 2011; Yuan and Yue, 2012). The non-specificity of the enzymes involved in dehydrogenases activity and fluorescein diacetate analysis such as protease and lipases (Guilbault and Kramer, 1964; Rotman and Papermaster, 1966), made them a good technique to determine the amount of active microbial biomass present. This study observed no significant difference ($p < 0.05$) between the intact

soil and the soil with dilution treatment, but a difference compared to autoclaved soil in both analyses. This indicates that the microbes in the soil with dilution treatment had no significant difference in their biomass activity when compared to intact soil.

In terms of species richness, the dilution treatment in soil microcosm showed a decrease in diversity of species detectable by the sequencing analysis **Figure 5-4** this agrees with Wertz *et al.*, (2006); Philippot, Spor, *et al.*, (2013) and Zhang and Zhang, (2015), where the species richness decreases progressively in microcosms with various dilutions of microbial suspensions. The species richness in the low-level dilution in our study (10^{-2} and 10^{-4}), such as *Nitrospirae* and *Ignavibacteriae*, did not appear to survive the dilution procedure. This is evident in **Figure 5-6**. However, Zhang and Zhang, (2015) suggested a unique perspective to how the dilution process may not have been the cause of the reduction in diversity observed in the low-level dilution microcosms (10^{-2} and 10^{-4}). They suggested that it could be that these species were transferred during the inoculation but did not persist in the soil and became non-detectable during the equilibration period. Our results also showed the different dynamics of bacterial interaction in the soil. This is evident when some rare species persisted in the microcosms with dilution, and some abundant species such as *Verrucomicrobia* became significantly less abundant on the brink of extinction. In addition, we observed the high proliferation of some bacteria compared to the control soil after the microbial disturbance caused by the autoclaving procedure. This change in the abundance could reflect that some microbes have a faster growth rate and are usually restricted by the presence of other bacteria in the soil. Also, the autoclaving process would have created a nutrient source from the dead microbes, which would aid the faster growth of bacteria inoculated into the soil. These different changes in the abundance of bacteria agree with Fierer, Bradford and Jackson, (2007); Maron *et al.*, (2018); Pascault *et al.*, (2013) and Tardy *et al.*, (2014). Here, just like in our study, there was a decrease in the abundance in some slow-growing bacteria

such as *Acidobacteria* and *Chloroflexi* (Fierer, Bradford and Jackson, 2007; Maron *et al.*, 2018; Pascault *et al.*, 2013; Tardy *et al.*, 2014) but an increase in the dominance of fast-growing phyla such as *proteobacteria* and *Firmicutes* (Fierer, Bradford and Jackson, 2007; Pascault *et al.*, 2013).

The overall analysis of the changes in the microbial composition due to the dilution treatment is represented in the beta diversity measures that calculated all the sample results using Bray-Curtis distance matrix for the ordination of PCOA **Figure 5-5**, as well as in **Figure 5-6** that looks at the relative abundance of the microbes at the phylum level. A detailed look at the PCOA **Figure 5-5** showed a significant ($p < 0.05$) shift in the microbial composition of the soil in the diluted soil compared to the intact soil. Noticeable is the similarity in the microbial composition between the lowest dilution factors 10^{-2} and 10^{-4}, and then the similarity between the two highest dilution treatments and the autoclaved soil. This pattern of grouping is also noticeable in their abundance measures **Figure 5-6**.

In summary, the patterns observed in the soil with dilution treatment that has equilibrated over 27 weeks agrees with the objectives that soil with a gradient of microbial communities has been created. Increasing dilution treatment led to a decrease in species diversity and a dissimilarity in species composition compared to the intact soil **Figure 5-4** and **Figure 5-5**. These agree with multiple studies focused on dilution methods (Wertz et al., 2006; Zhang and Zhang, 2015). We also observed the removal of rare species in the lower-level dilution treatment. As mentioned above, these rare species were likely present during inoculation but did not survive during the equilibration period. A way to resolve this problem and affirm that the rare species were transferred from the source soil would be to analyse the soil immediately after inoculation or to analyse the inoculant itself. The patterns observed for the biomass and bioactivity analysis also showed that the period of 27 weeks was adequate to allow all soil with dilution treatment to reach their maximum soil microbial carrying

capabilities when compared to the intact soil. This will ensure that the next step that focuses on microbial diversity's function will not have significant interference due to differences in microbial biomass.

Chapter 6 The impact of soil microbial diversity on *L. terrestris* and C cycle functions in maize residue-amended soil

6.1 Introduction

Soil organic carbon (SOC) is a key component of terrestrial ecosystems and represents a major pool of carbon in the global carbon cycle. The majority of SOC is composed of material of plant origin in various stages of decomposition (Castellano *et al.*, 2015). The mineralization of plant-derived inputs to soil is an important biological process that releases carbon dioxide (CO_2) into the atmosphere as well as making nutrients available in the soil (Li *et al.*, 2013; Mwafulirwa *et al.*, 2019; Raiesi, 2006). Mineralization rate and its extent depends on soil and plant residue physical, chemical and soil organism-related factors (Frouz *et al.*, 2014).

Earthworms are often referred to as ecosystem engineers and are considered effective decomposers that play a significant role in organic matter (OM) mineralisation. Earthworms influence the rate of OM turnover (Gómez-Brandón, Lores and Domínguez, 2012), through their actions of transporting OM residues, fragmenting, ingesting, integrating, and making them microbially accessible.

The role of soil microorganisms in soil ecosystem functioning to facilitate soil functions such as organic matter decomposition, as the primary decomposers, plant growth and carbon cycling is also crucial (Braga *et al.*, 2016; Coq *et al.*, 2007). It is considered that earthworms influence soil microbial community structure and diversity, and through this modification may influence microbially mediated functions (Medina-Sauza *et al.*, 2019). Earthworms can select and stimulate certain dormant microorganisms in their guts (Hoang *et al.*, 2017), using the intestinal energy-rich mucus in a phenomenon referred to as 'the sleeping beauty paradox' (Lavelle *et al.*, 1995; Brown, Barois and Lavelle, 2000; Hoang *et al.*, 2017), a process that stimulates key microorganisms necessary to carry out soil OM decomposition and hence, helps to increase the digestion of ingested SOM, since earthworms are not able to sufficiently produce the enzymes necessary for digestion (Fujii, Ikeda and Yoshida, 2012;

Lattaud *et al.*, 1998). Medina-Sauza *et al.*, (2019) have conceptualised the earthworm-soil microbial relationship in facilitating ecosystem functions such as nutrient cycling and nutrient mineralisation in soil (**Figure 6-1**). However, with many studies focusing on the influence of earthworms on soil microbes and microbial functions, reverse studies on the influence of soil microbial diversity on earthworm functions is lacking (**Figure 6-1**).

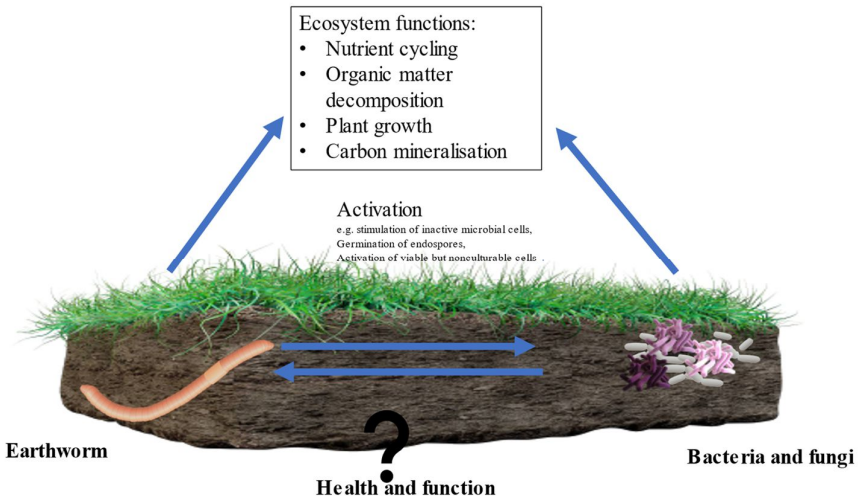

Figure 6-1. Summary of the hypothesis that soil microbial diversity influences the earthworm's health and functions. Adapted from Medina-Sauza *et al.*, (2019).

The interaction between the soil microbial community and earthworms with respect to OM mineralisation and the loss of carbon in terms of respiration has been vastly studied. A quantitative literature review by Lubbers *et al.*, (2013) looking at the effect of earthworm presence vs earthworm absence on soil respiration, showed that in, short-term studies, the presence of earthworms increased the respired CO_2 by 33 %. Many of the earthworm-soil microorganism studies are based on the assumption of 'healthy' soil with diverse soil microbial communities.

The previous work (thesis; chapter four) which investigated the influence of earthworm gut-associated microorganisms and soil microorganisms on the food preference of *L. terrestris*, showed that the manipulation of both the soil and earthworm microbiome, through the sterilisation or suppression of associated microbiomes, caused a change in *L. terrestris* feeding behaviour, altering both the choice and amount of litter consumed over the experimental period. Exposing earthworms to a completely eliminated soil microbiome is admittedly an extreme situation and not something that an earthworm would encounter in field soil. However, earthworms inhabit field soils that are variable in microbial diversity and community composition. Whether such variation in soil microbial diversity might influence the direct- and indirect- (mediated through soil microbial communities) earthworm-effects on soil functioning is yet to be investigated.

There have been many studies on the diversity of microbial communities on both the small and the large scale (Maron, Mougel and Ranjard, 2011). These studies systemically reveal that soil microbial diversity distribution is both heterogenous and spatially-structured (Maron, Mougel and Ranjard, 2011; Ranjard and Richaume, 2001). On a small scale, the heterogenous distribution of soil microbial diversity is generally determined by aeration, soil structure and soil organic carbon content (Ranjard and Richaume, 2001). On a large scale, pH is known to be the best predictor of soil microbial diversity and richness (Bååth *et al.*, 1995; Fierer *et al.*, 2005), as well as factors like soil organic status (Lejon *et al.*, 2007) , and soil management (Nicolardot *et al.*, 2007). All these factors inherently lead to changes in the microbial community diversity and composition which could inherently make changes to earthworms function.

The nature of the relationship between biodiversity and ecosystem function (B-EF) is one of the most researched topics in ecology and there is an increasing volume of studies with specific focus on the influence of soil microbial diversity on ecosystem functions such as OM decomposition and soil respiration (Bardgett and Van Der Putten, 2014; Fitter *et al.*, 2005; Heintz-Buschart *et al.*, 2020; Hunt and Wall, 2002; Philippot, Spor, *et al.*, 2013). Currently, a number of relationships between species richness and ecosystem function have been proposed (Nielsen *et al.*, 2011; Peter, 2011). One of the theories is that ecosystem function increases linearly with increases in biodiversity, implying that the ecosystem functioning is sensitive to any decline in biodiversity, as all species are required for a specific function to take place (Nielsen *et al.*, 2011). There is also the theory of functional redundancy, whereby the ecosystem can lose biodiversity, but ecosystem functions would continue, because there are other species capable of performing the same ecological functions without any consequences (Wertz *et al.*, 2006). The final relationship is idiosyncratic based on species with similar traits but differ in functioning (Peter, 2011). However, most of the studies conducted with plants and animals support a consensus view that most B-EF relationship follows a redundancy pattern (Bardgett and Van Der Putten, 2014; Cardinale *et al.*, 2011; Maron *et al.*, 2018).

Given that the main impact of earthworms on soil function is through stimulation of microbial communities to mineralize C through mixing, fragmentation, the kiss (priming) (Brown, Barois and Lavelle, 2000; Fujii, Ikeda and Yoshida, 2012; Hoang *et al.*, 2017; Lattaud *et al.*, 1998; Lavelle *et al.*, 1995; Lavelle and Gilot, 1994), and microbial C mineralization is not so sensitive to reductions in biodiversity being a function with a high degree of functional redundancy (Bardgett and Van Der Putten, 2014; Cardinale *et al.*, 2011; Maron *et al.*, 2018), it follows that the extent to which the earthworm-mediated stimulation of microbial C mineralization, and the potential nutritional feedbacks to earthworm health and

function, may also be similarly insensitive to reductions in biodiversity. On the other hand, if microbial functions provided to the earthworm extend beyond those related to provision of nutrients via general depolymerization of residue and SOM substrates and are more specialised (detoxification, for example), examining the relationship between soil microbial diversity and earthworm health and function may reveal more sensitive (linear) or idiosyncratic relationships.

The specific objectives were to use soil containing a gradient of microbial communities created using a dilution-to-extinction process (**Chapter five**) and *L. terrestris* as the test earthworm species to: (1) verify the positive influence of earthworms on soil organic matter mineralization in residue amended and non-amended soil; (2) determine if changes in soil microbial diversity influence: (i) soil respiration; (ii) the effect of earthworms on OM decomposition; (iii) the health status of earthworms that may feedback to ecosystem functioning.

Hypothesis:

1. The presence of earthworms will increase OM mineralisation irrespective of soil microbial diversity.
2. In the absence of earthworms, the soil microbial diversity -OM mineralization function relationship will suggest functional redundancy in soil microbial communities
3. The extent of the earthworm-induced increases in OM mineralization will depend on soil microbial diversity and be linked to microbial diversity impacts on earthworm health.

6.2 Material and methods

6.2.1 Soil

The soil used in this study was prepared as described in (Thesis: chapter four). Briefly, Hall farm soil was sieved to < 4 mm, homogenised and then sixty aliquots of 800 g of soil were autoclaved (121 ºC, 1 hour on three separate occasions). A further twelve 800 g aliquots were stored at 4 ºC and used as 'intact 'soil. Microbial soil suspensions of intact soil were made using serial dilution to create a dilution-to-extinction (10^{-2}, 10^{-4}, 10^{-6} and 10^{-8}) inoculant for 48 of the autoclaved aliquots. The remaining twelve autoclaved aliquots were non-inoculated and served as sterile controls. All aliquots (those that were sterilised, intact or with added microbial suspensions) were left to incubate at 70 % of the moisture holding capacity in the dark at 20 ºC for 27 weeks.

6.2.2 Residue preparation

Maize straw residues were collected from The University of Reading's Sonning farm in October 2019 after harvest and air dried. The residues were air dried for two weeks, and then chopped to approximately 1 cm in size to allow the homogenous mixing of residues with soil that increases the accessibility for earthworm consumption (Sizmur *et al.*, 2017) and mineralisation by soil microorganisms.

6.2.3 Earthworms

Specimens of *Lumbricus terrestris* were purchased from Worms Direct (Essex, UK). The earthworms were acclimated to the laboratory conditions for two weeks prior to the start of the experiment in a culture made from Kettering loam and Irish moss peat (2:1 ratio) in the

dark at 20 ± 2 °C (Arnold *et al.*, 2003; Sizmur *et al.*, 2011)and the earthworms were fed Irish moss peat at approximately 1 g earthworm^{-1} week^{-1} after one week of acclimation.

6.2.4 Experimental design

In a 52-day microcosm experiment, we quantified the effect of soil microbial diversity and the presence of earthworms on CO_2 emissions, after the addition of maize straw residue as source of organic matter input. The experiment had six replicates (4 replicates for chambers with residue present and 2 replicates of chambers with no residue) and it consisted of the treatments below **Table 6-1**:

Table 6-1. Table showing the experimental treatments containing maize straw, earthworm, and diversity distribution

Treatment	**Factor**			**replication**
	Diversity	**Earthworm**	**Residue**	
	Intact	+	+	4
	10^{-2}	+	+	4
	10^{-4}	+	+	4
	10^{-6}	+	+	4
	10^{-8}	+	+	4
	Autoclaved	+	+	4
	Intact	-	+	4
	10^{-2}	-	+	4
	10^{-4}	-	+	4
	10^{-6}	-	+	4
	10^{-8}	-	+	4

	Autoclaved	-	+	4
	Intact	+	-	2
	10^{-2}	+	-	2
	10^{-4}	+	-	2
	10^{-6}	+	-	2
	10^{-8}	+	-	2
	Autoclaved	+	-	2
	Intact	-	-	2
	10^{-2}	-	-	2
	10^{-4}	-	-	2
	10^{-6}	-	-	2
	10^{-8}	-	-	2
	Autoclaved	-	-	2

The treatments without earthworms were also set up to be able to quantify CO_2 emissions influenced by soil microbial diversity only. As well as control treatment with no earthworm or amended with maize residue.

Six hundred grams of soil (intact, autoclaved, 10^{-2}, 10^{-4}, 10^{-6} and 10^{-8}) was placed in a 1000 ml plastic wide-neck bottle; 1 g of maize straw was incorporated and slightly mixed with the soil on the surface. Although, this is different from the natural environment, the presence of litter on the surface will encourage the earthworms (*L. terrestris*) to feed as normal by coming to the surface through their burrows and dragging the litters into their burrows for decomposition (Bouché, 1977; Scheu *et al.*, 2002). For each treatment and replicate, one earthworm (*L. terrestris*) was added to the microcosm. Each microcosm was loosely covered

with its top for sufficient aeration but enough to stop the earthworm from escaping. All microcosms were incubated in the dark at 20 ºC. The moisture content was maintained gravimetrically by adding sterilised distilled water weekly for the first five weeks and afterward, on sampling day, after gas sampling.

Respiration of CO_2 was determined over a sampling time course (section 6.2.5), and, at the end of the 52-day experiment, earthworms were sampled to assess their health status through energy reserve measurements (section 6.2.6).

6.2.5 Headspace sampling and analysis for CO_2

On days 0, 1, 3, 6, 15, 20, 34, and 52 of the incubation, the bottles were opened and allowed to be flushed with ambient air for 30 min by leaving the lid off. The bottles were subsequently sealed with a rubber septum, the bottles were flushed with lab air, allow to incubate for 60 min before gas from the headspace was sampled by inserting a hypodermic needle with a 15 ml syringe through the septum and withdrawing 15 ml of the headspace air and then adding it to a 12 ml exetainer vials, creating overpressure, as the T_0 sample. Bottles were then closed for 1 hour (Shaaban *et al.*, 2016; Wu *et al.*, 2018) and then the gas was sampled again, as at T_0, to obtain a T_{60min} sample. After sampling, the bottles were loosely closed to allow aeration. The gas concentrations were analysed using gas chromatography equipped with a flame ionization detector, split/splitless front inlet (GC-7890B, Agilent Technologies, USA). Helium was used for carrier, FID makeup and the dilution of the sample. Air and H_2 were supplied to the FID. CO_2 calibration gases of 506, 2542 and 5163 ppm CO_2 supplied in nitrogen (SIP Analytical Ltd) were used as external standards.

6.2.6 Determination of *L. terrestris* energy reserves

After the last gas samples were collected, the earthworms were removed from the soil, cleaned, washed in sterilised water, weighed, depurated, weighed, and stored in plastic tube and kept at -20° C before destructively sampling. On the day of the sampling, the worms were taken out of the freezer and blended using a Dounce homogeniser. The earthworms' health status was determined using energy budget analysis through the quantitative measurement of total lipid, protein and carbohydrate content using methods modified from Bligh and Dyer, (1959); Amorim *et al.*, (2012); Albalasmeh, Berhe and Ghezzehei, (2013); Świątek and Bednarska, (2019). Each weighted frozen *L. terrestris* was added into a 50 ml glass Dounce homogeniser on ice and homogenised in a total volume of 1.8 ml distilled water. The homogenate was transferred into 15 ml centrifuge tubes for storage at -20° C until use.

For analysis of lipid content, 500 µl of homogenate was diluted 1:2 (v/v) by adding to 1000 µl distilled water in a 15 ml centrifuge tube. The mixture was thoroughly mixed using a vortex mixer. One hundred µl of the diluted homogenate samples or standard solutions (tripalmitate in chloroform; 0, 2, 4, 6, 10, 20 and 30 µg/ml) were transferred to Pyrex cell culture tubes and 250 µl of chloroform and 250 µl methanol were added. The samples were vortex mixed after the addition of each solvent. After centrifugation (1000 x *g*, 5 min, 20 ºC), the top phase (aqueous layer of methanol) was removed and 500 µL of concentrated H_2SO_4 was added to the remaining lipid extract (chloroform phase) which was then incubated in a heating block and the lipid extract charred for 15 min at 200 ºC in a fume cupboard. The Tube was removed and placed at room temperature for 15 seconds before being transferred to the ice bath for 5 minutes. The charred remains were reconstituted by the addition of 1.5 ml of distilled water, thoroughly mixed and placed on ice. The total lipid content was determined

after the tube had been left standing for 10 min and all bubbles had disappeared at an absorbance of 400 nm using tripalmitate (sigma) as standard.

For the total protein content, 300 µl of the prepared undiluted homogenate was added to an Eppendorf tube and 100 µl of 15 % trichloroacetic acid (TCA) was added and incubated at -20 ºC for 10 minutes. After the centrifugation (2500 x *g*, 4 ºC for 10 min) in a micro centrifuge, the supernatant was separated into a new Eppendorf tube and reserved for carbohydrate measurement. The pellet was re-suspended in 100 µl of 5% TCA, vortexed to mix, centrifuged, and the supernatant was collected for carbohydrate measurement, this collection was pooled with the previously collected sample. The pellet was re-suspended again in 500 µl of 1 M NaOH, incubated at 60 ºC for 30 minutes, and neutralised with 300 µl of 1.67 M HCl. The total protein content was assessed using 1 ml Bradford's reagent (Sigma, USA; Bradford, 1976; Applichem, 1990). The absorbance was measured at 595 nm using bovine serum albumin as a standard (Sigma, USA; 0, 10, 20,40,60,80,100,200,300,400 and 500 µg/ml) prepared in 1M NaOH and analysed alongside the unknown samples following the same steps.

To determine the carbohydrate content, 600 µl of concentrated H_2SO_4 was added to a 200 µl aliquot of the supernatant fraction (reserved from above) in an Eppendorf tube. The mixture was cooled on ice for 2 min to bring it to room temperature. The carbohydrate content quantification was performed by measuring absorbance at 315 nm against a standard curve of glucose (Sigma, USA; 10, 15, 20, 25, 30, 40, 50 and 60 µg/ml dissolved in distilled water) and treated as the samples. Because this method is not appropriate for samples that absorb in the UV range without any pre-treatment, the samples were pre-screened by measuring their absorbance at 315 nm at the start of the experiment without any treatment.

The different energy fractions (lipids, proteins, and carbohydrates) were converted into their energy equivalents by multiplying them with their energy of combustion (Gnaiger, 1983; Świątek and Bednarska, 2019): 17.5 KJ/g carbohydrate, 24 KJ/g proteins, and 39.5 KJ/g lipids.

6.2.7 Data analysis

The cumulative data for C respired as CO_2 was calculated by the summation of the C-CO_2 data as it grows with time. Repeated-measures analysis of variance (ANOVA) and mixed model repeated measures was performed in GenStat (18^{th} edition) to examine the effect of soil microbial diversity, earthworm presence and the interaction between microbial diversity and earthworm presence on respired CO_2. The data for energy reserves was analysed using two-ways analysis of variance (ANOVA) performed in GenStat (18^{th} edition). The data were checked for normality and lognormality test using Shapiro-Wilk test. The regression analysis carried out using linear regression analysis in excel.

6.3 Results

6.3.1 Impact of earthworms and residues on cumulative C-CO2 emissions in soils of differing microbial diversity

Figure 6-2 shows the effect of the impact of the presence of earthworms and residues on the observed cumulative emissions of C-CO_2 in soil with differing microbial diversity during the experiment period of 52 days.

Figure 6-2a – f and **Table 6-2** shows the mean cumulative C-CO_2 respired over the 52-day experiment across all diversity treatments, and the repeated measures analysis of variance of the data using 'earthworm', 'residue', and 'time' as factors. The overall C-CO_2 emissions in all the soil treatments during the 52 days period was positively affected by the time ($p <$ 0.05), earthworm ($p < 0.05$), and residue ($p < 0.05$). There was no significant effect of time * earthworm*residue interaction on cumulative C respired in intact soil and most of the soil with lower dilution of soil microbial diversity ($p > 0.05$). Also noticeable was that the effect of residue did not depend on the presence of earthworm in most of the different diversity treatments except in 10^{-6} and 10^{-8}. However, the effect of either the residue or earthworm was depended on time (time* earthworm ($p < 0.05$), time*residue ($p < 0.05$), across all diversity treatments.

Figure 6-2g shows the mean cumulative C-CO_2 respired over the 52-day experiment across all diversity treatments. The two-way analysis of variance with 'earthworm' and 'residue' as factors (**Table 6-3**) revealed that both earthworm ($p = <.001$) and residue ($p = <.001$) had a significant impact on cumulative C respired but that the effect of residue did not depend on the presence of earthworms (earthworm*residue interaction $p = 0.382$).

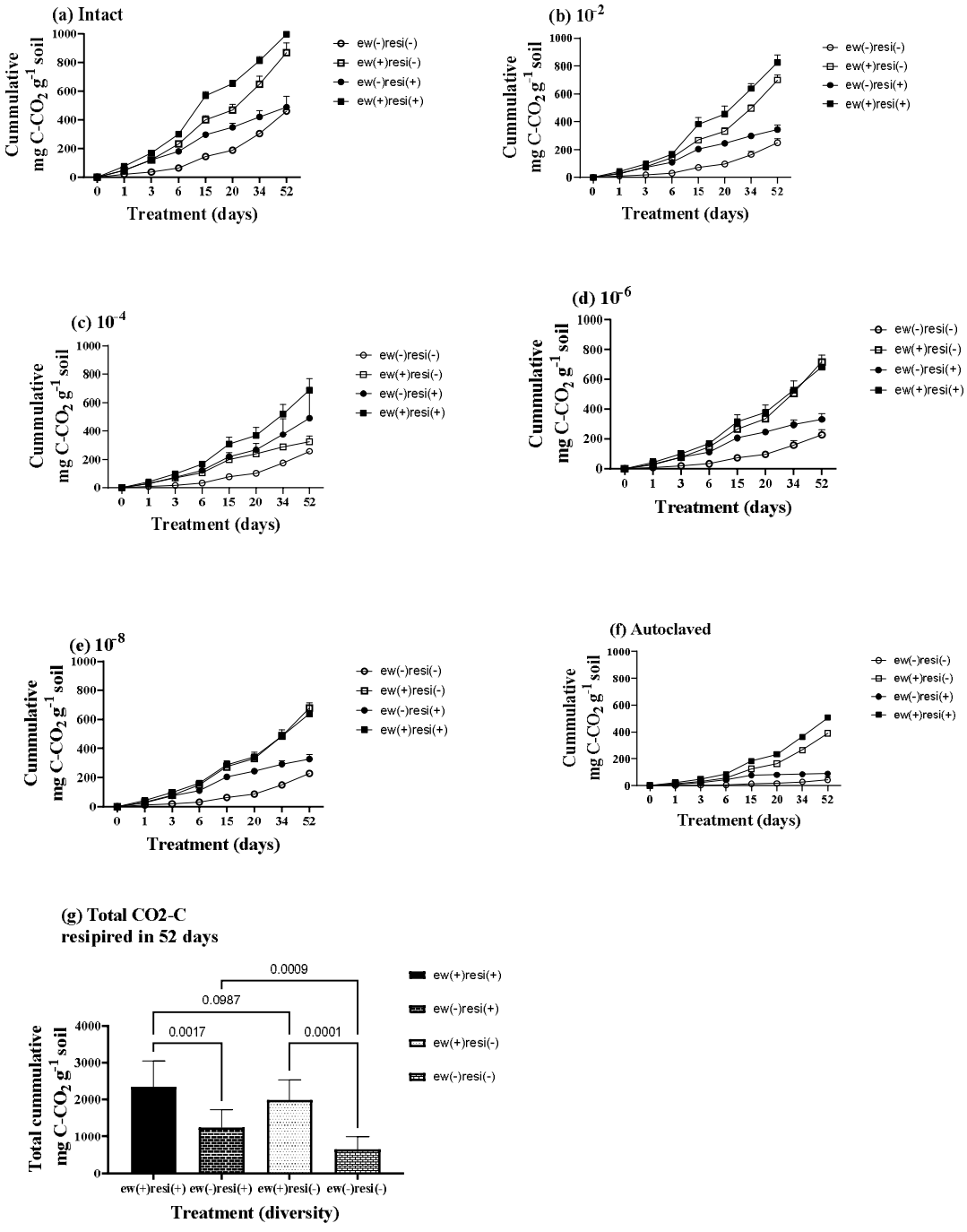

Figure 6-2. Graphs showing the impact of earthworms and residues on cumulative C-CO_2 emissions in soils of differing microbial diversity treatment ;(a) Intact diversity (b) 10^{-2}, (c)

10^{-4} (d) 10^{-6} (e) 10^{-8} (f) autoclaved (g) Total C-CO_2 respired in 52 days. The error bar represents standard errors of mean

Table 6-2. Repeated measures ANOVA analysis for the impact of earthworms and residues on cumulative C-CO_2 emissions in soils of differing microbial diversity treatment with earthworm, residue, and time as factors

Diversity	Source of variation	d.f	m.s	Fpr
	Earthworm	1	1004176.7	<.001
	Residue	1	173205.1	<.001
	Time	7	792806.6	<.001
	Earthworm*residue	1	725.6	0.533
	Time*earthworm	7	91245.5	<.001
	Time*residue	7	10229.5	<.001
Intact (A)	Time*earthworm*residue	7	1490.1	0.093
	Earthworm	1	670081.7	<.001
	Residue	1	80006.8	<.001
	Time	7	466959.5	<.001
	Earthworm*residue	1	714.3	0.54
	Time*earthworm	7	85941.7	<.001
	Time*residue	7	8376.1	<.001
10^{-2} (B)	Time*earthworm*residue	7	435.3	0.38
	Earthworm	1	407302.6	<.001
	Residue	1	65747.8	<.001
	Time	7	362556.3	<.001
	Earthworm*residue	1	7918.9	0.141
	Time*earthworm	7	53519.4	<.001
	Time*residue	7	3355.2	0.023
10^{-4} (C)	Time*earthworm*residue	7	404.1	0.395
	Earthworm	1	449676.6	<.001
	Residue	1	64622.2	<.001
	Time	7	373767.4	<.001
	Earthworm*residue	1	25313.5	0.013
	Time*earthworm	7	57117.1	<.001
	Time*residue	7	3156.4	0.022
10^{-6} (D)	Time*earthworm*residue	7	1809.9	0.08

	Earthworm	1	371408.9	<.001
	Residue	1	44655.9	0.002
	Time	7	338939.4	<.001
	Earthworm*residue	1	37285.5	0.004
	Time*earthworm	7	45609.3	<.001
	Time*residue	7	2438.2	0.018
10^{-8} (E)	Time*earthworm*residue	7	2675.3	0.014
	Earthworm	1	374066.33	<.001
	Residue	1	28592.86	<.001
	Time	7	110925.02	<.001
	Earthworm*residue	1	898.33	0.073
	Time*earthworm	7	59001.92	<.001
	Time*residue	7	2763.49	<.001
				<.001
Autoclaved (F)	Time*earthworm*residue	7	520.57	

Table 6-3. Two-way ANOVA analysis for mean cumulative C-CO_2 respired over 52 days with earthworm and residue as factors.

Source of variation	**d.f**	**m.s**	**Fpr**
Earthworm	1	25235756	<.001
Residue	1	3706507	<.001
Earthworm*residue	1	225230	0.382

6.3.2 The effect of soil microbial diversity on earthworm C cycle function in maize-amended soils

Focussing just on the data for the treatments receiving maize residues, **Figure 6-3** and **Table 6-4** examines the effect of soil microbial diversity, earthworm presence and the interaction between soil microbial diversity and earthworms on cumulative C respired.

The repeated measures analysis of variance with 'earthworm' 'diversity' and time as factors (**Table 6-4**) revealed that a significant effect of diversity treatment (p = <0.001), presence of earthworm (p = <0.001), and time (p = <0.001) on the cumulative C-CO_2 emissions. There was also a significant earthworm*diversity treatment interaction ($p < 0.05$) indicting that the effect of earthworm depended on diversity treatment. The analysis also revealed a significant impact on cumulative C respired by time*diversity*earthworm interaction (p = <0.001).

Two-way analysis of variance examination of the effect of earthworm and microbial diversity treatment on total C-CO_2 respired over the experimental period (**Figure 6-3**a; **Table 6-5**) also supported the significant impact of the presence of earthworm (p = <0.001) and diversity treatment (p = <0.001). However, there was no significant earthworm*diversity treatment interaction ($p = 0.693$).

Chao1 estimated total OTUs (chapter 5) for each sample within the microbial biodiversity treatments was used to examine the impact of earthworm presence on the relationship between microbial biodiversity (Chao1 richness)- and C mineralization function (**Figure 6-3** b-c) via linear regression.

The Pearson correlation for **Figure 6-3**b (with earthworm) suggests that the correlation between cumulative CO_2-C emissions and Chao1 richness is strong at 0.928. The regression model of between cumulative CO_2-C emissions and Chao1 richness indicates that the relationship is statistically significant, $p < 0.0075$. The Pearson correlation for **Figure 6-3**c

(without earthworm) suggests that the correlation between cumulative CO_2-C emissions and Chao1 richness is strong at 0.775. The regression model of between cumulative CO_2-C emissions and Chao1 richness indicates that the relationship is not statistically significant, $p < 0.071$.

Table 6-4. Repeated measures ANOVA analysis for the effect of soil microbial diversity on earthworm C cycle function in maize-amended soils with earthworm, diversity treatment and time as factor.

Source of variation	d.f	m.s	Fpr
Earthworm	1	1831397.3	<.001
Diversity treatment	5	337386.1	<.001
Time	7	1688929.7	<.001
Earthworm* diversity treatment	5	26877.5	<.001
Time*earthworm	7	247737.4	<.001
Time* diversity treatment	35	23924.3	<.001
Time*earthworm* diversity treatment	35	2708.5	<.001

Table 6-5. Two-way analysis for the effect of soil microbial diversity on earthworm C cycle function in maize-amended soils with earthworm and diversity treatment as factor.

Source of variation	d.f	m.s	Fpr
Earthworm	1	1831397	<.001
Diversity treatment	5	337386	<.001
Earthworm* diversity treatment	5	26878	0.693

Table 6-6. One way analysis for the effect of soil microbial diversity and organic matter mineralisation.

Source	**P-Value**
Diversity	0.000
Source of variation	**Grouping**
Intact	A
10^{-2}	B
10^{-6}	B
10^{-8}	B
10^{-4}	B
Autoclaved	C

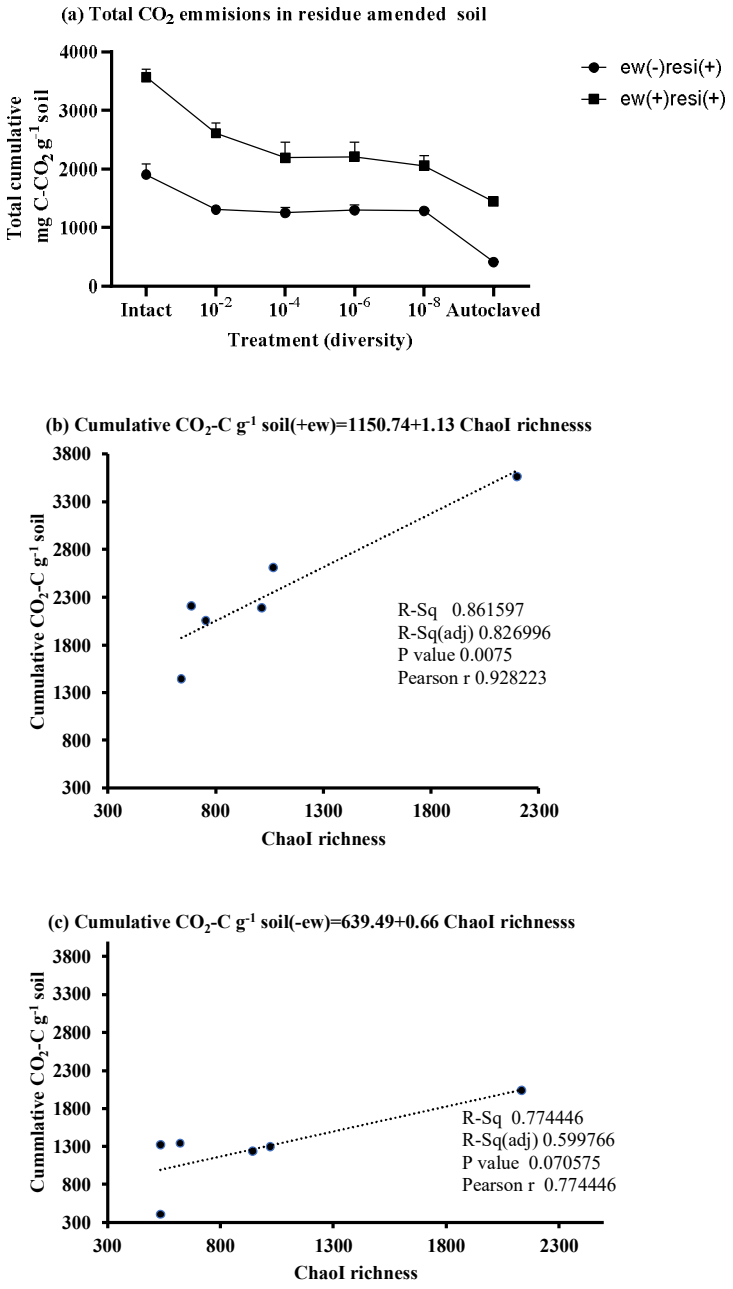

Figure 6-3. The effect of soil microbial diversity on C cycle function in maize residue-amended soils in microcosms: (a) cumulative CO_2-C emission with respect to dilution to extinction treatment in microcosms with maize amend soil in the presence and absence of earthworms (b) scatter plot and linear regression to examine the relationship between microbial diversity (as Chao1 richness) and cumulative CO_2-C emission for mesocosms with

earthworms present (c) scatter plot with linear regression data of cumulative CO_2-C emission vs Chao1 richness for data with earthworms absent.

6.3.3 The effect of soil microbial diversity on earthworm health in maize residue-amended soils

Figure 6-4 shows effect of soil microbial diversity treatment and microbial Chao1 richness on earthworm health in maize residue-amended soils, as determined by quantification of available energy reserves (E_a) as total lipid, protein, and carbohydrate content of earthworms. One-way ANOVA revealed that microbial diversity treatment had an overall significant impact on lipid (**Figure 6-4**a; p = <0.001), protein (**Figure 6-4**b; p = 0.019) and carbohydrate (**Figure 6-4**c; p = <0.001) reserves. Post-hoc analysis indicated that for the lipid energy reserves (**Figure 6-4**a), in the autoclaved soil, lipid E_a was lower and significantly different to the other soil treatments ($p < 0.05$). Protein and carbohydrate energy reserves (**Figure 6-4**b, c) were also lowest, and often significantly lower, in the autoclaved diversity treatment than in other treatments but no significant difference was observed between the carbohydrate contents of earthworms in intact soil and that of autoclaved soil. There was no statistical difference between intact and soil dilution (10^{-2}, 10^{-4}, 10^{-6} and 10^{-8}) diversity treatments for lipid (**Figure 6-4**a).

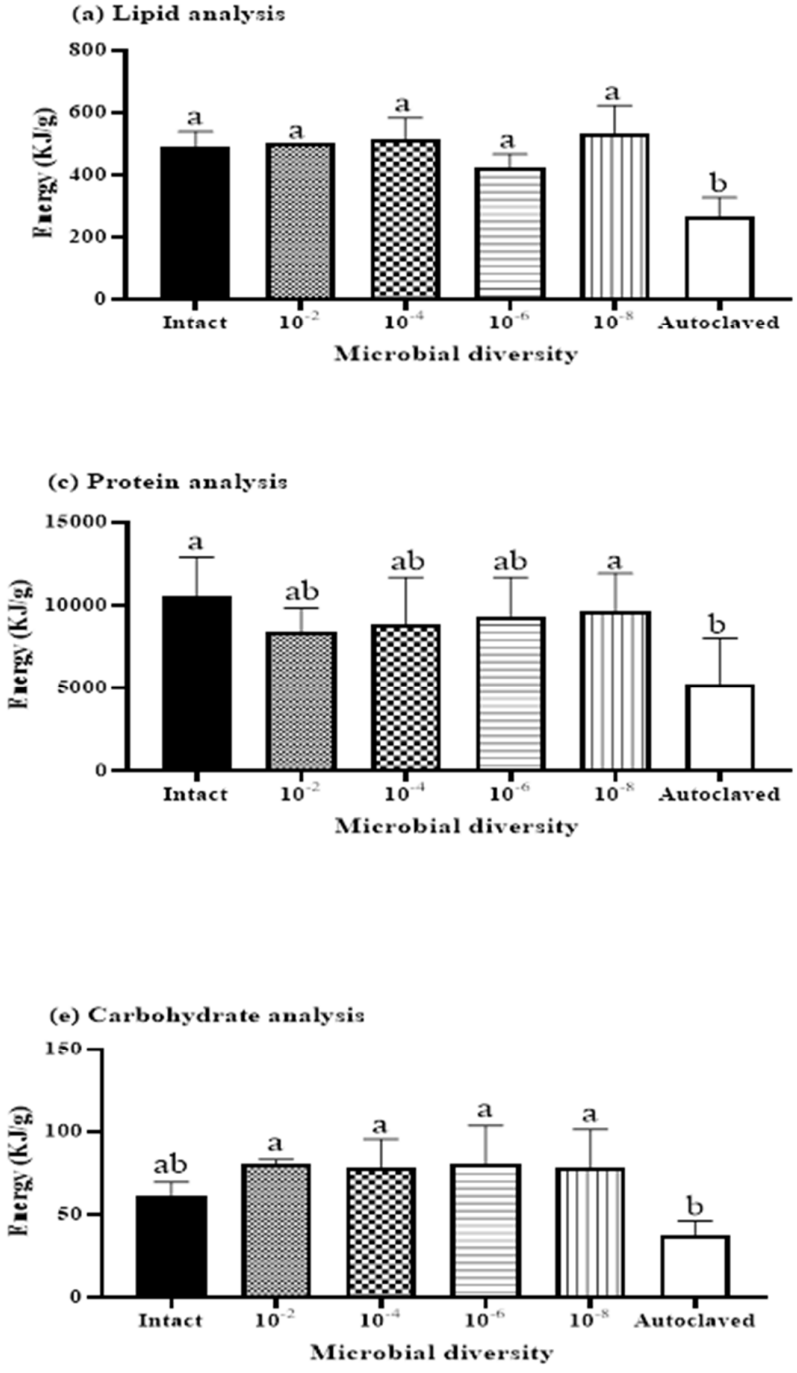

Figure 6-4: Total energy reserves (a)lipid (b) protein and (c) carbohydrate

6.4 Discussion

It is well established that earthworms are essential to ecosystem functioning (Bardgett and Van Der Putten, 2014; Philippot, Raaijmakers, *et al.*, 2013). A considerable amount of research has examined the impact of earthworms on soil microbial biomass, community composition and activity. However, research seldom focuses on the reverse interaction, such as the impact of soil microbial communities on earthworm-mediated function. Consequently, the experiment reported in this chapter aimed to examine the influence of soil microbial diversity on earthworm health and function in soil organic matter mineralisation. To fulfil this aim, we used the dilution-to-extinction soil created (in Chapter five) to obtain soils containing varying gradients of microbial diversity and richness.

Impact of earthworms and residues on cumulative CO_2-C emissions in soils of differing microbial diversity.

It was first hypothesised that the presence of earthworms would increase OM mineralisation irrespective of soil microbial diversity. The result showed that across all the different diversity treatments, **Figure 6-2**a- f and **Table 6-2**, the presence of earthworms in the microcosms had a significant effect ($p < 0.05$) on the cumulative C respired. This indicates that earthworms, either directly through contribution to total heterotrophic respiration or indirectly through the stimulation of microbial respiration by earthworm activity, contributed to the increase in cumulative C respired.

Earthworms can contribute to respiration directly through their own respiration (Nieminen *et al.*, 2015; Uvarov, 2016). In addition, earthworms add a large amount of easily degradable organic C (mucus) to the ingested material when they feed on organic matter (Angst *et al.*, 2019; Trigo *et al.*, 1999). This carbon of earthworm origin will be subject to microbial respiration and contributes to the cumulative C respired (passand Anderson, 1992; Lavelle

and Gilot, 1994; Bohlen *et al.*, 2002; Bityutskii, Maiorov and Orlova, 2012; Medina-Sauza *et al.*, 2019). The input of mucus C also might indirectly contribute to respired C through the stimulation of microbial respiration on SOM and residue-C. This indirect action has been termed the 'Sleeping Beauty Paradox', whereby the dormant microbes are activated by the presence of the mucus 'kiss' and triggers the surge of microbial processes with direct effects on increased soil respiration (Brown, Barois and Lavelle, 2000; Medina-Sauza *et al.*, 2019). Also, earthworms indirectly contribute to the respired carbon by stimulation of microbial decomposition of organic matter through fragmentation and transportation of fresh organic material into the soil (Edwards, 2004).

The experimental results on the impact of the presence of earthworms on soil respiration agrees with previous quantitative studies by Lubbers *et al.*, (2013), which reported on the influence of earthworm presence vs earthworm absence on the soil. Lubbers *et al.*, (2013) showed a strong effect of the presence of earthworms and an increase (33 %) CO_2-C emission from the soil over a short period < 30 days, compared with 46 % on average over 52 days in this present study. Other studies such as Frouz *et al.*, (2014); Lubbers, Pulleman and Van Groenigen, (2017) also agree with our study and indicate that the presence of earthworms increases the C respired from the soil. This is contrary to the studies by Guo *et al.*, (2019) that indicated that the presence of earthworms did not increase soil respiration. Variations in experimental design (e.g. numbers of earthworm individuals per mesocosm, earthworm species, soil type) may explain contrasting findings between studies, however, it is generally a consensus that the introduction of earthworms to ~~a~~ microcosm would increase the C respired over a short time but not significantly so after a long time (Hamamoto and Uchida, 2019; Lubbers *et al.*, 2013; Wu *et al.*, 2018).

In the absence of earthworms, the addition of maize residue also resulted in a significant ($p < 0.05$) increase in soil respiration when compared to the non-residue amended control (Table 2). This is in agreement with many other studies (Badagliacca *et al.*, 2017; Han *et al.*, 2015; Huang and Spohn, 2015; Wu *et al.*, 2018; Xu, Liu and Sayer, 2013) and is not surprising since the addition of litter residue provides a source of decomposable substrates for soil microbes which are catabolised to CO_2. The addition of the fresh residue also increased the availability of labile carbon that may then stimulate the decomposition of older organic matter compounds; the process referred to as 'priming' (Kuzyakov, Friedel and Stahr, 2000; Sayer, Powers and Tanner, 2007).

In contrast to the mesocosms without earthworms, there was weaker evidence for a residue addition effect on total respired C in treatments with earthworms. This agrees with the findings by Bossuyt, Six and Hendrix, (2005), but was contradictory to the findings by Zhang and Hendrix, (1995) and Zareitalabad *et al.*, (2010), where there was a significant increase in C respired in the treatment with earthworms and litter residue. Their increase in C respired can be explained by the enhanced contact between litter and soil microbes (Uyl, Didden and Marinissen, 2002).

However, the results for this current study can be attributed to competition for labile residue C between the earthworm and soil microbes and the production of macroaggregate by the earthworm that makes the labile carbon less accessible for soil respiration by microbes (Bossuyt, Six and Hendrix, 2005) that might counter-balance potential stimulatory effects of earthworms on respiration from both residue and native SOM through 'the kiss'.

Effect of soil microbial diversity on earthworm C cycle function in maize residue-amended soils.

A second hypothesis for this study is that, in the absence of earthworms, the soil microbial diversity-OM mineralisation function relationship will suggest functional redundancy in soil microbial communities.

Although the function of respiration (decomposition) and the amount of respiration is different, and a lower C respired does not equate to lower functioning, the data in this study is an approximation. Measurement of soil respiration is one of the widely acceptable ways to measure carbon and energy flows in detritus ecology (Singh and Gupta 1977).

The one-way analysis of variance and Tukey analysis data **Table 6-6** observed from the microcosm experiments did not support the hypothesis that the soil microbial diversity-OM mineralisation function relationship will suggest functional redundancy in soil microbial communities., because the cumulative C respired differs significantly between the intact soil and that of the diversity treatments (10^{-2} -10^{-8}). Similarly, the relationship between Chao1 richness and cumulative C respired in **Figure 6-3**c, showed a strong correlation between the two variables (as the Chao1 richness increases, the cumulative C respired increases), but not a significant relationship ($p = 0.07$) between the two variables. These findings contradict the most beliefs that most soil microbial communities are characterised by functional redundancy (Nannipieri *et al.*, 2003).

Also, the biomass result obtained in (Chapter five), which shows no significant differences between the biomass of intact soil and manipulated soil after the equilibration period, eliminated the argument that differences in microbial biomass was the driving force for function instead of varying diversity and richness. It also supports Kemmitt *et al.*, (2008), which has previously suggested that microbial biomass is not necessarily related to microbial respiration. Instead, this current result showed some inconsistency in the diversity

relationship. Comparing all the data, primarily the intact and the manipulated soil diversity, highlight an idiosyncratic relationship with functional consequences because of diversity loss due to the dilution-to-extinction ratio.

However, assessing the manipulated soil containing varying gradients of microbial diversity alone (10^{-2} -10^{-8}) indicate that despite the progressive reduction in microbial diversity, OM mineralisation did not differ significantly between the treatment and showed a redundancy relationship, and support the hypothesis. This result from the manipulated soil only data is consistent with (Griffiths *et al.*, 2001; Wertz *et al.*, 2006) and highlight the low sensitivity of soil microbial communities functioning to diversity erosion.

This study also hypothesised that the extent of the earthworm-induced increase in OM mineralisation would depend on soil microbial diversity. **Figure 6-3**a shows the positive effect of earthworm addition on CO_2 mineralization across all microbial diversity treatments, however, the relative magnitude of this earthworm-induced increase showed little variation with microbial diversity as supported by the lack of a statistically significant interaction effect (earthworm* diversity treatment; $p>0.05$; **Table 6-5**). This suggests that the stimulation of OM mineralisation by earthworm is insensitive to soil microbial species richness and is contrary to the prediction in hypothesis 2.

Effect of soil microbial diversity on earthworm health in maize residue-amended soils

The commonly known roles of the microbiome to their hosts, such as earthworm, for their health and function, is the role of nutrition provision, aiding digestion, and creating immunity (Brestoff and Artis, 2013; Buffie and Pamer, 2013; Nyholm and Graf, 2012). Microbes associated with the mucosal lining of their host can help stimulate the production of

antimicrobial peptide and provide resistance to fight against invading pathogens (Koch and Schmid-Hempel, 2011; Kwong, Mancenido and Moran, 2017).

Perturbation of this healthy system can reduce the availability of some of the microbes that can potentially lead to a reduction in the immune function of the host, and thereby makes the host susceptible to pathogens, unable to carry out their basic functioning, and consequently death (Motta, Raymann and Moran, 2018).

Naturally, earthworms live in soil with a high diversity of microbes. The gut of earthworms has relatively been well described. It is known that most of these gut microbiome is composed of either transient bacteria associated with their ingested food and soil (Zeibich, Schmidt and Drake, 2019) or bacteria that are tightly associated with the intestinal wall (Singleton *et al.*, 2003; Thakuria *et al.*, 2010). These microbes are also known to confer immunity and protection against the pathogen in a system not fully understood (Cooper and Roch, 2003). Any loss of some of the earthworm symbiotic microbes through any means, such as pollution, can reduce the host's fitness (Pass *et al.*, 2015; Viana *et al.*, 2018). This reduction in fitness could feedback to earthworm functioning in terms of altered physiological or behavioural interactions with residue and SOM-C. Hence, this study hypothesised that the relationship between soil microbial diversity and the extent of earthworm-induced increases in OM mineralisation is linked to microbial diversity impacts on earthworm health. This was tested by assessing the differences between the available energy reserve based on carbohydrate, protein, and lipid analysis in earthworms exposed to the dilution-to-extinction soil microbial diversity gradient.

Energy reserve analysis can be used as an indicator of general wellbeing of an organism (Bednarska, Stachowicz and Kuriańska, 2013; Stachowicz and Lebiedzińska, 2016; Świątek and Bednarska, 2019). Energy reserves for an organism are fixed. Any changes to the

organism system can cause a change in how these reserves are used and diminish the amount available for other essential maintenance of the organism, such as growth and reproduction (Calow, 1991). These reductions can be directly determined using energy reserve analysis (De Coen and Janssen, 1997). A few studies have used this energy budget approach to measure the effect of metal and toxic stress on soil invertebrates (Bednarska, Stachowicz and Kuriańska, 2013; Moolman, Van Vuren and Wepener, 2007; Świątek and Bednarska, 2019), but this method, to our knowledge has rarely been used in this situation with reduced microbial diversity caused by the dilution-to-extinction process. It is postulated that changes in the average microbial diversity of the soil caused by reduced microbial richness and diversity would potentially remove taxa used by the earthworms for general (e.g nutrition) or a more specialised purpose such as immunity and detoxification. This would eventually result in a stressed earthworm, preventing them from carrying out essential functions such as OM mineralisation.

In this present study, the energy reserves of the earthworm were measured at the very end of the soil C respiration experiment. Therefore, it is impossible to measure the temporal variation during this experimental period.

The findings in this study revealed that the earthworms in soil with the most eroded soil microbial diversity (autoclaved soil) consistently had the lowest energy reserves measured **Figure 6-4** despite the access to an equal source of native soil organic matter and added maize residue. This could be related to the availability of microbial biomass as a source of nutrition. In Chapter five, the autoclaved soil with the most eroded microbial richness was observed to have the smallest microbial biomass present after the equilibration period, whilst every other treated soil recovered their microbial biomass comparable to the intact soil. It could also be due to the reduction in microbial diversity and richness, resulting in the loss of microbes with a specialised role for OM mineralisation by the earthworm.

In conclusion our experiment reveals that, both addition of earthworm and plant residues to soil stimulate soil respiration. However, the actions of an earthworm in a soil during OM mineralisation is not influenced by the presence of litter residue. We also observe a correlation between bacterial species richness and C respiration. Our data showed evidence for a redundant biodiversity – C mineralisation relationship in the absence of earthworms. We also observed the importance of the presence of microbial biomass and diversity to earthworms carrying out their ecological function and their health in soil with severely eroded microbial community. This is because, the lowest energy reserves measured in lipid, protein and carbohydrate was from the soil with the most eroded microbial biomass and diversity. Our findings clarify the importance of the presence of microbes for earthworms health and function. However, due to the largely redundant relationships between bacterial species richness and earthworm health and earthworm functional impacts on SOM decomposition, there was no evidence for a bacterial role in providing non-redundant, specialised functions to the earthworm host that could influence feedbacks between C cycle function and health at low levels of biodiversity loss.

Chapter 7 General discussion and Conclusion

7.1 General discussion

In recent years, there has been a growing interest in the gut-associated microbiome of soil-dwelling organisms, on their functionalities and their importance for their host (Drake and Horn, 2007; Liu *et al.*, 2018). This has in part been fuelled by the rise in global challenges which has led to a reduction in soil microbial biodiversity, from which microbiomes might be partially derived, that is pivotal to ecosystems and services (Bardgett and Van Der Putten, 2014; Wagg *et al.*, 2014). Some research has indicated that gut-associated microbiomes of invertebrates such as earthworms form a symbiotic relationship with the host, which plays a vital role in the health and functioning of the host (Kostic, Howitt and Garrett, 2013). For example, it is thought that the microbiome symbiotic relationship with the host provides digestion and nutritional benefits (Fujii, Ikeda and Yoshida, 2012) and influences the host's reproduction and maturation (Viana *et al.*, 2018). Although many studies demonstrate the advantages of the microbiome symbiotic relationship as mentioned above, there is still a lack of understanding about the impact of these microorganisms on their earthworm hosts and the potential for general and more exquisite functional roles of earthworm transient soil microorganisms. The knowledge of high microbial diversity in the soil and prominent levels of microbial functional redundancy has highlighted a knowledge gap for further research into the role of this diversity for the earthworm microbiome's greater functionality and its support of ecosystem processes, as well as earthworm's health.

The overall aim of this thesis (Chapter 1) was therefore to further understanding of the importance of the earthworm - earthworm microbiome relationship in supporting critical soil ecosystem process such as organic matter mineralisation, explicitly paying attention to the feedback of the presence of the earthworm microbiome to the health and function of earthworms in supporting ecosystem processes.

To address this aim, manipulations and experiments were carried out to examine the impacts of different levels of perturbation to both (tightly- associated) earthworm and (transient-ingested) soil microbiomes. The key findings addressing the objectives posed in chapter 1 are discussed below.

Assessing the efficacy of antibiotic treatment to produce earthworms with a suppressed microbiome.

The objective for Chapter 3 was to develop and evaluate an antibiotic-based procedure for producing 'axenic' specimens of earthworms (*E. fetida* and *A. chlorotica* and *L. terrestris)*. The creation of 'axenic' earthworms, or at least earthworms with substantially reduced microbial burden, was deemed an essential first step to understanding the role of both: (i) the (tightly-associated) earthworm microbiome; and (ii) the (transient) soil-derived microbiome. For the former (i), it was hypothesized that antibiotic treatment would impact the earthworm microbiome directly. In the case of (ii), future studies examining the role of (ingested) soil microbiome diversity required earthworms with an irradicated/ reduced microbiome to minimise the earthworm-mediated re-introduction of microorganisms to soil, and therefore disruption to soil-based experiments involving sterile or low microbial diversity treatments.

Antibiotics, added singularly, depending on the antibiotic added, or in a cocktail mixture, were found to be effective in reducing the abundance of earthworm-associated culturable microorganisms. Some antibiotics added singularly were ineffective, possibly due to antibiotic resistance or the antibiotic spectrum of activity relative to the taxa that were earthworm-associated. For example, in the analyses on the two types of agar used, the microbes were consistently resistant to penicillin, a beta-lactam antibiotic. There is common knowledge of widespread resistance in soil bacteria to penicillin (Demanèche *et al.*, 2008; Armalytė *et al.*, 2019). In both *E. fetida* (NA) and *A. chlorotica* earthworm species, antibiotic

cocktail reduced the microbial abundance below the detection limit. This finding strengthens the evidence from other studies that antibiotics can create earthworms (*E. fetida* in this case) that are considered 'axenic' based on a lack of observable culturable colony forming units (Hand and Hayes, 1987; Whiston and Seal, 1988).

Whilst antibiotic treatment of *E. fetida* (NA) and *A. chlorotica* reduced the culturable bacteria below the limit of detection, the culturable microbiome of *L. terrestris,* although significantly reduced, was not completely eradicated, suggesting that the efficacy of antibiotics in creating 'axenic' earthworms might be dependent on the earthworm species. Species differ in burrowing and feeding behaviour and other possible qualities not the focus of this experiment. *Lumbricus terrestris* is a species noted to harbour many antibiotic-resistant microorganisms (Liu *et al.*, 2019; Zhu *et al.*, 2019), and some of its culturable bacteria have shown resistance to the effect of the antibiotics. Although the antibiotic agar volume was scaled up for *L. terrestris*, it was also possible that the earthworm did not explore and ingest agar to the same extent as the *E. fetida* and *A. chlorotica* earthworms. Also, due to the location of the nephridia, the nephridial symbiont might have been shielded from the antibiotics.

The interpretations of the efficacy the antibiotic treatments applied in chapter 3 should be conscious of the culture-based method used to assess microbial abundance and as the basis for amplicon sequencing to determine effects on bacterial community composition. The use of a culture-based method only limits the detection of potentially significant but non-culturable fractions that were sensitive, resistant or escaped antibiotic treatment (Walsh and Duffy, 2013). However, if earthworm tissues were used directly as the basis for DNA extraction and culture-independent microbial characterization, the possibility that the amplicon analysis would not distinguish between the DNA of viable bacteria versus those recently killed under the influence of the antibiotic treatment could not be ruled out.

It was evident in the amplicon sequencing results that although the antibiotic treatment reduced the abundance of the microorganisms enumerated on the agar plates, a significant impact on richness was not detectable, which may be partly due to the variability of the bacterial richness between individual earthworms before the initial incubation process. This finding strengthens other studies suggesting the high variability of host-associated microbes found in earthworms (Aira, Pérez-Losada and Domínguez, 2018; Sapkota *et al.*, 2020; Swart *et al.*, 2020). The analysis of beta diversity, based on presence/absence, showed a subtle effect of antibiotic treatment on community composition with the antibiotic treatments most dissimilar in composition to the control being those with the most significant reduction in bacteria enumerated on agar plates.

In support of hypothesis 1 (Chapter 1), this experiment has shown that it was possible to use antibiotics-based procedure to produce 'axenic' earthworm specimen. However, the efficacy of the method to create 'axenic' earthworm depends on the antibiotic used, the types of agar used for microbial enumeration, and the earthworm species. The experiment also provided a insight into the composition of the culturable microbiome of *L. terrestris*, its sensitivity to antibiotics and the high variability of the microbial composition of earthworms. Future developments to improve the assessment of the efficacy of the method and method efficacy comprise: (i) Including a greater number of replications to control for the initial variability in microbial richness in individual earthworms; (ii) Initial characterization of the community composition of the earthworm microbiome to help make an informed decision on the antibiotics to be used that are likely to be most efficacious; (iii) Varying the antibiotic exposure time to take account of differences in earthworm size and therefore potential differences in gut transit and antibiotic exposure.

Investigating the influence of earthworm tightly-associated microbiome and the soil microbiome on the food preference of *Lumbricus terrestris*

The objective of chapter 4 was to assess the role of the earthworm tightly-associated microbiome and the soil microbiome in influencing the earthworm's feeding activity and choice.

Out of the three earthworm species investigated in Chapter 3, *L. terrestris* was chosen for the focus of future experiments in the thesis. This choice was based on wanting to work on a representative of an ecological group that interacted with soil through burrowing, given the focus on soil microbial diversity. The ease of experimentation as *L. terrestris* is available commercially. Finally, when considering the influence in the C cycle, *L. terrestris* (an epi-anecic earthworm specie), is quite influential type in terms of incorporation and fragmentation of litter.

In response to chapter 3, which concluded that antibiotics could reduce the abundance of earthworm-associated culturable microorganisms to create 'axenic' earthworms, the response of earthworm feeding behaviour based on the earthworm's and soil's microbial status was assessed. A food choice chamber experiment involving oak, ash and ryegrass litter species was used to investigate the effect of the microbial community in influencing the earthworm's feeding preferences and behaviours. The food choice experiment involved determining the plant litters removed from the feeding tubes by the earthworms (intact/near-axenic microbiome) in a chamber containing soil (intact/ autoclaved microbiome) over two weeks and in two batches to allow for an overall six replicates for each treatment and reduce the high effect of biological variability. This replication was decided based on the information gained in Chapter 3 regarding the high variability of initial microbiome composition for *L. terrestris*. Apart from observing the plant litter removed by the earthworms, the combined

cast and depurate, as soil most intimately in contact with the earthworm individual, was analysed using high-throughput amplicon sequencing.

The alpha and beta diversity from the amplicon sequencing data showed a clear separation between the cast + depurate communities from chambers with autoclaved versus intact soil. However, there was no detectable impact of the introduced earthworm microbiome on the composition of the soil microbiome.

This choice chamber design was used to test the following main hypothesis: Litter feeding behaviour (both quantity of litter consumed (feeding activity) and preference for litter of differing quality) will depend on soil and earthworm's microbial status. When considering feeding activity, the loss of either soil or earthworm microbiome had an impact, thus supporting the hypothesis. However, the combined loss of both soil and earthworm microbiome did not have a further additive effect on feeding activity. This non-additive antagonistic interaction between microbiomes might imply that soil and earthworm microbiomes are functionally redundant when it comes to outcomes that affect feeding activity. In the case of feeding preference, only the combined loss of both microbiomes was important, with feeding in the double-microbiome impacted earthworms showing a reduced preference for oak at the expense of ash and ryegrass. This implies that either there is a non-additive synergistic interaction between soil and earthworm microbiomes such that the function for the host can only occur through the activity of both microbiomes working together, or, that one microbiome can compensate for the absence of the other. For example, it might be that it is the tightly-associated earthworm microbiome that provides functions that influence food preference, but in its absence, the soil microbiome can ‘step in’, potentially recolonize the earthworm to fill tightly-associated niches and to provide this function.

Whilst previous studies have examined the effect of microbiome on host health and behaviour (Hosokawa *et al*., 2010; Rosengaus *et al*., 2011), chapter 4 reports the first study to examine the effect of the transient soil-derived and more tightly earthworm-associated microbiome on the feeding behaviours of the earthworm. The results obtained in chapter 4 reveal potential synergistic and antagonistic microbiome interactions with outcomes influencing earthworm feeding behaviour. This raises new questions regarding the nature of the microbiome functions provided to the host. Since, effects on food preference involved the reduction in preference for a litter (oak) thought to be less desirable due to high tannin and polyphenol content (Hendriksen, 1990; Satchell and Lowe, 1967) in most microbially compromised earthworms, it is possible that the function provided by the synergistic or compensatory interaction of microbiomes relates to the earthworm ability to resist chemical irritation or derive nutrition from such less desirable litters. The findings in Chapter 4 also raise a further question in relation to the mechanism of the feedback between microbiome(s) and earthworm that result in the altered feeding choices for the host.

Creating a gradient of microbial diverse soil using the dilution to extinction approach

In response to chapter 4, that addresses the influence of soil derived gut and earthworm whole microbiome in earthworm feeding behaviour, this chapter and the subsequent one focuses on the soil derived microbiome alone. This was primarily due to the similarities in the food preference and the amount eaten by earthworm with intact microbiome and manipulated soil derived microbiome or manipulate 'axenic' earthworm microbiome and intact soil derived microbiome (chapter 4). A significant change to the litter preference was when both earthworm microbiome and soil derived microbiome were compromised (chapter 4). The soil derived microbiome and earthworm microbiome appear to be compensatory for one another and soil microbiome is easier to manipulate and more relevant since variability in soil microbiomes will be encountered by the worm in the face of environmental and land

management change. Also, because it is in assumption that the soil derived microbiome does impact the host the most**,** this chapter and the subsequent chapter focuses on soil derived microbiome.

The objective of chapter 5 was to create soil with a gradient of microbial richness using the dilution-to-extinction approach, primarily due to the need to understand the link between microbial diversity and ecosystem function. This was the focus in the next chapter (chapter 6). It can be challenging to determine which microbial species are responsible for certain ecosystem functions. However, this issue has been addressed with several techniques that can manipulate microbial communities before addressing their functional roles. Some of these techniques have already been described in (**Chapter 5: Introduction**), however, the most adopted approach is the dilution-to-extinction approach used in this chapter because it considers all the soil microbial groups present in the soil and does not underrepresent indigenous soil microbial communities. This method also ensured that the reduction in species is random rather than selective when compared to techniques such as chloroform fumigation.

After the dilution-to-extinction approach that created soil containing a range of reduced microbial diversity through inoculating sterile soil with serial dilution of soil suspension, the experimental soils were then equilibrated for several weeks to obtain soil with equal biomass with differing microbial diversity.

The alpha diversity showed that the dilution-to-extinction approach created soil with various levels of richness, suggesting that the method has successfully created gradients of microbial richness based on the dilution of the inoculant added to the sterile soil before the equilibration period. The beta diversity showed a clear difference in the bacterial community between the control and the various dilution also shows that the bacterial composition is similar in the

lower dilution (10^{-2} and 10^{-4}), which are different to the bacteria in the higher dilutions (10^{-6}, 10^{-8}) and the autoclaved soil. This result showed that the dilution process inherently created soil with different bacteria communities grouped depending on if it originated from an initial lower dilution or higher dilution.

The core microbiome at the genus level was also examined to assess how the dilution treatment difference may affect the abundance of the taxa. The soil with lower dilution treatment (10^{-2} and 10^{-4}) showed similar abundance and presence of key taxa and are different to the abundance and presence of the taxa in the higher dilution treatments. Some taxa were also noticeably absent in the lower dilution treatment when compared to the control, and further absence observed as the dilution increased. Also noticeable is the increase in abundance and reduction in some abundance depending on the dilution treatment. This suggests that the dilution treatment resulted in the loss of rare species as the dilution increases and the proliferation of fast-growing species given the absence of competition from other lost species through the dilution treatment.

This chapter also assessed the biomass of the soil using fumigation, fluorescein diacetate analysis and dehydrogenase activity in soil with different dilution treatments. The results after the 27 weeks showed that the control soil results were not significantly different in soils with different dilution treatments. This suggests that over 27 weeks, the microbes in the soil reached their maximum biomass capacity and therefore were not significantly different to the control. This biomass measurement is essential for the next chapter looking at soil diversity on earthworm function. This ensured that the bias that could have been introduced through different biomass instead of diversity is removed.

The impact of soil microbial diversity on *L. terrestris* health and C cycle functions in maize-amended soil

In this final experimental Chapter (**Chapter 6**), the soil created from chapter 5 with a gradient of microbial diversity was carried forward and used here to determine to what extent the earthworm-induced increase in OM mineralisation is dependent on soil microbial diversity and whether this can be explained via a feedback from soil microbial diversity to earthworm health. The experimental factors were soil microbial diversity manipulation (intact, autoclaved, autoclaved and re-inoculated with 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8} dilutions) and earthworm (*L. terrestris* present or absent). Corresponding treatments without maize residue addition were also included. Respired C-CO_2 (at eight time points over 52 days) and earthworm energy reserves (at the conclusion of the time course) were the variables measured.

When considering the impact of earthworms and residue on the cumulative CO_2-C emission in soil with varying gradients of microbial communities, the cumulative C respired in the presence of earthworms was significantly higher ($p<0.05$) compared to the microcosms with no earthworms. This suggests that earthworms, directly through their own respiration, or, indirectly through stimulation of microbial respiration, contributed to the cumulative C respired. This agrees with the hypothesis (**Chapter 6; Hypothesis 1**) that the presence of earthworms will increase OM mineralisation irrespective of soil microbial diversity

In the absence of earthworms, the relationship between microbial richness and organic matter respiration exhibited a low sensitivity of respiration function to diversity erosion. This is compatible with the hypothesized (**Chapter 6; Hypothesis 2**) functional redundancy in soil microbial communities for functions such as respiration of soil organic matter. This chapter also examined whether any effect of microbial richness on the earthworm role in carbon cycle functioning could be linked to microbial diversity impacts on earthworm health. Soil respiration is an indicator of healthy soil. An unhealthy environment can cause a reduction in microbial respiration (Bastida *et al.*, 2008; Cardoso *et al.*, 2013). Aside from the specific

result of the reduction in cumulative C respired observed in the soil with the most eroded microbial diversity (autoclaved soil), which also had the least biomass measured, there was no significant correlation between the microbial diversity and the energy reserves measurement. This suggests that the health of earthworms is mostly negatively impacted when the soil microbial diversity is severely eroded.

Overall, this research highlights the importance of redundant biodiversity for OM mineralisation in the absence of earthworms. It also highlights the importance of the presence of earthworm in OM mineralisation which shows an elevated cumulative C respired in the presence of earthworms, most likely due to the earthworm priming effect of the soil microbes to encourage mineralisation. It has also highlighted the importance of the presence of soil microbes for improving the health of the earthworm when the most eroded soil consistently measures the lowest respiration value.

7.2 General conclusion

In investigating the overall hypothesis highlighted in the introduction (**Chapter 1**) for this thesis: '*Earthworm health and function with respect to the decomposition of added organic matter sources depends on soil microbial biodiversity*' the experiments conducted have informed the following concluding comments:

- *L. terrestris* was chosen as the species of focus due to its specific feeding habit. Different aspects of the role of the *L. terrestris* x soil microbiome interaction in determining the fate of added and native soil organic matter were investigated. Chapter 4 examined the impact of the soil microbiome (alone, and in combination with the tightly-associated earthworm microbiome) on the feeding activity on, and food preference for, litter inputs whereas Chapter 6 examined the impact of soil microbial diversity in determining *L. terrestris* -induced increases in OM

mineralization. When soil microbial abundance and diversity was extremely impacted through complete initial elimination by autoclaving, this resulted in a reduced feeding activity (**Chapter 4**) and also, consistent with this, a negative impact to earthworm health as judged by analysis of earthworm energy reserves (**Chapter 6**). Complete initial elimination of the soil microbiome also resulted in an altered feeding preference (**Chapter 4**), but only when earthworm individuals also had a perturbed tightly-associated microbiome. These findings point to an important relationship between soil microbiome, earthworm health and earthworm feeding behaviour but due to the extreme effects of soil autoclaving it was not possible to determine from this autoclaved perturbation alone which property, or combination of properties, of the microbiome (microbial abundance, activity or richness) underpins this relationship. However, examining a dilution-to-extinction gradient of soil microbial richness (**Chapter 6**), spanning a range of (Chao 1 -estimated) richness OTUs for soils that had statistically equal abundance (biomass) and activity revealed no effect of microbial diversity on earthworm functional impacts on SOM mineralization or earthworm health and therefore does not support the overall hypothesis for this thesis. Taken together, the findings currently suggest that the correlated properties of the soil microbiome that are important for earthworm health and functional role are abundance (biomass) and activity rather than the richness of species present or the presence of specific species or combinations of species. The insensitivity of both earthworm health and function to bacterial species loss suggests that the nature of the function provided by the ingested soil bacterial microbiome to its host is a functionally redundant one.

7.3 Wider implications and further research

To the best of my knowledge, the research explained in this thesis is the first to examine the influence of the soil microbiome and soil microbial diversity on the health and function of an earthworm (*L. terrestris*) host. The evidence that erosion of the soil microbiome might only impact the health and function of the earthworms in the extreme case of (near-) eradication, where biomass, activity and diversity are all severely reduced, suggests that the earthworm dependence on the soil microbiome and the outcome in terms of supporting critical soil ecosystem processes such as organic matter mineralisation is a robust one that might not be affected by management or environmental change induced perturbations to soil microbial diversity.

The outcome of the research in this thesis also raised many questions and relied on assumptions, the investigation and verification of which inform suggestions for further research:

As a result, a few key areas where additional experiments are needed have been recommended. These are:

(a) A controlled experiment similar to the one set up in Chapter 4, with more litter with a broader range of different chemical properties and more soil chemical properties analysed to eliminate responses that could lessen the distinctive effect of the microbial properties on earthworms' feeding behaviour.

(b) A controlled experiment similar to the one set up in Chapter 6, with extra analysis of the respired C and C distribution among soil organic matter fractions using Isotope Ratio Mass Spectrometry (IRMS). The maize straw was intentionally chosen during the experimental design because it is a C4 plant distinct from most of the other carbon sources present in this soil which had a history supporting the growth of C3 plants and

therefore receiving C3- derived inputs. The distinctive C4 carbon isotopic ratio of maize would enable the maize-derived C to be traced using IRMS to both respired C and to organic matter physical fractions (free-light, intra-aggregate light and organomineral- associated C). This would enable the long-term effects of earthworms on the fate of maize straw C and the interactions with native soil organic carbon via priming effects and how this was influenced by soil microbial diversity. This further experiment would be based on the work done in both chapters 5 and 6 of this thesis but with further analyses.

These are discussed in more detail below.

- In chapter four, the results obtained showed potential synergistic and antagonistic microbiome interactions with outcome influencing earthworm feeding behaviour. It also raises new questions because the host's microbiome function affects it's food preference based on the desirability of the litters due to their high tannin and polyphenol content. There was a reduction in preference to oak mostly by the microbially compromised host. However, the differences in the chemical properties between the litters used in this study although statistically significant, may have been trivial in terms of biological meaning (as the difference in phenol concentration between oak and ash litter was less than 5.14%), and lessen the interpretation of a distinctive effect of microbial function on earthworms' feeding behaviour in relation to litter quality. A future experiment could follow the litter choices employed by Hendriksen, (1990), where the chemical properties of the litters chosen varied as followed: C: N (between 14.2 and 47.0); polyphenol content (between 0.29 and 2.62) and lignin % (not measured in this current work, between 12.9 to 34.8).

- In chapter 6, the impact of soil microbial diversity on *L. terrestris* health and C cycle functions was determined in the maize-amended soil. The results showed that the earthworms directly or indirectly contributed to the cumulative carbon respired. The results also showed that the relationship between microbial richness and organic matter respiration in the maize-amended soil exhibited low sensitivity to diversity erosion in the absence of earthworms. However, it was impossible to determine the proportion of the carbon emission that was due to the respiration of the added carbon source (maize) against respiration of native soil organic carbon. Plant residue decomposition is one of the significant components of C cycling in soil and a significant regulator of C emissions from soil (Li *et al*., 2013) through their priming effect. The native C derived from the soil, and the plant-derived C was not differentiated in this study, which could have given a better insight into the precise estimate of the decomposition rate of the residue (Gorissen and Cotrufo, 2000) and the impacts of residue addition on respiration of native C via priming effects and the influence of earthworm and earthworm x microbiome interactions on such effects.
- Apart from the role of residue decomposition in C emission, plant residue is also essential for maintaining and accumulating soil organic matter (Kong and Six, 2010; Lugato *et al*., 2014). The stabilisation of soil organic matter is possible through several mechanisms, such as physical protection of the OM within the soil aggregates (Angst *et al.,* 2019; Don *et al*., 2008), with soil aggregation influencing soil C sequestration and SOM cycling (Angst *et al*., 2019; Six *et al*., 1998). Earthworms ingest plant residues and mineral particles that are then mixed and excreted as casts (Parmelee *et al.,* 1990). These casts are often more stable than their surrounding soil and contain organic matter that is not respired and emitted into the atmosphere (Bossuyt, Six and Hendrix, 2005; Edwards and Bohlen, 1996; Marinissen, 1994;

Scullion and Malik, 2000). The aggregates formed might increase the physical protection of the carbon from microbial mineralisation (Brown, Barois and Lavelle, 2000). To determine the fate of the carbon source (maize) with respect to soil aggregation, a second experiment that examines C sequestration using quantification of the distribution of maize and native SOC across soil physical fractions is advised.

- Using the particle size, physical soil fractionation based on the procedure described and used by Rovira and Ramón Vallejo, (2002); Garcia-Pausas *et al*., (2012) and Mwafulirwa *et al.,* (2019), the residue and SOM-derived C retained in the soil can be determined.

Chapter 8 Reference

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Chapter 9 Supplementary materials

Appendix 1: supplementary table

Chapter 4

Supp table 4-1 Weights of earthworms (intact/near-axenic microbiome) in soil (intact/autoclaved microbiome) at the beginning and the end of the choice chamber experiment.

Earthworm	Soil	Initial(wt.)	Final (wt.)	final -initial (wt.)
Near -axenic	Intact	4.92	3.63	-1.29
Near -axenic	Intact	3.6	3.41	-0.19
Near -axenic	Intact	4.5	4.19	-0.31
Near -axenic	Intact	7.15	dead	dead
Near -axenic	Intact	4.95	5.2	0.25
Near -axenic	Intact	6.71	4.1	-2.61
Near -axenic	Autoclave	7.27	4.79	-2.48
Near -axenic	Autoclave	5.04	5.02	-0.02
Near -axenic	Autoclave	4.52	2.32	-2.2
Near -axenic	Autoclave	6.96	3.9	-3.06
Near -axenic	Autoclave	5.82	5.4	-0.42
Near -axenic	Autoclave	7.15	dead	dead
Intact	Intact	5.7	6.34	0.64
Intact	Intact	7.16	8.71	1.55
Intact	Intact	4.6	5.33	0.73
Intact	Intact	6.07	6	-0.07
Intact	Intact	5.3	4.6	-0.7
Intact	Intact	6.65	7.05	0.4
Intact	Autoclave	3.7	3.09	-0.61
Intact	Autoclave	4.78	dead	dead
Intact	Autoclave	5.2	5.47	0.27
Intact	Autoclave	6.6	6.8	0.2
Intact	Autoclave	5.86	5.3	-0.56
Intact	Autoclave	4.86	5	0.14