The short term influence on the soil food web base of regenerative pastoral systems in New Zealand

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ABSTRACT

Food production systems are coming under increasing societal pressure to improve sustainability and reduce environmental impacts. This has led to the development of alternative agricultural practices, such as regenerative agriculture (RA). New Zealand's economy relies heavily on its pastoral agriculture production, while also being an important contributor to global food production. Healthy soils are essential for productive and sustainable agriculture and are a key part of the RA concept. Soil biota is a crucial component of soil health. Monitoring soil health is important for improving the sustainability of agriculture and for testing whether RA achieves its objectives.

The current study is part of the Whenua Haumanu research program. This involves comprehensive farmlet studies investigating the effects of RA management and diverse pastures within New Zealand's pastoral farming system on a wide range of outcomes, including soil health. Treatments on the farmlets consist of combinations of contemporary New Zealand farming management practices vs RA management and standard New Zealand pasture vs diverse pasture. This is implemented across two farm systems: drystock sheep farmlets and dairy farmlets. These are both located near Palmerston North, New Zealand, but are on different soil types. The current study investigates the effects of RA management and diverse pasture treatments on three different components of soil biota, focusing on the soil microbiome and the base of the soil food chain. The physiological state of soil bacteria and the community structure of the soil microbiome was investigated. The effects of the treatments on the soil food web were monitored by an analysis of soil nematodes. Soil moisture and temperature were measured for each sample, as these factors are known to have an influence on soil biota.

Analysis of the physiological states of soil bacteria was carried out using epifluorescent microscopy in combinations with differential stains that allowed the active, potentially active, dormant and dead pools of soil bacteria to be enumerated. The microbial community composition was investigated by analysis of soil phospholipid fatty acid (PLFA) biomarkers. This allowed the proportions of major microbial groups in the soil to be determined, as well as provided a proxy of total microbial biomass. Analysis of the 16:1ω5 biomarker in the neutral lipid fraction, via neutral lipid fatty acid (NLFA) analysis was used to measure arbuscular mycorrhizal fungi (AMF). Total nematode abundance and the relative abundance of each trophic group was analysed. Soil temperature was measured in the paddock at the time of sampling while soil moisture was determined by oven drying and calculating the gravimetric water content.

On the sheep farmlets, diverse pasture under contemporary management (Div-Con) had higher dead bacteria than the other treatments, while on the dairy farmlets, standard pasture under contemporary management (Std-Con) had higher active bacteria compared to diverse pasture under regenerative management (Div-Reg). The sheep farmlets had

higher total bacteria counts. The results from the epifluorescent microscopy indicated that the staining procedure used to enumerate total bacteria was unreliable and, by comparison with published results and the PLFA analysis results of the current study, was shown to underestimate the total bacteria present by an order of magnitude. This limits the conclusions that can be drawn from these results, especially regarding the proportions of active and dead bacteria. Due to the methodological limitations and lack of consistent treatment effects between the sheep and dairy farmlets, generalised conclusions on the effects of the different treatments on soil bacteria physiological state cannot be made.

The PLFA analysis results showed that there was no difference in the structure of the microbial communities, the NLFA 16:1ω5 biomarker concentration, or total PLFA concentration between treatments. However, on the dairy farmlets, the fungi to bacteria ratio was highest on the Div-Reg treatment. The total PLFA concentration was higher on the sheep farmlets compared to the dairy farmlets, and there was a difference in PLFA concentration between the soil types on the dairy farmlets. The community composition also differed between the soil types on the dairy farmlets and between the sheep and the dairy farmlets, which are also on different soil types. This indicates the importance of accounting for differences in soil type when measuring soil biota.

Total nematode abundance was higher on the sheep farmlets compared to the dairy farmlets, and the trophic group composition differed between the farmlet systems. The total abundance of nematodes was higher under contemporary management than under regenerative management on the sheep farmlets, although the same trend was not observed on the dairy farmlets. The nematode community was dominated by bacterivorous and herbivorous nematodes. No differences in the trophic group composition were observed on the sheep farmlets, however, on the dairy farmlets the Div-Reg treatment had a higher portion of bacterivorous nematodes than the Std-Con treatment. The observed differences in nematode abundance and community composition between the contemporary and regenerative managed treatments on the sheep and the dairy farmlets are likely due to differences in the grazing management between contemporary and regenerative practices which changes nematode food source availability. As the nematode community contains a large portion of herbivorous nematodes, their high abundance is likely detrimental to plant growth.

The results from the analysis of soil moisture and soil temperature indicated that both diverse pastures and regenerative management reduce soil temperature, potentially buffering it from fluctuations. Similarly, there was evidence that diverse pastures and regenerative management increase soil moisture. However, more data is needed to draw definitive conclusions.

Overall, the results show that the soil food webs across all treatments are dominated by the bacterial decomposition pathway and the bacterial and herbivore energy channels. As differences in the soil biota measurements were inconsistent between treatments and across the farmlet systems, drawing general conclusions on the effects of diverse pasture and regenerative management is difficult. The largest and most consistent differences where between soil types and the farmlet systems. As the two farmlets systems were on different soil types, the differences between them could also be due to soil type differences.

This has important implications for correctly controlling for soil type differences in studies on soil biology. However, both the soil type and the farmlet systems were fully confounded with different sampling dates, and therefore the possibility that this contributed to observed differences cannot be eliminated.

At the time of this study, the soils had only been under their respective treatments for two and a half years. As soils often take a long time to adapt to changes, it is possible that differences between them will become more apparent as the research program continues. It is also important to acknowledge that all farmlet treatments are comparing stable pasture systems which have had minimal historic soil cultivation. These conditions are known to promote healthy soil and soil biota, so it is possible that against this back drop, subtle changes in management and the addition of more diverse pasture species are not resulting in clear differences between treatments. Therefore, although there is some evidence to suggest that diverse pastures and RA management is influencing soil biology in the current study, the nature of this effect is difficult to determine. Continued monitoring is required to confirm whether the treatments have an effect on soil biology and to determine what the impacts are and the possible mechanisms.

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Contents

LIST OF FIGURES

LIST OF TABLES

COMMON ABBREVIATIONS

The abbreviations are defined at the first use in each chapter and then without definition throughout this thesis.

AMF	Arbuscular mycorrhizal fungi
ANOVA	Analysis of variance
AOA	Ammonia oxidising archaea
AOB	Ammonia oxidising bacteria
C	Carbon
DM	Dry matter
DNA	Deoxyribonucleic acid
Div-Con	Diverse pasture under contemporary management
Div-Reg	Diverse pasture under regenerative management
FAME	Fatty acid methly ester
FDA	Fluorescein diacetate
FITC	Fluorescein iso-thiocyanate
MAOM	Mineral associated organic matter
MI	Maturity index
N	Nitrogen
NLFA	neutral lipid fatty acid
P	Phosphorus
PCR	Polymerase chain reaction

PI	Propidium iodide
PLFA	Phospholipid fatty acid
POM	Particulate organic matter
PPI	Plant parasitic index
RA	Regenerative agriculture
RNA	Ribonucleic acid
SOM	Soil organic matter
Std-Con	Standard pasture under contemporary management
Std-Reg	Standard pasture under regenerative management

CHAPTER 1: INTRODUCTION

Pastoral agriculture is an essential component of New Zealand's economy. Primary industries make up 79% of New Zealand's goods exports, and 55% of its total exports, with the majority of this coming from the dairy and the red meat industries (Beef and Lamb New Zealand, 2024; Infometrics, 2024; New Zealand Foreign Affairs and Trade, 2024). Over 95% of the livestock diet in New Zealand is from grazing and forage crops with around 40% of New Zealand's land area in grazed pastures, highlighting the importance of pastoral agriculture to these industries (Chapman et al., 2025; Morris & Kenyon, 2014). Globally, New Zealand is the 16th largest agricultural exporter (New Zealand Foreign Affairs and Trade, 2024). It is the second largest dairy exporter and accounts for 47% of total global lamb meat trade (Morris, 2009; New Zealand Foreign Affairs and Trade, 2024). Therefore, as well as being an essential component of New Zealand's economy, pastoral agriculture in New Zealand is also an essential component of global trade and food production. The climate in New Zealand is suitable for a pasture based agricultural system allowing for efficient and relatively low cost food production (Caradus et al., 2023; Chapman et al., 2025; Morris & Kenyon, 2014). New Zealand's pastoral agriculture system relies on introduced plant species, with the majority of intensively grazed farms containing pastures consisting predominantly of perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) (Chapman et al., 2025).

Pastoral farming in New Zealand causes a number of environmental and sustainability issues. The geology of New Zealand means that many of the soils are relatively young, and large areas of agricultural land is on highly erodible unconsolidated cover rock, while the high rainfall climate provides an effective mechanism for erosion (Basher, 2013; Grelet et al., 2021). Removal of the native vegetation and replacement with shallow rooting pasture species has increased the susceptibility of this land to erosion (Basher, 2013). The loss

of the fertile soil to erosion affects farm productivity. The eroded soils ultimately end up in streams and rivers, causing increases in water turbidity and deposition of sediment on the stream beds, affecting aquatic wildlife (Ryan, 1991; Vale et al., 2023). Transport of nutrients from fertilised pasture via leaching and surface runoff causes eutrophication of waterways and negatively impacts water quality (Beeckman et al., 2018; Joy et al., 2022). Although low per unit of agricultural product relative to other countries (Beef and Lamb New Zealand, 2024; Ledgard et al., 2011; Ledgard et al., 2020), due to the scale of the primary industries in New Zealand, agriculture makes up the majority of the country's greenhouse gas emissions (Ministry for the Environment, 2025). Most of this comes from enteric methane, however, nitrous oxide from fertilised pastures and carbon dioxide from farm machinery also play a role (Ministry for the Environment, 2025). The agriculture systems in New Zealand, like internationally, have come under increasing pressure to improve farm sustainability. As New Zealand's primary industry is based on global exports, it relies on how it is perceived by consumers internationally. Therefore, improving the sustainability of farming in New Zealand is important to protect the environment as well as for maintaining the social licence to farm and upholding the reputation of sustainable farming that gives New Zealand's agricultural produce an edge in international markets.

Regenerative agriculture (RA) has gained increasing attention in recent years, both within the scientific community and among farmers, for its potential to increase the sustainability of the food production industry (Giller et al., 2021; Newton et al., 2020). RA aims to improve environmental sustainability while maintaining farm productivity and profitability (Khangura et al., 2023; Newton et al., 2020; Schreefel et al., 2020). A key component of RA is improving soil health, which involves enhancing soil physical characteristics, increasing soil carbon and biodiversity and integrating soil microflora and fauna into the farming system for nutrient cycling (Giller et al., 2021; Khangura et al., 2023; Schreefel et al., 2020). The soil related outcomes RA claims to achieve, based on various reviews, are displayed in Table 1.1. RA is gaining momentum in New Zealand and is being adapted

Table 1.1: Soil related outcomes associated with regenerative agriculture.

Category	Outcome	Citation
Biological	Increase soil biodiversity	Schreefel et al. (2020); Grelet et al. (2021); Giller et al. (2021)
	Increase soil carbon content and sequestration	Schreefel et al. (2020); Newton et al. (2020); Grelet et al. (2021); Giller et al. (2021)
	Increase soil organic matter	Newton et al. (2020); Grelet et al. (2021)
	Increase rooting depth	Grelet et al. (2021)
	Increase biological activity	Grelet et al. (2021)
	Increase total worms and fungi	Grelet et al. (2021)
Chemical	Increase soil chemical fertility	Newton et al. (2020); Grelet et al. (2021)
	Increase total soil nitrogen and nutrient cycling	Grelet et al. (2021)
Physical	Improve soil physical quality and structure	Schreefel et al. (2020); Grelet et al. (2021); Newton et al. (2020)
	Increase water holding capacity and infiltration	Schreefel et al. (2020); Grelet et al. (2021)
	Reduce compaction	Grelet et al. (2021)
General	Reduce soil erosion	Schreefel et al. (2020); Grelet et al. (2021)
	Flood and drought resilience	Grelet et al. (2021)

to suit the unique environment and sustainability issues of New Zealand (Grelet et al., 2021). However, the lack of empirical evidence to support the claimed benefits of RA is impeding its widespread adoption, and the effectiveness of RA practices vary between different agroecological regions (Khangura et al., 2023). Therefore, in order for the potential benefits of RA to be realised in New Zealand and to ensure that New Zealand's agricultural products remain competitive in the global market, more research into RA is required (Grelet et al., 2021).

The current study investigates whether some of the biological aspects of soil health

are realised under RA practice, as well as monitors the general impact RA has on soil biota within New Zealand's pastoral farming system. The study is part of the Whenua Haumanu research program run by Massey University, New Zealand. This program involves comprehensive farmlet studies investigating the effects of diverse pasture and RA management, within New Zealand pastoral agriculture, on a wide range of outcomes across all aspects of the farming system, including soil, pasture, animal, production, environmental and financial outcomes over seven years. As the Whenua Haumanu research program had only been established for two and a half years at the time samples were collected, the current study only determines whether implementing RA practices causes measurable changes in the short term. The Whenua Haumanu research program places special emphasis on the impact that diverse pasture has in the New Zealand pastoral system, with this being treated as a separate treatment factor to other RA practices. Therefore throughout this thesis the effects have been reported separately for diverse pasture and RA management. However, it is important to note that diverse pasture is essentially another RA management practice, and the reporting of these effects separately does not imply that diverse pastures is not a component RA management, but rather that this practice has been considered separately from the other RA practices in the current study.

Three components of soil biota were investigated in the current study, focusing on the soil microbiome and the base of the soil food web. Whether diverse pasture and RA management increases soil biological activity was determined by measuring the physiological state of soil bacteria. This was carried out using epifluorescent microscopy and differential stains to determine the number of soil bacteria that are active, potentially active, dormant and dead. The impacts of diverse pasture and RA management on the microbial community structure were monitored through phospholipid fatty acid (PLFA) analysis. This allows the relative portions of major microbial groups to be measured to determine if there are any changes in the community composition, and is also a proxy

for the total living microbial biomass present. Analysis of soil nematode abundance and trophic groups provided insight on the impacts to the wider soil food web.

Healthy soils are essential for productive, sustainable agriculture. The importance of soils is well appreciated within RA (Giller et al., 2021; Khangura et al., 2023; Schreefel et al., 2020), however, even within wider agricultural systems, soils are increasingly being acknowledged (Rashid et al., 2019). Soil biota is a crucial component of healthy soils. It drives most of the processes required for plants to thrive and thereby for productive agriculture, as well as the processes involved in mitigating the negative environmental impacts of agriculture (Tomazelli et al., 2023). Soil biology will likely also play an important role in addressing climate change through carbon sequestration (Stockmann et al., 2013). Therefore, improving our understanding of soil biota is crucial for improving farm sustainability and reducing environmental impacts. It is also essential in order to test whether RA achieves its objectives of improved soil health. Despite this, soil biota remains elusive and difficult to monitor. The current study looks at three different components of soil biota in an attempt to understand how it is affected by pastoral farming and to test whether RA practices applied within the New Zealand pastoral agriculture system have an impact on soil biota.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Soil biota is crucial for healthy soil and the proliferation of the plants growing in them. It plays an important role in the productivity and sustainability of farming, as almost all forms of agriculture depend on the primary production of plants. Being able to monitor soil biota is essential for investigating the impact farming practices have on it. This also allows the management practices that optimise the benefits soil biota can provide, to be determined. This is difficult due to the microscopic size of many important soil organisms and the opaque nature of soil making direct observation of the soil biota almost impossible. A range of methods have been developed to overcome this. This literature review will provide a description of the major groups of soil organisms, their function in the soil, and the factors that have an impact on them, with a focus on New Zealand's pastoral agricultural soils, as well as an overview of the methods that can be used to investigate soil microflora and microfauna.

Section 2.2 describes the key organisms present in New Zealand's agricultural soils. All of the organisms found in the soil interact with each other in some way, either directly or indirectly. Trophic interactions are complex and can be displayed in a soil food web. At the base of the soil food web are the decomposers, which break down organic matter and detritus from plants. These in turn provide a food source for larger and more complex soil organisms which together carry out many of the important soil processes required to sustain the plants that ultimately feed them. Section 2.3 provides an overview of the key interactions occurring between the organisms described in section 2.2 that make up the soil food web. The soil conditions and agricultural management practices that influence

soil biota are discussed in section 2.4, including a review of regenerative agriculture (RA) and how it impacts soil biota. An overview of methods and techniques for measuring the composition and function of the soil microflora and microfauna is provided in section 2.5.

2.2 Soil biota

Soil biota is made up of organisms varying in size by orders of magnitude. The microflora consists of organisms less than 5 μm and includes bacteria, archaea and fungi (Crotty, 2020). Microfauna is between 5 and 10 μm and consists of protozoa, nematodes and tardigrades, while mesofauna contains organisms with a body width less than 2mm and includes spring tails (Collembola), mites (Acarina) and other microarthropods (Crotty, 2020). Macrofauna has a body width greater than 2mm and includes earthworms (annelids), arthropods and molluscs (Crotty, 2020). Megafauna includes vertebrates that live in the soil, such as rabbits, moles and rodents (Crotty, 2020). This review will focus on the microflora and microfauna, with other groups of fauna not being further considered, although brief descriptions of the mesofauna and macrofauna are included in this section to provide a comprehensive overview of the biota in soils.

2.2.1 Microflora

The microflora in soil collectively carries out many important soil processes and functions. They also make up the base of the soil food web as primary consumers, with 90% to 95% of all nutrient flow to higher trophic levels passing through them (Kennedy, 1995).

2.2.1.1 Bacteria and archaea

The diversity of bacteria and archaea in soils is huge, with Anthony et al. (2023) estimating that 4.3×10^8 and 3.6×10^4 species of bacteria and archaea, respectively, exist in the soil, while also acknowledging that this could be a considerable underestimation. Globally, the dominant phyla present in agricultural soils are Proteobacteria, Acidobacteria, Actinobacteria and Verrucomicrobia (Janssen, 2006; Rossmann et al., 2020; Tomazelli et al., 2023). Bacteroidetes, Chloroflexi, Planctomycetes, Gemmatimonadetes, and Firmicutes are also commonly found in soils, at lower abundances (Janssen, 2006). In New Zealand grassland soils, these same phyla are common with similar relative abundances, however Planctomycetes is among the dominant phyla (Hermans et al., 2020), rather than being a minor component as found by Janssen (2006).

Bacteria and archaea involved in the nitrogen (N) cycle are common in the soil and have been extensively studied. Ammonia oxidising bacteria (AOB) and ammonia oxidising archaea (AOA) are involved in the first step of the nitrification reaction, with the former consisting of 3 genera (Nitrosomonas (β-proteobacteria), Nitrosospira (β-proteobacteria) and Nitrosococcus (γ-proteobacteria)) and the latter found within the Crenarchaeota phylum (Hayatsu et al., 2008). AOB have been shown to be more important than AOA in oxidising ammonia in soils (Di et al., 2009; Zou et al., 2022). The second step of nitrification is carried out by nitrite oxidising bacteria from four genera (Nitrobacter (α-proteobacteria), Nitrospina (δ-proteobacteria), Nitrococcus (γ-proteobacteria) and Nitrospira (class Nitrospira, phylum Nitrospirae)) (Hayatsu et al., 2008). Certain bacteria in the phylum Planctomycetes have been shown to carry out anaerobic oxidation of ammonium and nitrate directly to N gas, known as anammox (Hayatsu et al., 2008). Although this has not been documented in pasture soils, it has been observed in paddy soil (Ding et al., 2021; Sun et al., 2022; Yang et al., 2015), and as Planctomycetes bacteria are abundant in the soil it is possible that this reaction is taking place (Hayatsu

et al., 2008). Bacteria of the genus *Nitrospira*, that can oxidise ammonium directly to nitrate in one step (comammox), have been shown to be present in New Zealand pasture soils (Chisholm et al., 2023). There are also diverse bacteria undertaking denitrification (Hayatsu et al., 2008). Bacteria of the genera *Rhizobium* and *Frankia* are important in symbiotic N fixation for leguminous and non-leguminous plants, respectively (Santi et al., 2013), with *Rhizobium leguminosarum* bv. *trifolii* being the bacteria that form a symbiotic association with white clover (Wakelin et al., 2018). Free living bacteria and archaea not associated with plants also contribute to N fixation in soils (Reed et al., 2011). Bacteria involved in methane production and utilisation have also been well studied. Methanogens are a phylogenetically diverse group of archaea, while methanotrophs are a phylogenetically diverse group of bacteria (Topp & Pattey, 1997). Bacteria and archaea involved in the N cycle and methane evolution have been extensively studied, and therefore more is known about which microorganisms are involved in these processes, however the soil contains many important and diverse groups of microbes undertaking various functions.

The sulphur cycle is driven by microbial processes (Eriksen, 2009; Vidyalakshmi et al., 2009). Sulphur is mineralised from organic matter through microbial activity as well as through enzymatic hydrolysis by sulfatases, produced mainly by bacteria and fungi, but also plant roots (Eriksen, 2009). Sulphur oxidising bacteria play an important role in converting inorganic sulphur into bioavailable forms (Vidyalakshmi et al., 2009). Species in the genus *Thiobacillus*, including *Thiobacillus thiooxidans*, *T. ferrooxidans*, *T. thioparus*, *T. denitrificans* and *T. novellus*, play an important role in oxidation of sulphur in soil (Vidyalakshmi et al., 2009). However, a range of other genera of bacteria are also capable of oxidising sulphur as well as archaea in the order Sulfolobales and some fungi (Vidyalakshmi et al., 2009).

Soil bacteria influence the phosphorus (P) cycle (Chen et al., 2023). A significant portion of soil P is contained within the soil microbial biomass (Richardson & Simpson, 2011). This

temporally protects the P from immobilisation in the soil inorganic phase and is eventually returned to the soil solution, and thereby made plant available, through microbial turnover (Richardson & Simpson, 2011). Soil bacteria also contribute to mineralisation of P from organic matter through decomposition (Richardson et al., 2009). Bacteria that are able to solubilise inorganic P have been isolated from the soil, including species from the genera *Actinomycetes*, *Pseudomonas*, *Bacillus*, *Arthobacter* and *Streptomyces* (Richardson & Simpson, 2011; Zheng et al., 2019). They do this by releasing protons to acidify the soil and secreting organic anions which competitively chelate with metal ions (Richardson & Simpson, 2011; Zheng et al., 2019). Along with P solubilising bacteria, fungi are also important for P cycling in soil (Richardson et al., 2009).

Soil bacteria and archaea consist of a phylogenetically and functionally diverse group of microorganisms that drive the carbon (C) and nutrient cycles in the soil and undertake many essential roles. Tomazelli et al. (2023) identified 24 different functional groups in soils, with the most abundant being chemoheterotrophic, aerobic chemoheterotrophic, parasites or symbionts, predatory or exoparasitic and cellulolytic. The abundance of organisms in the chemoheterotrophic and cellulolytic groups indicates that a large portion of the microorganisms in the soil are involved in organic matter cycling. The parasites or symbionts category likely contains the mycorrhizal fungi, which are also abundant in the soil. The minor categories, found to be present at less than 1%, were involved in N, sulphur or iron cycling, among other things, indicating that organisms that are less abundant still carry out many important processes in the soil (Tomazelli et al., 2023).

2.2.1.2 Fungi

Soil fungi are divided into two groups: saprophytic and mycorrhizal (Crotty, 2020). Saprophytic fungi obtain their energy and C by decomposing organic matter in the soil and include the phyla Ascomycota, Chytridiomycota and Basidiomycota (Rossmann et

al., 2020). Mycorrhizal fungi form symbiotic relationships with plant roots and obtain their energy and C source directly from living plants. They are a monophyletic group belonging to the phylum Glomeromycota (Parniske, 2008). Mycorrhizal fungi will be discussed further below (section 2.3.1.2). Common fungi associated with plant debris or free living in New Zealand soils include *Paecilomyces carneus*, *Gliocladium roseum*, *Cladosporium* sp., *Fusarium oxysporum*, *Sporotrichum* sp. and *Trichoderma sporulosum*, as well as species in the genera *Humicola* and *Penicillium* (Jackson, 1965). However, it should be noted that these species were identified before the use of metagenomics, so it is likely that the diversity of fungi in the soil is much higher.

Fungi carry out many important functions in soils. A major role is in C cycling due to their ability to break down complex substances that are difficult for other microorganisms to decompose, such as cellulose and lignin (Kennedy, 1995). They are also involved in nutrient release from soils, soil weathering and formation of stable soil aggregates (Fan et al., 2022; Kennedy, 1995). Some fungi have even been found to undertake aerobic heterotrophic denitrification, therefore contributing to the N cycle (Hayatsu et al., 2008).

2.2.1.3 Fungi to bacteria ratio

Soil bacteria and fungi are the primary decomposers in the soil and are a food source for the other soil organisms in the detrital food chain. As bacteria and fungi have distinct ecological and physiological characteristics, such as differences in C:N ratio, decomposition process and responses to environmental conditions, they have a strong influence on the higher trophic levels of soil fauna (Ingham et al., 1989; Wang et al., 2019). This causes the consumer community to differ between the bacterial and fungal decomposition pathways (Ingham et al., 1989; Wang et al., 2019). As certain conditions favour either fungi or bacteria, and these in turn influence which soil fauna are present, the relative abundance of these two groups is a useful indicator of both the soil conditions

and the soil biota present. This is frequently measured as the fungi to bacteria ratio.

Various methods for measuring fungi to bacteria ratio have been used, including direct microscopy, phospholipid fatty acid (PLFA) analysis, respiration analysis and quantitative polymerase chain reaction (PCR) (Wang et al., 2019). It is also measured indirectly, by measuring the influence bacteria and fungi have on the higher trophic groups that feed on them, including comparing fungal and bacterial feeding nematodes and comparing the respective energy channels, which is the total consumer biomass stemming from either of the two food sources (Ferris, 2010b; Wang et al., 2019). Results from different methods cannot be directly compared due to inherent limitations (de Vries et al., 2006; Wang et al., 2019). For example, direct microscopy tends to overestimate fungal biomass due to the inability to distinguish between living and dead hyphae, while PLFA analysis tends to underestimate fungal biomass due to the lower surface area to volume ratio of hyphae resulting in less PLFAs per unit biomass as well as the limited number of fungal PLFA biomarkers compared to bacterial PLFA biomarkers (de Vries et al., 2006; Ohtonen et al., 1999; Wang et al., 2019). In addition to this, Wang et al. (2019) argues that the fungi to bacteria ratio provides only a snapshot of the relative abundances and does not take into account the cumulative contribution of fungi and bacteria to energy flow through the food web, which is more ecologically relevant. They argue that “a species with low biomass and a high turnover may have a greater impact on the functioning and stability of the food web compared to that with high biomass and a low turnover” (Wang et al., 2019), and therefore the microbial assimilation, measured by microbial production and respiration rate, needs to be taken into account to determine the actual impact of differences in the microbial community. However, they also state that even if an ecosystem is dominated by one microbial group, it does not indicate that the other is of lesser importance, as they are usually non-substitutable in ecosystems since they play different roles and are dependent on each other in the decomposition process.

As biotic and abiotic conditions impact fungi and bacteria differently, the ratio of fungi

to bacteria varies between different ecosystems. Bacteria are favoured in soils with low organic matter and high nutrient availability while fungi are favoured in acidic soils with high organic matter and low nutrient availability (Wang et al., 2019). Forest ecosystems tend to be dominated by fungi while grassland ecosystems tend to have more bacteria (Ingham et al., 1989; Zhao & Neher, 2014). An increase in the fungi to bacteria ratio was also found along a primary succession transect ranging from barren glacial till to a 100 year old forest, which was attributed to a change in plant community with high quality litter favouring bacteria to one with low quality litter favouring fungi (Ohtonen et al., 1999; Wang et al., 2019). Intensive management of grassland systems along with high inputs of fertiliser, especially N, has been found to reduce the bacteria to fungi ratio (Bardgett & McAlister, 1999; de Vries et al., 2006; Parfitt et al., 2010). A higher fungi to bacteria ratio has been suggested to be indicative of a more sustainable agricultural system that more closely resembles natural ecosystems and self regulates through relying on biological processes (Bardgett & McAlister, 1999; de Vries et al., 2006; Ingham & Slaughter, 2004; Parfitt et al., 2010; Yeates et al., 1997). Soil cultivation has also been shown to negatively affect fungi, and thereby reduce the fungi to bacteria ratio (Beare et al., 1997; de Vries et al., 2006; Frey et al., 1999; Wang et al., 2010).

2.2.1.4 Carbon cycling by microflora

Microbes play a key role in the C cycle, being responsible for both decomposition of organic matter and its incorporation and storage in the soil. The soil microflora utilises the organic matter present in the soil as a C and energy source and is therefore responsible for its loss from the soil via respiration. However, they also control the processes involved in organic matter stabilisation in the soil and the sequestration of the C within it. In order for C to be sequestered in soil organic matter (SOM), it must in some way be protected from decomposition and mineralisation by the microbial community (Stockmann et al., 2013). The inclusion of SOM into soil aggregates and interactions between SOM and metal ions

play an important role in SOM stabilisation (Lehmann & Kleber, 2015; Lützow et al., 2006; Stockmann et al., 2013). Both of these processes are driven by microbial activity and microbial necromass is an important component of SOM (Hu et al., 2023).

SOM is commonly divided into two pools, known as particulate organic matter (POM) and mineral associated organic matter (MAOM) (Angst et al., 2023; Lavallee et al., 2020; Underwood et al., 2024; Yu et al., 2022). POM consists of largely undecomposed, mainly plant derived, light weight organic matter fragments that have minimal protection for microbial decomposition and therefore have relatively short turnover times in the soil. By contrast, MAOM consist of low molecular weight compounds of both microbial and plant origin that form complex interactions with soil minerals which protects them from further decomposition (Lavallee et al., 2020; Underwood et al., 2024). While POM mainly exists unprotected in the soil, it can be protected from mineralisation by inclusion into soil aggregates, which provides a barrier to the soil microbes, making it physically inaccessible as well as limiting oxygen availability, which the microbes require to respire and metabolise (Kimble, 2007; Lehmann & Kleber, 2015; Naresh et al., 2017; Stockmann et al., 2013). Decreases in molecular size of the SOM through microbial activity increases the ability of SOM to be included in the soil aggregates and stable microaggregates (Lehmann & Kleber, 2015). This also causes an increase in ionisable groups and polarity of the molecules, thereby increasing the solubility of the compound and its reactivity with metal ions, inorganic soil components or other SOM (Lehmann & Kleber, 2015; Lützow et al., 2006). These intermolecular interactions allow the formation of MAOM and make the organic matter more stable and resistant to microbial decomposition (Lavallee et al., 2020; Underwood et al., 2024). Fungal hyphae and extracellular polysaccharides produced by bacteria bind soil particles to form water stable soil aggregates and contribute to the protection of SOM (Kennedy, 1995). The inherent chemical recalcitrance towards decomposition of some compounds, such as lignin, chitin and glomalin, also contributes to SOM stability, as these organic compounds are difficult or impossible for microbes to

break down and as a result are able to stay in the soil long term (Lützow et al., 2006; Stockmann et al., 2013). These are often compounds produced by microbes.

In addition to its long-term storage, microbes also control the decomposition and mineralisation of SOM, which is an important process for maintaining soil fertility, as this provides nutrients for plants growing in the soil (Lavallee et al., 2020). Generally, labile SOM that is rapidly turned over is considered to consist predominantly of POM, while MAOM makes up the majority of long-term stable SOM (Lavallee et al., 2020). However, this concept has been challenged, with Yu et al. (2022) suggesting that MAOM contributes significantly to the short-term SOM mineralisation and Angst et al. (2023) arguing that POM can be a stable long term C store. This indicates that these two pools are not discrete, and are interlinked with overlapping compositions and functions (Angst et al., 2023; Lavallee et al., 2020). The cycling of organic matter in soils, both its decomposition and the formation and long-term stability of SOM in the soil, is tightly controlled by biological processes.

2.2.2 Microfauna

2.2.2.1 Protists

Protists are single celled eukaryotes and include flagellates, amoebae and ciliates in the soil (Amaresan & Chandarana, 2024; Ingham et al., 1986). These prey on bacteria, preforming an important role in controlling bacteria numbers (Ingham et al., 1986; Kennedy, 1995). The protozoa also prey on each other. Ciliates prey on both amoebae and flagellates, while amoebae also feed on flagellates, with some giant amoebae being able to engulf nematodes (Ekelund & Rønn, 1994; Ingham et al., 1986). Mycophagous protozoa also exist in the soil, feeding on yeast and fungal spores, although amoebae that feed on fungal hyphae have also been observed, and obligate mycophagous ciliates,

flagellates and amoebae have been isolated (Ekelund & Rønn, 1994). Soil protozoa are often assumed to be solely bacterivorous, with the mycophagous protozoa often being overlooked in soil food webs, however Geisen et al. (2016) suggests that it is common among soil protozoa and should be included in the study of soil ecosystems.

2.2.2.2 Nematodes

Nematodes are an important part of the soil food web, being the major link between the microorganisms and the higher trophic states (Heijboer et al., 2017). Nematodes control microflora populations through predation, and thereby also assist in cycling nutrients immobilised in microbial biomass (Ferris, 2010a; Ingham et al., 1985; Yeates, 2003). They also provide an important food source for higher level soil fauna (Ferris, 2010a).

Nematodes can be classified into eight trophic groups: 1) plant feeder, 2) hyphal feeder, 3) bacterial feeder, 4) substrate ingester, 5) predator of animals, 6) unicellular eucaryote feeder, 7) dispersal or infective stage of parasites, and 8) omnivore (Yeates et al., 1993). Of these, five are commonly used, including bacterivores, fungivores, herbivores, predators and omnivores (Kennedy, 1995; Ritz & Trudgill, 1999). This wide range in food sources means nematodes are present in each heterotrophic level of the soil food web (Ritz & Trudgill, 1999). They have a range of life history strategies, ranging from high fecundity with short life spans (r-strategists) to low fecundity with long life spans (K-strategists) (Ritz & Trudgill, 1999). Nematodes found in New Zealand pasture soils are diverse, with between 30 and 40 taxa identified, including species from the orders Tylenchida, Rhabditida, Teratocephalida, Araeolaimida, Monhysterida, Enoplida and Dorylaimida (Parfitt et al., 2010; Schon et al., 2010; Yeates, 1978; Yeates, 1981). Nematode communities vary considerably under pasture and are strongly influenced by management, however, tend to be dominated by bacterial and plant feeding nematodes (Hu et al., 2015; Lazarova et al., 2021; Mills & Adl, 2011; Schon et al., 2010). Intensive

grazing and synthetic N application has been shown to favour herbivorous nematodes and reduce higher trophic levels (omnivorous and predatory) as well as fungivorous nematodes (Bardgett et al., 1997; Herren et al., 2020; Hu et al., 2015; Parfitt et al., 2010). In contrast, multispecies pastures have been found to support more stable food webs with more abundant nematodes of higher trophic levels (Eisenhauer et al., 2011; Lazarova et al., 2021).

2.2.3 Mesofauna

Soil mesofauna includes Enchytraeidae, mites (Acarina), springtails (Collembola) and other microarthropods (Crotty, 2020; Lavelle et al., 2006). Mites are the dominant mesofauna in soils, while springtails are next most abundant (Crotty, 2020; Ren et al., 2024). Mites are functionally diverse, including predators, fungivores and detritivores (Crotty, 2020). Larger species of springtails tend to be detritivores, while smaller species feed on bacteria and fungi, with a few being herbivorous (Bell & Willoughby, 2003; Crotty, 2020). Mites and springtails play an important role as predators and decomposers, respectively, within the soil food web.

2.2.4 Macrofauna

2.2.4.1 Annelids

Introduced Lumbricidae species make up the majority of earthworms in pasture soils in New Zealand (Springett, 1992). Earthworms can be assigned to one of seven ecological categories (anecic, endogeic, epigeic, epi-anecic, endo-anecic, epi-endogeic and intermediate), however, only the first three are commonly used (Bottinelli & Capowiez, 2021). These ecological categories have often been used as proxies for functional groups, however Capowiez et al. (2024) argues this inappropriate and has described six

functional groups that can be used instead. Earthworms have many important ecological roles in the soil. These include soil formation, both physical weathering and organic matter cycling, improving soil structure due to burrowing activity, thereby influencing porosity and aggregate size distribution, which in turn affects water infiltration, runoff and improves water storage by allowing the soil to hold water at a wide range of potentials (Andriuzzi et al., 2015; Blouin et al., 2013). Earthworms are also involved in nutrient cycling, organic matter mineralisation and C sequestration (Blouin et al., 2013). Earthworms have been shown to increase plant productivity in agricultural systems through increased mineralisation of N (van Groenigen et al., 2014).

2.2.4.2 Arthropods

A wide range of arthropods are present in soils. The dominant macro arthropods include Isopoda, Myriapoda and Insecta (Culliney, 2013). Isopoda (woodlice) are terrestrial crustaceans that feed primarily on detritus. Myriapoda include Diplopoda (millipedes) and Symphyla, which are mainly detritivorous, however some millipedes will also feed on fungi (Culliney, 2013). This subphylum also includes centipedes (Chilopoda), which are predominantly carnivorous (Lewis, 1981). Examples of insects found in the soil include termites (Isoptera), ants (Formicidae) and beetles (Coleoptera) (Culliney, 2013). Arthropods influence nutrient cycling in the soil through feeding on litter and its comminution, thereby making it available for soil microbes, as well as by mineralisation of nutrients through predation on lower trophic groups (Brown et al., 2010; Culliney, 2013; Forgie et al., 2018; Lavelle et al., 2006). They also improve soil structure by mixing the soil, creating pores and contributing to aggregate formation (Brown et al., 2010; Culliney, 2013; Forgie et al., 2018; Lavelle et al., 2006). The distribution of microbes in the soil is aided by arthropod activity, as microbes or their propagules are often transported on or in the bodies of arthropods (Culliney, 2013). Some soil arthropods are important agricultural pests in New Zealand, that live in the soil during the larval stage and variously feed on

pasture plant roots or foliage (Ferguson et al., 2019). Arthropods play an important role in the soil food web, both as decomposers of organic material as well as predators of the mesofauna.

2.2.5 Plants

The majority of pastoral agriculture in New Zealand is based on perennial ryegrass and white clover swards. However, mixed pastures are becoming more common, with around 55 pasture plant species commercially available in New Zealand (Cosgrove et al., 2022). The most common ones include a wide range of species and cultivars from the families Fabaceae (legumes, including lucerne and a range of clovers) and Poaceae (grasses) (Ministry for the Environment, 2022). Herbs from the families Asteraceae (chicory and yarrow), Plantaginaceae (plantain), Polygonaceae (sheep's sorrel) and Rosaceae (sheep's burnet) are also present in New Zealand pastures (Ministry for the Environment, 2022). Plants are important as they are the primary producers in the soil food web. Inputs into the soil food web include both plant litter at the soil surface and belowground inputs via roots through rhizodeposits, which consists of excreted C compounds and sloughed root cells and other dead material (Berendsen et al., 2012; Eisenhauer & Reich, 2012). Between 12 - 18% of photosynthetically fixed C is released by roots into soil (Barber & Martin, 1976). When grazers are present, plant material is also returned to the soil indirectly through faeces. Under grazed pasture in New Zealand, between 34,000 and 42,000 kg DM ha^{-1} yr^{-1} of plant inputs, either directly through roots and litter or indirectly through dung, are added to the soil (Schon et al., 2010). Lange et al. (2015) found that under ungrazed pasture, between 850 and 1,200 kg C ha^{-1} yr^{-1} were added to the soil organic C stock, although this site had previously been used for arable cropping and therefore had low initial soil C.

2.3 Soil biological interactions

2.3.1 Plant-microbe interactions

2.3.1.1 Rhizosphere

The rhizosphere is the area of soil that is within centimeters of the root surface and is influenced by the plant root (Berendsen et al., 2012; York et al., 2016). Microbial activity here is heightened due to the constant input of organic matter, both via sloughed cells and other dead material and active excretion of C compounds by the plant, which can be up to 40% of the plants photosynthates (Berendsen et al., 2012; Mendes et al., 2013). These are collectively known as rhizodeposits and are utilised by the microbes (Mendes et al., 2013; Raaijmakers et al., 2009). Organisms present in the rhizosphere include protozoa, viruses, fungi, algae, bacteria, archaea, oomycetes, nematodes and arthropods (Mendes et al., 2013; Raaijmakers et al., 2009). These provide many benefits to the plant, including protection from parasites and diseases and improved tolerance to stress, nutrient acquisition and growth and development (Mendes et al., 2013). However, organisms deleterious to plant growth are also present in the rhizosphere, including pathogenic fungi, oomycetes, bacteria, and nematodes (Mendes et al., 2013; Raaijmakers et al., 2009).

Protection of plants from pathogens by the rhizosphere microbiome occurs via competitive exclusion of disease-causing organisms, as well as the production of antimicrobial secondary metabolites, hyper parasitism of pathogens and stimulation of the plant's immune system (Berendsen et al., 2012; Raaijmakers et al., 2009). Plants are able to actively select microbes present in their rhizosphere, as indicated by differences in the microbial composition of the rhizosphere and the bulk soil, with the former tending to have lower diversity (Berendsen et al., 2012). Plants are able to influence their rhizosphere microbiome by excreting specific root exudates to select for certain microbes as well

as producing secondary metabolites that inhibit others (Berendsen et al., 2012). They are also able to produce compounds that interfere with bacterial communication through quorum sensing, allowing plants to manipulate the gene expression of the bacteria (Berendsen et al., 2012). Plants can also recruit specific microbes in response to a pathogen attack, as well as stimulate antifungal activity in the rhizosphere microbes (Berendsen et al., 2012). Interactions between plants and their rhizosphere microbiome are complex, however a healthy microbiome benefits both the plant and the microbes.

2.3.1.2 Mycorrhizae

Mycorrhizal fungi are a group of fungi that form a symbiotic relationship with the majority of land plants, including pasture plant species (Crotty, 2020). The plant provides photosynthates as a C and energy source to the fungus, and in return the fungus provides the plant with greater access to nutrients through increased soil volume exploration as well as specific uptake of nutrients otherwise unavailable to plants, such as P (Crotty, 2020). Six species of mycorrhizal fungi, in the genera Glomus, Gigaspora, Acaulospora, and Sclerocystis, were found to be present in New Zealand soils, with *Glomus fasciculatus* and *Acaulospora laevis* being the most common (Powell, 1977). However, this was determined by identifying spores in a soil sample, meaning non sporing were not accounted for, which are likely to be important in agricultural soil (Powell, 1977; Rosendahl & Stukenbrock, 2004). *Glomus tenuis*, *Gigaspora margarita* and *Glomus fasciculatus* were all found to form mycorrhizal associations with both ryegrass and white clover when introduced into New Zealand pastures, variably increasing their P uptake and growth, with *Glomus tenuis* being the most efficient (Powell, 1979; Powell & Daniel, 1978b, 1978a). Xiao et al. (2023) has also found that mycorrhizal fungi have a symbiotic interaction with free living N fixers, although the details of this are not yet clear.

2.3.1.3 Symbiotic nitrogen fixation

Symbiotic N fixation in pasture occurs dominantly between legumes, especially clover and rhizobium bacteria (Shi et al., 2023). The bacteria receive a C and energy source from plant photosynthates, as well as an environment protected from both predators and oxygen, as nitrogenase is extremely sensitive to oxygen (Abd-Alla et al., 2023; Stein & Klotz, 2016). The plant in return receives biologically available N, which is often limiting growth (Abd-Alla et al., 2023). As only prokaryotes are able to fix N, this relationship allows plants to access the atmospheric N pool, that would otherwise be unavailable to them, to supplement a potentially limited soil N pool (Stein & Klotz, 2016).

2.3.2 Soil food web

Bacteria and fungi are near the base of the food web with most of the energy and nutrients flowing through them to higher trophic states (Ingham et al., 1986). Protozoa and nematodes prey on the microbes, collectively being a major link between the microflora and the larger soil fauna. Mesofauna, including mites and springtails feed on fungi as well as nematodes and in turn provide prey for larger fauna (Crotty, 2020). The interaction between larger invertebrates and microbes is indirect. Macrofauna selectively activate microbial activity as well as aid microbes through increasing food availability by comminution of organic matter and incorporating it into the soil where it is accessible for the microbes (Lavelle et al., 2006).

2.4 Factors affecting soil biological activity

2.4.1 Edaphic factors

Climatic conditions and rainfall influence soil biota, affecting soil temperature and moisture. Soil biota has complex responses to differences in soil moisture and soil temperature and often depends on an interaction of these two variables (Butenschoen et al., 2011; Thakur et al., 2018). Generally, increasing soil moisture and temperature increases microbial activity, however contrasting effects can be obtained under different combinations of these factors, and when other factors are considered (Butenschoen et al., 2011; Siebert et al., 2023; Thakur et al., 2018). Similarly, soil nematodes are affected by moisture and temperature, with the former having a greater influence on total abundance while the latter has stronger effects on diversity (Bakonyi & Nagy, 2000). Nematode abundance tends to have negative correlations with soil moisture, although the optimum differs between species and therefore will affect them differently (Bakonyi & Nagy, 2000). They tend to increase with temperature, although this will depend on interactions with soil moisture (Renčo et al., 2010; Yeates, 1981). Soil detritivores have been shown to have similar responses as soil microbes (Siebert et al., 2023; Thakur et al., 2018).

Soil pH has a strong influence on soil biota (Banerjee & van der Heijden, 2023). As fungi are able to tolerate low pH better than bacteria, reducing soil pH causes bacterial biomass to decrease and therefore the fungi to bacteria ratio to decrease (de Vries et al., 2006). Low pH causes the species richness and abundance to decrease (Wei et al., 2022). Changes to the bacterial community structure occur in response to soil pH, with lower pH favouring Acidobacteria, higher pH promoting Actinobacteria and Bacteroidetes while near neutral pH is optimum for β- and γ- proteobacteria (Lauber et al., 2009). Arbuscular mycorrhizal fungi (AMF) are also negatively affected by low pH (Edlinger et al., 2022; Svenningsen et al., 2018). Similarly, pH affects soil nematodes, with an increase in pH causing the total

nematode abundance to increase, although individual species have different responses (Yeates, 1976; Zhao Jie et al., 2015). Fungivorous nematodes are reduced with increasing pH, which is thought to be due to bottom-up effects caused by changes to the fungal community (Zhao Jie et al., 2015). These changes also have flow on effect to the fauna of the wider soil food web (Wei et al., 2022).

Soil organic matter content also affects microbial activity, as it is the main C and energy source for many microbes, as well as influencing soil physical conditions (Banerjee & van der Heijden, 2023). Soil physical factors, such as structure, compaction, porosity, and texture modify the soil environment which influences water infiltration, water holding capacity and aeration, which in turn affect microbial activity. Soil microbial biomass and the C content of soils is strongly positively correlated (Banerjee & van der Heijden, 2023; Kallenbach et al., 2016).

2.4.2 Farm management factors

Agricultural management practices can directly influence the composition and functionality of soil biota (Banerjee & van der Heijden, 2023; Tomazelli et al., 2023). This includes practices that physically disturb the soil, the application of synthetic inputs and manipulation of the vegetation growing in the soil.

Mechanical disturbance of soils, such as tillage for soil cultivation, decreases fungal diversity and abundance and although initially promotes bacterial growth by aerating the soil and exposing organic matter, long term soil cultivation decreases bacterial diversity (Crotty, 2020; Kabir, 2005). Soil cultivation also alters the physical properties of the soil, indirectly affecting soil biota by modifying the soil environment. In pastoral agriculture, physical disturbance of the soil can occur through soil compaction due to stock and vehicle traffic. Treading damage has been shown to reduce both bacterial and fungal biomass (Hiltbrunner et al., 2012). While increases in soil bulk density caused

by both high stocking rate and heavy machinery did not affect nematode abundance, it did increase the dominance of herbivorous nematodes and reduce the abundance of bacterivores, omnivores and predators (Bouwman & Arts, 2000; Schon et al., 2010). This has been attributed to reduced habitable pore space and reduced plant root penetration to depth, causing an increase in root density near the surface and thereby increasing the food source for herbivorous nematodes (Bouwman & Arts, 2000; Schon et al., 2010).

Synthetic inputs, via agrichemicals and mineral fertilisers, can influence soil biota. Pesticide application often impacts more than just the target species and can cause microbial biomass to decrease (Banerjee & van der Heijden, 2023; Crotty, 2020). Fungicide application negatively impacts beneficial AMF, reducing their ability to supply plants with P (Edlinger et al., 2022). Application of mineral fertilisers also weakens mycorrhizal associations with plant roots by reducing the necessity of this interaction (Crotty, 2020). This is especially true in the case of P fertilisers, which has been shown to decrease root colonisation by AMF (Fornara et al., 2020; Lu et al., 1994; Ryan et al., 2000; Valentine et al., 2001). However, P fertiliser application to pasture in New Zealand has also been shown to have no effect or increase total AMF abundance and diversity, although root colonisation was not measured in these studies (Parfitt et al., 2010; Wakelin et al., 2012). Wakelin et al. (2012) indicated that the increase in AMF they observed could be due to changes in the botanical composition of the pasture community induced by the P fertilisation, rather than a direct response to the P addition. Application of N fertiliser reduces both fungal and bacterial biomass, however, it has a larger effect on the former and causes the microbial community to shift towards a lower fungi to bacteria ratio, while also favouring certain bacterial populations, such as nitrifying bacteria (de Vries et al., 2006; Hayatsu et al., 2008). Fertiliser application increases nematode abundance, which was attributed to higher net primary production, resulting in an increased food source (Parfitt et al., 2010). However, application of N fertilisers shifts the nematode community towards being herbivore dominated with reduced abundance of omnivorous

and predatory nematodes (Herren et al., 2020). Agricultural intensification tends to select for organisms with short life spans and high fecundity (r strategists), herbivorous nematodes and disrupted, simplified food webs (Lazarova et al., 2021).

Plant species diversity in pasture has been associated with increased soil biodiversity (Crotty, 2020; Lange et al., 2015). Higher pasture species diversity causes higher C inputs into the soil via root exudates which stimulate microbial activity and increase C storage in the soil (Lange et al., 2015). The denser vegetation of diverse plant communities promotes microbial activity by reducing evaporation from the top soil (Lange et al., 2015). This causes more active, abundant and diverse microbial communities (Lange et al., 2015; Scherber et al., 2010). However, the presence of legumes in diverse pasture causes decreased C storage in the soil, despite their ability to increase soil N, which was attributed to lower root biomass and therefore reduced below-ground C inputs. Increased pasture diversity also increased nematode community diversity and abundance (Eisenhauer et al., 2011; Scherber et al., 2010). Under diverse pastures, nematode food webs are more complex, with an increased abundance of higher trophic levels, while low diversity pastures are dominated by herbivorous nematodes and communities that are generally detrimental to plant growth (Eisenhauer et al., 2011). The effect of diverse pastures on fungivorous nematodes is conflicting, having been shown to have both positive and negative effects (Eisenhauer et al., 2011; Scherber et al., 2010). Diverse pastures influence the below ground food web through bottom-up controls, having the greatest influence on the primary consumers with effects passed on to the higher levels along trophic cascades (Scherber et al., 2010).

2.4.3 Regenerative agriculture

RA is an alternative form of agriculture that was developed in the 1980's, however, has recently gained increasing attention for its potential to improve the sustainability of

food production systems (Giller et al., 2021; Newton et al., 2020). There is currently no widely agreed upon definition for RA, however those that do exist are defined by either management practices, outcomes achieved or a combination of both (Newton et al., 2020). The outcomes RA aims to achieve generally involve improving soil health and environmental sustainability, while maintaining farm productivity and profitability (Newton et al., 2020; Schreefel et al., 2020). Management practices RA practitioners implement include, minimising external inputs, fertiliser and pesticide use and tillage, integrating livestock and use of cover crops, perennials, trees and manure and compost (Newton et al., 2020; Schreefel et al., 2020). However, a wide range of other activities are also implemented, including use of windbreaks, silviculture and intercropping (Newton et al., 2020; Schreefel et al., 2020). In RA, much focus is placed on improving soil health, which involves increasing soil C and biodiversity and improving soil physical characteristics. Soil flora and fauna should be integrated into the farming system for nutrient cycling, with plant production being based on biological interaction and substances that disrupt this (such as agrichemicals and synthetic fertilisers) being discouraged (Giller et al., 2021). Therefore, RA practices have the potential, and are claimed, to have a strong influence on soil biota. However, the lack of a universal definition of RA makes testing its effectiveness difficult (Newton et al., 2020). Although, this lack of definition also prevents RA from being restricted and confined to a set of rules, and is considered a key concept of RA (Grelet et al., 2021).

RA in the context of New Zealand agriculture is unique in that many of the negative consequences of farming that RA attempts to resolve are the result of management practices not implemented in New Zealand (Grelet et al., 2021). However, New Zealand has its own unique sustainability issues involving the highly erodible soils found across large areas under agricultural management and an extreme contrast between the species used in agriculture and the native biodiversity, making it difficult to integrate native biodiversity into the farming system, which is a key component of RA (Grelet et al., 2021).

Therefore Grelet et al. (2021) argues that New Zealand should develop its own narrative regarding RA, that allows it to build on its current agricultural system and address its own unique challenges. The increasing popularity of RA in New Zealand means this will likely become an important management approach that influences biota in agricultural soils.

2.5 Methods for measuring soil microflora and microfauna

2.5.1 Total microbial biomass

2.5.1.1 Culture dependent methods

Culture dependent methods involve making a serial dilution of a soil suspension and growing the bacteria on plates or microtiter plate by incubating on various media under different conditions to select for organisms with specific metabolic capabilities (Foght & Aislabie, 2005). Bacteria are enumerated through plate counts or the most probable number method. This method is known to severely underestimate the number and diversity of microorganisms present (Blagodatskaya & Kuzyakov, 2013; Foght & Aislabie, 2005; Ingham & Slaughter, 2004). This is due to cultivation bias, as the majority of soil microorganisms are known to not be cultivable and will not grow on the agar plates (Blagodatskaya & Kuzyakov, 2013; Foght & Aislabie, 2005; Ingham & Slaughter, 2004).

2.5.1.2 Direct microscopic count

Direct microscopic count involves making dilutions of a soil suspension and staining the microorganisms before observing the sample under a microscope. The number of bacteria present are counted, and the length of fungal hyphae measured. The original method, developed by Jones et al. (1948), involved making a suspension of the soil sample in 1.5% agar, preparing agar films of a known volume on a haemocytometer before staining in

water-soluble aniline blue and observing under a light microscope. Several modifications of the methods have since been made, including the use of various dyes for staining, preparation methods and microscopy types. The use of specific stains to determine the portion of active bacteria and fungi in the sample will be further discussed in section 2.5.2. A description of the various dyes used is given in Blagodatskaya & Kuzyakov (2013). Sample preparation to calculate hyphae length was optimised by Thomas et al. (1965), however an alternative method using filtration of the sample to extract the hyphae has been developed, which is less labour intensive (Hanssen et al., 1974). The filtration method was optimised by West (1988), finding that epifluorescent microscopy with the fluorescent brightener stain, gave the best results. Bååth & Söderström (1979) indicated that hyphal diameter needs to be measured along with hyphal length to give accurate estimations of fungal biomass. In general, direct microscopic counts are labour intensive, as at least 20 microscope fields per slide, on 4 different slides, should be counted for accurate estimation (Foght & Aislabie, 2005; Jones et al., 1948). This, as well as needing to be carried out on fresh soils, limits this methods' use for analysing a large number of samples. The use of direct microscopic counts has been criticised. Issues pointed out by Stahl et al. (1995) include observer subjectivity in measuring, microbes obscured by soil aggregates or particles and variability in results, suggesting that microscopic counts should be used in conjunction with other methods.

2.5.1.3 Biomarkers

PLFA analysis can be used to measure microbial biomass in soils. They are found in the membranes of all living organisms, while also being found exclusively in living cells, making them a good marker for live biomass (Blagodatskaya & Kuzyakov, 2013; Gabriel, 2010). Certain PLFAs are found predominantly or exclusively in specific microbial groups (Joergensen, 2022). This allows the various fractions of the PLFAs present in a sample to be associated with different bacterial and fungal groups and thereby allowing a high level

analysis of the microbial community structure (Joergensen, 2022). As the amount of a specific PLFA in a sample is proportional to the microbial biomass, PLFA analysis provides a quantitative measure of soil microbes (Blagodatskaya & Kuzyakov, 2013). The method involves chemical extraction of the PLFAs from a freeze dried soil sample, chromotography purification and detection with a mass spectrometer or flame ionisation detector (Gómez-Brandón et al., 2010; Joergensen, 2022). Neutral lipid fatty acid (NLFA) analysis is similar to PLFA, however involves analysing the neutral lipid fraction of soil lipids. These are energy storage compounds in eukaryotes and, since they do not use lipids as an energy store, are rarely found in bacteria (Bååth, 2003; Joergensen, 2022; Olsson et al., 1995). The NLFA 16:1ω5 is found predominantly in AMF, as it contains large amounts in its spores and vesicles as energy stores (Bååth, 2003; Olsson et al., 1995). This makes it a useful indicator of AMF in soil (Frostegård et al., 2011; Joergensen, 2022).

Biomarkers specific for bacteria or fungi, or subgroups of these, have also been used to determine the biomass of specific components of the soil microbiome. Ergosterol is the main sterol found in most fungi and has been used as an indicator of total fungal biomass and growth (Blagodatskaya & Kuzyakov, 2013; Gabriel, 2010). Chitin is a component of fungal cell walls and can also be used as a biomarker specific to fungi, which allows both the living and dead fungal biomass to be measured (Adamczyk et al., 2020). Muramic acid, glucosamine, and diaminopimelic acid are used as specific biomarkers for prokaryotes, while teichoic acid and components can be used to detect gram positive bacteria (Gabriel, 2010).

2.5.1.4 Microbial biomass carbon

Total microbial biomass C is commonly used to measure the total amount of microbes present in the soil. The most widely used technique is based on the work of Jenkinson & Powlson (1976b), in which chloroform fumigated soil is incubated for 10 days, during

which time the oxygen gas (O_2) utilised and carbon dioxide (CO_2) produced is measured and compared to a control. The amount of CO_2 produced is used as an indicator of the total microbial biomass present in the soil (Jenkinson & Powlson, 1976a). An adjustment of the method by Jenkinson & Powlson (1976b) was made by Vance et al. (1987), which replaced the 10 day incubation with dichromate digestion.

2.5.1.5 Extractable carbon

Water extractable C can be extracted on dried soil samples with hot or cold water. Hot water extractable C has been shown to be a useful proxy of total microbial biomass (Ghani et al., 2003). This simple method involves first extracting the water soluble C from a sample, before extracting the labile C by heating the sample in water at 80 ^{O}C for 16h (Ghani et al., 2003). The C content of the extracts can then be analysed. Cold water extractable C follows the same method as described by Ghani et al. (2003), however instead of heating the sample to 80 ^{O}C for 16 h, it is kept at 20 ^{O}C for 16h (Ćirić et al., 2016). In a comparison of the two methods, Chantigny et al. (2014) found that extracted C reached a maximum after 1 hour at 20 ^{O}C and after 4 hours at 80 ^{O}C, with longer extractions causing a compositional change in the sample due to microbial activity. They recommended limiting the extraction time to 1 and 4 hours for cold and hot water extractable C, respectively.

The amount of labile C present in the soil can also be determined by the amount of C oxidised by potassium permanganate. The method described by Blair et al. (1995) involves adding 15 ml of 333 mM potassium permanganate to a soil sample and mixing for 1 hour before diluting the samples and measuring the absorbance at 565 nm on a photospectrometer. The amount of C oxidised is determined by the change in concentration of the potassium permanganate. This method overestimates the amount of active C present (Blair et al., 1995; Dell, 2009). It was modified by Weil et al.

(2003), involving a simplified method with a lower concentration (20 mM) of potassium permanganate and 100 mM calcium chloride as a flocculation agent. A field kit, with a hand-held colorimeter, using this method can be used for on site measurements of labile C (Weil et al., 2003). This method, with a few minor modifications, has become the dominant potassium permanganate test (Culman et al., 2021). Despite this, Dell (2009) suggests that using a very dilute (2.5 mM) potassium permanganate solution gives more accurate results. Blair et al. (1995), Weil et al. (2003) and Dell (2009) all noted that the contact time between the soil sample and the potassium permanganate, as well as its concentration, affected results, so standardised methods are required. Another limitation of this test is that the potassium permanganate stock solution must be carefully prepared and stored to prevent its decomposition, which will alter the concentration and affect the test (Blair et al., 1995). However, it provides a much more rapid measure of labile C than laboratory incubations (Culman et al., 2021; Dell, 2009).

2.5.1.6 Dehydrogenase activity

Dehydrogenase is an enzyme that is found in all living microbial cells, but do not accumulate outside of the cells in the soil (Wolińska et al., 2012). This means they can be used as an indicator of overall microbial activity and is proportional to the total microbial biomass in the soil (Wolińska et al., 2012). Dehydrogenase activity can also be used as an indicator of soil conditions, increasing under anaerobic conditions as well as being positively correlated with total organic C and temperature. Adaptions of the method originally described by Casida et al. (1964), are the most common methods for measuring dehydrogenase activity (Camiña et al., 1998; von Mersi & Schinner, 1991). It involves adding triphenyltetrazolium chloride to a soil sample and incubating for 24 hours. The dehydrogenase enzyme acts on the triphenyltetrazolium chloride, converting it to triphenylformazan, which causes a colour change from colourless to red (Wolińska et al., 2012). The triphenylformazan is then extracted by filtration and the colour intensity

measured with a photospectrometer (Casida et al., 1964).

2.5.2 Active microbial biomass

Blagodatskaya & Kuzyakov (2013) grouped soil microorganisms into four physiological states (active, potentially active, dormant and dead) which together make up the total microbial biomass. The active state was defined by them as the organisms involved in substrate utilisation and biochemical reactions, making up 0.1% to 2% of total microbial biomass. The dormant state includes microorganisms with strongly reduced physiological activity and do not contribute to turnover processes. The potentially active state consists of dormant microorganisms that can rapidly (within minutes to hours) switch to the active state and make up 10% to 60% of the total microbial biomass. Since the microorganisms in the active state carry out the microbial related soil processes, Blagodatskaya & Kuzyakov (2013) emphasised the importance of determining the active microbial biomass in soils, rather than just the total microbial biomass.

2.5.2.1 Respiration analysis

Respiration analysis involves analysing the CO_2 produced or O_2 used from a soil sample. Basal respiration measures the CO_2 or O_2 evolution of an unamended soil sample. This does not represent the active microbial biomass, as many factors including substrate limitation, physiological state of the microbes and maintenance requirements influence basal respiration, making it an indication of integrated metabolic activity (Blagodatskaya & Kuzyakov, 2013). Substrate induced respiration (SIR) analysis, originally described by Anderson & Domsch (1978), involves adding a readily available substrate, often glucose, to the sample and measuring the CO_2 evolved. While this can be used to calculate the total microbial biomass (Anderson & Domsch, 1978; Mingorance & Peña, 2016), on its own, it is an indicator of the active microbial biomass (Blagodatskaya & Kuzyakov, 2013).

Measuring the CO_2 evolution of a substrate amended sample in real time for 24 - 48 hours, allows growth rate models to be applied and substrate induced growth rate calculated (Wutzler et al., 2012). From this, the active and potentially active portions of the original sample can be calculated (Wutzler et al., 2012). Selective respiratory inhibition works on the same principle as respiration analysis, however the activity of bacteria or fungi is selectively inhibited by antibiotics to allow respiration from the other to be determined (Anderson & Domsch, 1973). This can be used to determine the fungal to bacterial respiration ratio, however, cannot be used to measure active bacteria or fungi individually, as the inhibitors used are rarely fully selective and the results often not fully additive (Blagodatskaya & Kuzyakov, 2013; Lin & Brookes, 1999; Susyan et al., 2005; Velvis, 1997). Several methods exist for measuring respiration, including using automated respirometers (Mingorance & Peña, 2016), including electrolytic microrespirometers (Scheu, 1992), CO_2 analysers, infrared gas analysers or gas chromatography (Anderson & Domsch, 1978; Hsieh et al., 2020; Mingorance & Peña, 2016). Techniques have been developed to measure respiration without laboratory equipment in real time, such as the MicroRes developed by Hsieh et al. (2020). Similarly, CO_2 flux from the soil can be measured in real time using a LiCOR chamber (LI-COR Environmental, 2025). The ratio of basal respiration to substrate induced respiration can be used to infer the metabolic state of the soil.

2.5.2.2 Microscopy and staining

Staining a sample with specific stains that can differentiate between viable and non-viable cells can be used to determine the portion of active microbes in a sample. Stains that bind to cell components and can cross intact cell membranes will stain all cells, both living and dead (Blagodatskaya & Kuzyakov, 2013). Stains that bind to nucleic acids but are unable to cross the membrane of living cells can be used to identify dead cells, while stains that are taken up by active transport or metabolised in the cell shows cells that

Table 2.1: Stains used in fluorescent microscopy to distinguish different physiological states of soil microbes (Blagodatskaya & Kuzyakov, 2013).

Stain	Target	Properties	Physiological state stained
Acridine orange	Nucleic acids	Cross membranes of living cells	All
SYBR Green I	Nucleic acids	Cross membranes of living cells	All
4,6-diamidino-2-phenylindole (DAPI)	Nucleic acids	Cross membranes of living cells	All
Europium chelate	Nucleic acids	Cross membranes of living cells	All
Fluorescein iso-thiocyanate (FITC)	Proteins	Cross membranes of living cells	All
Phenol aniline blue	Cell wall polysaccharides	Cross membranes of living cells	All
Phenolic tryptophan blue	Cell wall polysaccharides	Cross membranes of living cells	All
5-4,6dichlorotriazinyl aminofluorosceine (DTAF)	Cell wall polysaccharides	Cross membranes of living cells	All
Propidium iodide (PI)	Nucleic acids	Cannot cross intact cell membranes	Dead
Ethidium bromide	Nucleic acids	Cannot cross intact cell membranes	Dead
Fluorescein diacetate (FDA)	Cytoplasm	Converted to fluorescent compounds by metabolically active cells	Active
5-cyano-2,3-ditolyl-tetrazolium chloride (CTC)	Cytoplasm	Converted to fluorescent compounds by metabolically active cells	Active
2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT)	Cytoplasm	Converted to fluorescent compounds by metabolically active cells	Active
Calcofluor white	Fungal cell wall	Fungi specific	Fungi
FB28 brightener	Fungal cell wall	Fungi specific	Fungi

are active (Blagodatskaya & Kuzyakov, 2013). Blagodatskaya & Kuzyakov (2013) gives examples of specific dyes within each of these categories (Table 2.1). Dual staining with dyes that reveal different physiological states of the cells can be used to determine the portion of each state. Using ribonucleic acid (RNA) based fluorescent in situ hybridisation (FISH) in combination with staining for total microbial biomass also allows the active portion of the microbial population to be determined (Blagodatskaya & Kuzyakov, 2013). These techniques require the stained samples to be manually counted with either light or fluorescent microscopy, depending on the stain used, and therefore suffer from the same draw backs as mentioned in section 2.5.1.2.

2.5.3 Mycorrhizal colonisation

The most widely used technique for determining the colonisation of plant roots with mycorrhizae is staining and microscopy (Kokkoris et al., 2019). This involves viewing stained root sections under a microscope and counting the number of colonisation sites. Various stains can be used, however Chlorozol black E has been found to give the best stain (Rajapakse & Miller, 1992). Methods for counting colonisation vary, but the two most commonly used are those developed by Trouvelot (1986) and (Kokkoris et al., 2019; McGonigle et al.; 1990). These methods involve observing stained root sections of random subsamples and scoring the portion of roots colonised (Kokkoris et al., 2019). Issues with the Trouvelot method include observer subjectivity and lack of standardisation, meaning results cannot be compared across labs (Kokkoris et al., 2019). The McGonigle method is more objective, removing most of the observer variation, however, fails to account for colonisation intensity (Kokkoris et al., 2019). Both methods tend to overestimate colonisation (Kokkoris et al., 2019). The NLFA 16:1ω5 is found exclusively in arbuscular mycorrhizal fungi (Heijboer et al., 2017). It is correlated with the amount of mycorrhizae present in the soil and has been used as an indicator of total mycorrhizae present (Barceló et al., 2020; Olsson et al., 1995). It has also been correlated with the length of root colonised with arbuscular mycorrhizal fungi (Barceló et al., 2020; Sharma & Buyer, 2015).

2.5.4 Protists

Enumeration of total protozoa can be done with the most probable number method (Amaresan & Chandarana, 2024). This technique was originally developed by Singh (1946) and has since been modified, with the method described by Amaresan & Chandarana (2024) involving making serial dilutions of the soil suspension in a 96 well

microtiter plate, along with a suitable bacterial food source, and scoring growth in each well using an inverted microscope. Protozoa can also be enumerated with staining and microscopy to measure total and active protozoa (Griffiths & Ritz, 1988). Amaresan & Chandarana (2024) also describes methods for measuring abundance of specific protozoa, as well as techniques for identification and classification.

2.5.5 Nematodes

Nematode analysis involves using a microscope to enumerate nematodes in a sample and classify them into family groups. These families can then be used to calculate various nematode indices which give an indication of food web structure, soil disturbance and pollution. The original index was developed by Bongers (1990) and is known as the maturity index (MI). This is used to give an indication of the environmental disturbance of the soil ecosystem and is based on the life history strategies of the nematodes. Obligate plant feeding nematodes were excluded from this index and were instead used to calculate a separate index known as the plant-parasite index (PPI) (Bongers, 1990). This was based on the grounds that herbivorous nematodes were more dominant in nutrient enriched soils, correlate with increased primary productivity and depend on plant establishment to colonise disturbed soils, which causes them to have a delayed reaction compared to non herbivorous nematodes (Bongers, 1990). This causes the MI and the PPI to have an inverse relationship, with the former decreasing under increasing soil disturbance while the latter increases (Bongers et al., 1997).

Yeates (1994) argued that herbivorous nematodes should be included in the MI, adapting it to the ΣMI. The indices developed by Ferris et al. (2001) and Ferris (2010b) are based on Bongers (1990) MI, but have been elaborated to include trophic classifications, grouping nematodes into functional guilds which can then be used to infer the state of the soil food web and ecosystem. Ferris et al. (2001) described the structure, enrichment

and channel indices. The former two indicate the primary enrichment of the food web and the structure of the food web respectively, together giving an indication of the overall soil food web health. The channel index compares bacterial feeding nematodes to fungal feeding nematodes to determine the dominant decomposition pathway present. These were extended to quantify the ecosystem functions and services provided by each component of the nematode community by including metabolic footprints (Ferris, 2010b). This provides a measure of the turnover of the different microbial groups.

Ritz & Trudgill (1999) indicated that useful information can be obtained by studying nematodes without the need to classify them into family groups. The feeding type can be determined by observation under a microscope, as most nematodes can be assigned to functional group based on the structure of their feeding apparatus, and lifestyle strategy can be inferred from their size (Ritz & Trudgill, 1999). Samples can be easily extracted from soil, observed with limited equipment and stored in simple preservatives such as formaldehyde (Ritz & Trudgill, 1999). Nematode analysis has been used extensively in New Zealand to measure effects of pastoral agriculture on soil health (Parfitt et al., 2010; Schon et al., 2010; Yeates, 1976, 1994; Yeates et al., 1999; Yeates, 2003).

2.5.6 Molecular techniques

Methods for studying microbial deoxyribonucleic acid (DNA) in soil consist of metabarcoding and metagenomics (Semenov, 2021). These involve extracting and sequencing DNA from soil samples and using computational biology to determine microbial community structure. Metagenomics involves sequencing and analysing the entire DNA sample of a soil. Metabarcoding amplifies only a specific gene, known as a barcode. This is often the 16S for prokaryotes, ITS for fungi, and 18S for eukaryotes (Anderson & Cairney, 2004; Semenov, 2021). Metabarcoding allows the composition of the microbial community to be determined, while metagenomics also allows inferences to

be made about their metabolic functions. For instance, Dos Santos et al. (2012) identified a minimum gene set that can be used to determine N fixation capabilities. However, DNA based analyses are generally not quantitative.

While analysis of DNA from soil samples allows the community composition and its potential functions to be determined, in order to establish what processes and functions are actually occurring, analysis of the RNA or metatranscriptomics is required. This involves extracting, purifying and sequencing the mRNA of the soil sample, to determine which genes are being actively transcribed by the soil microbial community, allowing inferences to be made on the biochemical processes that are actually occurring in the soil (Wang et al., 2012). Obtaining high quality and high yield of RNA from soil samples is difficult, however advancements in extraction methods and sequencing technology are helping overcome limitations (Poursalavati et al., 2023; Wang et al., 2012). Care must be taken when interpreting results from RNA analysis. Although it can provide a lot of information around gene functioning, it is ultimately the proteins that carry out the functions and mRNA levels do not always correspond to protein level and provide no information on the kinetics of enzyme activity (Wang et al., 2012). Combining the genomics and transcriptomics with proteomics, interactomics and metabolomics can help overcome some of these limitations (Wang et al., 2012).

2.6 Knowledge gaps

Soil biota is a crucial component of healthy soils and is essential for productive and sustainable agriculture (Crotty, 2020; Kennedy, 1995; Rashid et al., 2019). However, its importance has long been overlooked and has only relatively recently become the focus of scientific research and considered in farm management (Crotty, 2020; Kennedy, 1995). Therefore, a lot remains unknown about soil biota and how it is influenced by agriculture, as well as which methods are most suitable and practical for measuring soil biological

health.

As RA was developed overseas, there is a need to determine its relevance within the New Zealand agricultural sector (Grelet et al., 2021). Grelet et al. (2021) highlight the importance of researching RA within the New Zealand context and adapting it to suit our unique situation. Due to its potential to improve farm sustainability, it is important that the effects of RA are tested and the underlying mechanisms of the impact it has determined, so that proven, effective practices can be implemented in New Zealand's farming industry (Grelet et al., 2021). This will also ensure New Zealand has access to any potential market premiums that may come from this in the future, to ensure New Zealand's agricultural exports remain competitive in the global market, which is crucial for an economy that relies on its agricultural exports (Grelet et al., 2021). After social wellbeing, soil health, including physical, chemical and biological health, was considered the second most important component of RA by the New Zealand agricultural sector, and the dominant topic that current RA practitioners in New Zealand called for more research on (Grelet et al., 2021). Impacts on soil health was identified as one of the current knowledge gaps regarding RA in New Zealand, with interest in whether RA can enhance biological activity in soils, the mechanisms and impact this has on ecosystem function, as well as surrounding the ability of RA to increase soil C and improve nutrient cycles (Grelet et al., 2021). This highlights the need for research on soil biota under RA management in New Zealand.

2.6.1 Research aims

In contrast with mainstream agriculture, where soil health had often been neglected, the RA movement emphasises the importance of soil health and the contribution of soil biota to this, however, often taking it to the opposite extreme, with bold claims of what soil biota can do (Schreefel et al., 2020). There is a short fall of scientific literature to back

up these claims, and while this does not mean they are untrue, it does cause them to be regarded with scepticism from the scientific community and many people outside of the RA community. Therefore, there is limited knowledge about soil biota in agricultural systems in general, however, there is further shortfall of evidence regarding the effects of RA on soil biota. Gaining a better understanding of how farm management impacts soil biota, and how it can be manipulated, is important for improving farm productivity and sustainability.

This thesis aims to address the limitations in understanding of the effects of RA on soil biota in New Zealand's pastoral agricultural sector. It investigates how changing farm management to RA practices in the short term impacts soil biota, with a particular focus on microbiome. To test whether RA enhances soil biological activity, changes in the physiological state of soil bacteria is assessed via fluorescent microscopy. To determine the influence RA has on total microbial biomass and the microbial community composition analysis of soil PLFAs is conducted. Whether RA has effects on the wider soil food web was investigated by analysing the soil nematode community.

The research study objectives were to determine whether changing farming practices to diverse pasture and regenerative management in the short term influences:

1. the physiological state of soil bacteria in New Zealand pastoral agricultural systems.
2. the abundance and community composition of the soil microbiome in New Zealand pastoral agricultural systems.
3. the abundance and community composition of soil nematodes in New Zealand pastoral agricultural systems.

CHAPTER 3: GENERAL METHODS

This chapter provides an overview of the experimental design and site descriptions that applies to all of the subsequent chapters.

3.1 Experimental sites

The study was conducted as part of the Whenua Haumanu research program, across two sites around Palmerston North, New Zealand. The first is a sheep farm located on the Tokomaru marine terrace 5.3 km southeast of Palmerston North. The other is a dairy farm located on the flood plain of the Manawatū river just south of the city. The detailed description of each site is given in sections 3.1.1 and 3.1.2 below. The broader Whenua Haumanu study investigates the impact of implementing regenerative management practices and diverse pastures in New Zealand's pastoral farming system on a range of soil, pasture, animal, production and environmental outcomes.

On each farm different treatments were applied as self-contained farmlets, which were managed independently as fully operational farms according to set treatment criteria. Each farmlet on the dairy farm site was 12 ha in area, while on the sheep farm site, each farmlet was 3 ha in area. The farmlets were established at the beginning of the 2022 farming season. In New Zealand the farming season starts on the 1st of June (southern hemisphere winter), as most dairy farms in New Zealand calve seasonally and this is when the cows are dry (not lactating). All pastures were sprayed out with glysophate together with saflufenacil on the dairy farmlets and thifensulfuron on the sheep farmlets and direct drilled with the pasture species shown in Table 3.1 in April of 2022. During the first year after the farmlets were established, selected paddocks received targeted mineral nutrient

applications and all paddocks received lime across all treatments to adjust any background differences in soil nutrient levels. The farmlets were set up and operational by the start of the calving season in July to August of 2022. Therefore, at the time of this study, the farmlets had been operating under their respective treatments for two and a half years. The treatments applied on the farmlets consisted of a combination of two factors each with two levels.

The type of pasture sown was one factor; the two pasture types were standard pasture and diverse pasture. Standard pasture consisted of ryegrass with red and white clover, as is the standard practice in New Zealand. Diverse pasture contained a mix of different pasture species, including grasses, legumes and herbs. Table 3.1 shows the pasture species sown when the farmlets were established and the pasture species actually present in the year of sampling for this study (2024). This shows that many of the species sown in the diverse pastures did not persist, while some of the species that were not sown in the standard pasture are now present, presumably due to dispersal from the diverse pasture paddocks. However, these self sown species tended to be a low percentage of the pasture composition.

The other factor was the farm management practices implemented. The two levels were contemporary management and regenerative management. Contemporary management was defined as following the best practice grazing advice of Beef and Lamb New Zealand (Geenty, 1994/2018) on the sheep farmlets and the Dairy NZ best practice grazing guidelines on the dairy farmlets (DairyNZ, 2024a, 2024d, 2024c, 2024b), with mineral fertilisers and agrichemicals used as required. A boom spray was used for weed control. Between 20 to 40 kg ha^{-1} of nitrogen (N) fertiliser had been applied on the sheep farmlets in previous years depending on requirements, while on the dairy farmlets between 60 and 80 kg ha^{-1} of N fertiliser had been applied. Therefore, this treatment replicated current standard practices of conventional sheep and dairy farms in New Zealand.

Table 3.1: Pasture species present in each pasture type, showing which species were sown when the pastures were established, in April 2022. Species in bold were still present in the pasture at greater than 1% of the total count at the time of this study (November-December 2024), based on monitoring of botanical composition conducted as part of the Whenua Haumanu program. Species underlined were not sown but were present in the pasture in November-December 2024.

Pasture type	Functional group	Pasture species
Standard pasture (both farmlets)	Grass	**Diploid perennial ryegrass (*Lolium perenne*), Tetraploid perennial ryegrass (*Lolium perenne*)**, Cocksfoot (*Dactylis glomerata*)
	Legume	**Red clover (*Trifolium pratense*), White clover small leaved (sheep farmlets) or large leaved (dairy farmlets) (*Trifolium repens*), White clover medium/large leaved (*Trifolium repens*)**
	Herb	Plantain (*Plantago lanceolata*)
Diverse pasture (sheep farmlets)	Grass	**Diploid perennial ryegrass (*Lolium perenne*), Tetraploid perennial ryegrass (*Lolium perenne*), Meadow fescue (*Lolium pratense*), Cocksfoot (*Dactylis glomerata*)**, Timothy (*Phleum pratense*)
	Legume	**Red clover (*Trifolium pratense*), White clover small leaved (*Trifolium repens*), White clover medium/large leaved (*Trifolium repens*)**, *Lotus corniculatus*, Sainfoin (*Onobrychis vicifolia*), Balansa clover (*Trifolium michelianum*), Persian clover (*Trifolium resupinatum*), Arrowleaf clover (*Trifolium vesiculosum*), Subterranean clover (*Trifolium subterraneum*), Strawberry clover (*Trifolium fragiferum*), Vetch (*Vicia sativa*)
	Herb	**Chicory (*Cichorium intybus*), Plantain (*Plantago lanceolata*)**, Sheep's Burnett (*Sanguisorba minor*)
Diverse pasture (dairy farmlets)	Grass	**Diploid perennial ryegrass (*Lolium perenne*), Tetraploid perennial ryegrass (*Lolium perenne*), Meadow fescue (*Lolium pratense*), Cocksfoot (*Dactylis glomerata*)**, Timothy (*Phleum pratense*), Tall Fescue (*Lolium arundinaceum*), Phalaris (*Phalaris aquatica*), Prairie grass (*Bromus catharticus*)
	Legume	**Red clover (*Trifolium pratense*), White clover large leaved (*Trifolium repens*), White clover medium/large leaved (*Trifolium repens*)**, Balansa clover (*Trifolium michelianum*), Persian clover (*Trifolium resupinatum*), Crimson clover (*Trifolium incarnatum*), Vetch (*Vicia sativa*), Lucerne (*Medicago sativa*)
	Herb	**Chicory (*Cichorium intybus*), Plantain (*Plantago lanceolata*)**

Regenerative management was defined by having longer grazing intervals and higher post-grazing residuals compared to contemporary management (Table 3.2) and minimal mineral fertiliser and agrichemical use. Minimal use meant that in exceptional circumstances mineral fertiliser and agrichemicals could be used on regeneratively managed farmlets. Synthetic N fertiliser could be applied at typically lower rates than contemporary, if the pasture was N limited and there was a feed shortage, to ensure there was sufficient food for the stock due to animal welfare concerns. Weeds were controlled mechanically, through chipping or mowing, however spot spraying can be used if these methods fail to keep the weeds under control. At the time of the study, these circumstances had not occurred since establishing the farmlets, and therefore no agrichemicals had been used on regeneratively managed farmlets. In the first and second year after establishment one half and one quarter, respectively, the usual rate of N fertiliser were applied to regenerative farmlets to gradually accustom the pasture to reduced N inputs, however, no additional mineral fertilisers were used. Fish hydrolysate, seaweed, lime flour and amino acids have been applied on the regenerative farmlets. Although there was no target pre-grazing pasture cover on the regenerative treatment, this often occurs due to the longer rotation lengths and higher post grazing residuals (Table 3.2). On the dairy farm, target average pasture cover on balance day (when pasture growth rates match the feed demand of grazing live stock in spring) was 1800 - 2200 kg DM ha^{-1} under contemporary management while it was 3000 kg DM ha^{-1} under regenerative management. Also, during spring and summer, paddocks with pasture cover above 3000 - 3600 kg DM ha^{-1} were cut for baleage under contemporary management while only paddocks over 4700 kg DM ha^{-1} were cut under regenerative management. This illustrates that an objective of regenerative management was to have higher pasture covers before grazing and more pasture was left following grazing. Stocking rates were similar across all treatments, although did differ slightly in some farming seasons in response to anticipated pasture growth and feed supply (Table 3.3).

Table 3.2: Grazing rotation under each management type on the sheep and the dairy farmlets. Post grazing residual for the sheep farmlets are minimum post grazing residuals while on the dairy farmlets they are the target post grazing residuals.

		Contemporary		Regenerative	
Farmlet system	Season	Rotation length (days)	Post-grazing residual (kg DM ha^{-1})	Rotation length (days)	Post-grazing residual (kg DM ha^{-1})
Sheep	Spring	12-24	1400	24-48	1400
	Summer	30-40	800-900	50-60	1100-1200
	Autumn	50-70	700-800	80-100	700-800
	Winter	60-75	900-1200	80-100	900-1200
Dairy	Spring	20-30	1500-1800	40-50	2000-2200
	Summer	30-40	1700-1800	50-60	2200-2400
	Autumn	40-60	1500-1800	60-80	2000-2200
	Winter	40-60	1300	60-80	1300

Therefore, four possible treatments exist, consisting of all combinations of the levels of the factors described above. They are: standard pasture under contemporary management (Std-Con), standard pasture under regenerative management (Std-Reg), diverse pasture under contemporary management (Div-Con) and diverse pasture under regenerative management (Div-Reg). Each farmlet contains just one of these four treatments, with the different treatments applied between the farmlets. Note that while all four treatments are present on the sheep farmlets (Table 3.4), on the dairy farmlets the Std-Reg treatment was not implemented (Table 3.4) due to funding constraints and industry priorities. The treatment that contains a combination of standard pasture and contemporary management (Std-Con) imitates the current standard farm system used in New Zealand and therefore acts as the control treatment. These treatments are part of the Whenua Haumanu research program. The current study is a component of the Whenua Haumanu research program, specifically investigating soil biology.

Table 3.3: Stocking rate of each treatment across both the sheep and the dairy farmlets each farming season since the farmlets were established. Stocking rate on the sheep farmlets is in ewes ha^{-1}, while on the dairy farmlets it is in cows ha^{-1}.

Farmlet system	Treatment	Stocking rate (stock ha^{-1}) in the season starting: 2022	2023	2024
Sheep (ewes ha^{-1})	Std-Con	15.0	14.0	14.0
	Std-Reg	15.0	12.0	14.0
	Div-Con	15.0	13.0	14.0
	Div-Reg	15.0	12.0	14.0
Dairy (cows ha^{-1})	Std-Con	2.0	2.5	2.5
	Div-Con	2.0	2.5	2.0
	Div-Reg	2.0	2.5	2.0

Table 3.4: Treatments present on the experimental sites, indicating the pasture type and management type they contain, and whether they are present on either of the two farmlet systems.

Treatment	Pasture type	Management type	Sheep farmlets	Dairy farmlets
Std-Con	Standard	Contemporary	Present	Present
Std-Reg	Standard	Regenerative	Present	Absent
Div-Con	Diverse	Contemporary	Present	Present
Div-Reg	Diverse	Regenerative	Present	Present

3.1.1 Sheep farmlets

The sheep farmlets are located 5.3km south east of Palmerston North, New Zealand (40°23'20.1"S 175°36'40.8"E), and have an elevation of 53 m above sea level. The long term average monthly soil temperatures at 10cm depth (2002- 2024) ranged from 7.4 ^{o}C in July to 18.9 ^{o}C in January, and the long term average annual rainfall (2002 - 2023) was 977 mm (Earth Sciences New Zealand, 2023, Weather station 21963, 40°22'48.0"S 175°36'36.0"E). As this weather station is not situated on the same soil type as is present on the sheep farmlets, the soil temperatures may vary slightly. However, this is the closest weather station to the study site and will therefore provide an indication of the likely soil temperature range expected. The farmlets are on the Tokomaru marine terrace. Only one soil type is present on the sheep farmlets site. The soil type is Tokomaru silt loam (Smap sibling name Mairaki_33a.1), with a soil classification of argillic-fragic perch-gley pallic soil (Landcare Research, 2025). Figure 3.1 shows a map of the sheep farmlets site.

All paddocks on the sheep farmlets were sampled for soil chemical analysis on 23 May 2024 to a depth of 7.5 cm. For each paddock, 20 cores were taken on a diagonal transect across the paddock and bulked together before being air dried at 40 ^{o}C and then ground and sieved to <2 mm. The samples were commercially analysed at Hill Laboratory, Hamilton, New Zealand, using their standard protocols. A brief overview of each method is provided here. pH was determined in a 1:2 (v/v) soil:water slurry. Olsen P was determined using Olsen extraction followed by molybdenum blue colorimetry. Total P was measured using a nitric/hydrochloric digestion followed by inductively coupled plasma optical emission spectrometry (ICP-OES). Extractable organic sulphur was extracted in 0.02 M potassium phosphate and measured by near infrared spectroscopy (NIR). Total N was measured by Dumas combustion. Potassium was extracted in 1 M neutral ammonium acetate and measured with ICP-OES. Cation exchange capacity (CEC) was determined by the summation of extractable cations (K, Ca, Mg, Na), measured by

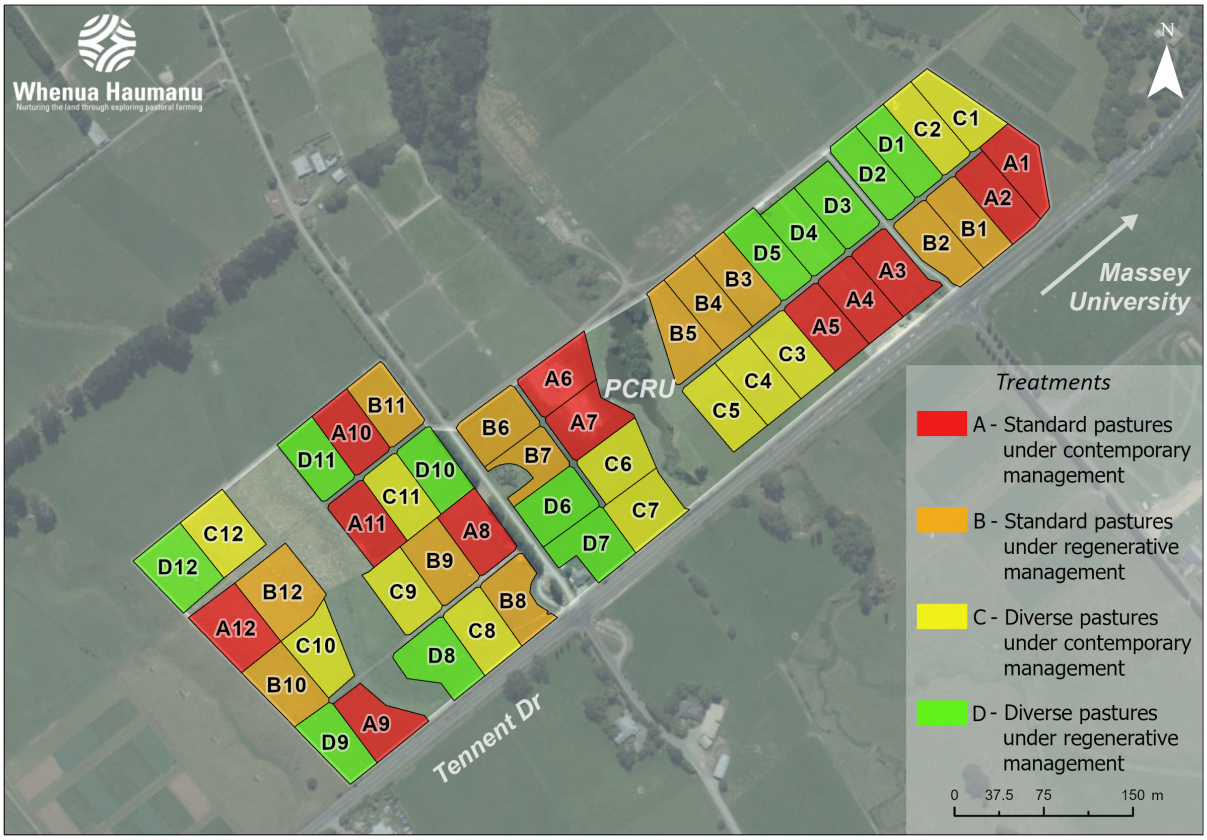

Figure 3.1: Map of the sheep farmlets site. Different colours indicate the different farmlets. On each farmlet a separate treatment was applied. Each farmlet was self contained and independent of the other farmlets.

Table 3.5: Soil chemical fertility averaged across each treatment on the sheep farmlets, measured in May 2024 (0-7.5 cm depth).

Soil series	Treatment	pH	Olsen P	Total P	Org-S[1]	Total N	K	CEC[2]	Base[3]	HWEC[4]
			(mg/l)	(mg/kg)	(mg/kg)	(%)	(me/100g)	(me/100g)	(%)	(mg/kg)
Tokomaru	Std-Con	6.0	30.8	691.2	7.8	0.3	0.5	14.0	72.5	2074.2
	Std-Reg	6.2	38.2	720.8	8.5	0.3	0.6	14.5	75.5	1988.2
	Div-Con	6.3	34.8	769.5	8.5	0.3	0.7	14.8	80.0	2067.2
	Div-Reg	6.2	40.8	731.8	8.0	0.3	0.6	14.2	76.8	1833.8

[1]Org-S = Extractable organic sulphur, [2]CEC = Cation Exchange Capacity, [3]Base = Total base saturation, [4]HWEC = Hot water extractable carbon;

P = Phosphorus, S = Sulphur, K = Potassium, N = Nitrogen, pH measured in water

extraction in 1 M neutral ammonium acetate and detection with ICP-OES, and extractable acidity. Total base saturation was calculated from extractable cations and CEC. Hot water extractable carbon was measured by extracting the sample at 80 ^{o}C for 16 hours followed by infra red spectroscopy (IR) detection. Table 3.5 shows the average fertility for paddocks in each treatment included in this study.

3.1.2 Dairy farmlets

The dairy farmlets are located on the southern bank of the Manawatu river (40°22’31”S 175°36’58”E), bordering the city of Palmerston North, New Zealand. It is 33m above sea level. The long term average monthly soil temperature at 10cm depth (2002- 2024) ranged from 7.4^{o}C in July to 18.9^{o}C in January and the long term average annual rainfall (2002 - 2023) was 977 mm (Earth Sciences New Zealand, 2023, Weather station 21963, 40°22’48.0”S 175°36’36.0”E). As the dairy farmlets are located on the banks of the Manawatu river, a large portion of the farmlets are in the river flood plain and are at risk of frequent flooding. This caused regular deposits of alluvial sediment, with the last major deposit being in 2004. Multiple soil types are present on the dairy farmlet

site (Figure 3.2). Samples for the current study were collected from only two of the soil types. The soil types of the sampled paddocks are Manawatu sandy loam (Smap sibling name Awatere_24a.1) and Rangitikei loamy sand (Smap sibling name Shalimah_8a.1). The classification of these soils is typic fluvial recent soil and fluvial raw soil, respectively (Landcare Research, 2025). Figure 3.3 shows a map of the dairy farmlets site.

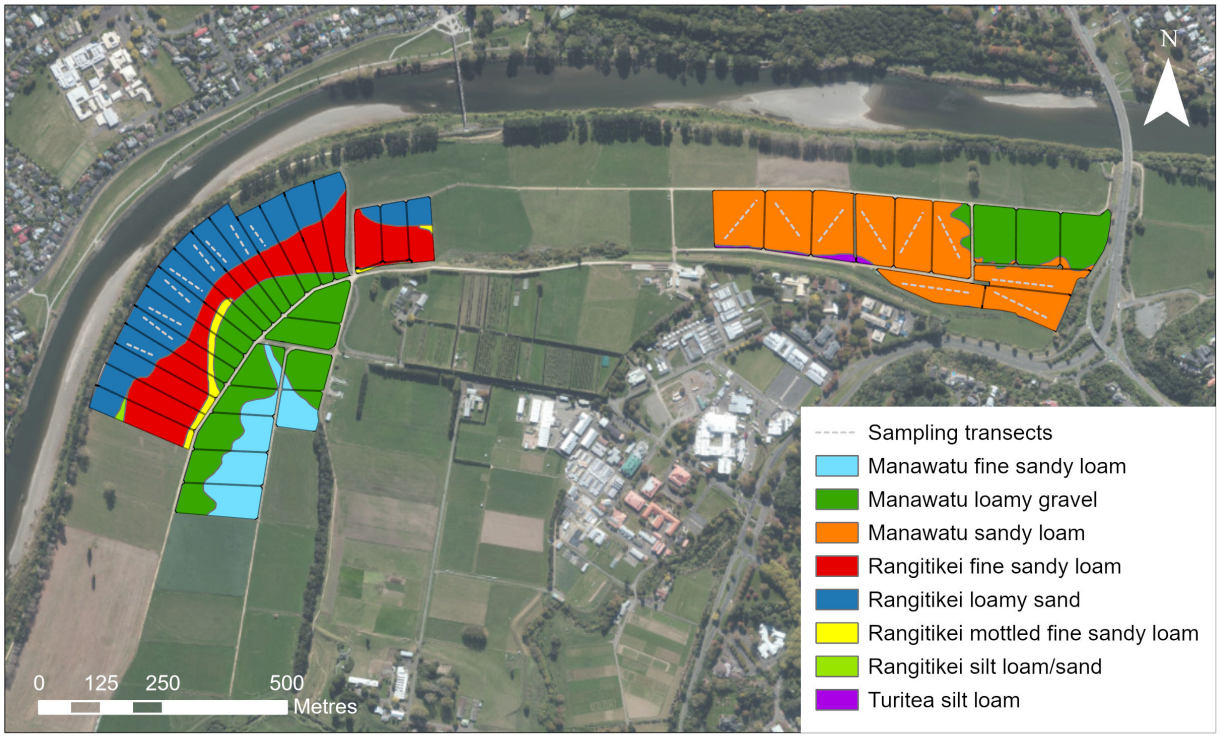

Figure 3.2: Map of the soil types present on the dairy farmlets site. For the current study, samples were only collected from the paddocks with the Manawatu sandy loam soil (orange), and the portion of the paddocks with the Rangitikei loamy sand (dark blue), as shown by the dashed sampling transects.

All paddocks on the dairy farmlets were sampled for soil chemical analysis on 23 May 2024 as described in section 3.1.1 for the sheep farmlets. The samples were commercially analysed at Hill Laboratory, Hamilton, New Zealand, using their standard protocols. See section 3.1.1 for an overview of the methods. Table 3.6 shows the average fertility for each treatment in the paddocks included in the current study on the dairy farmlets.

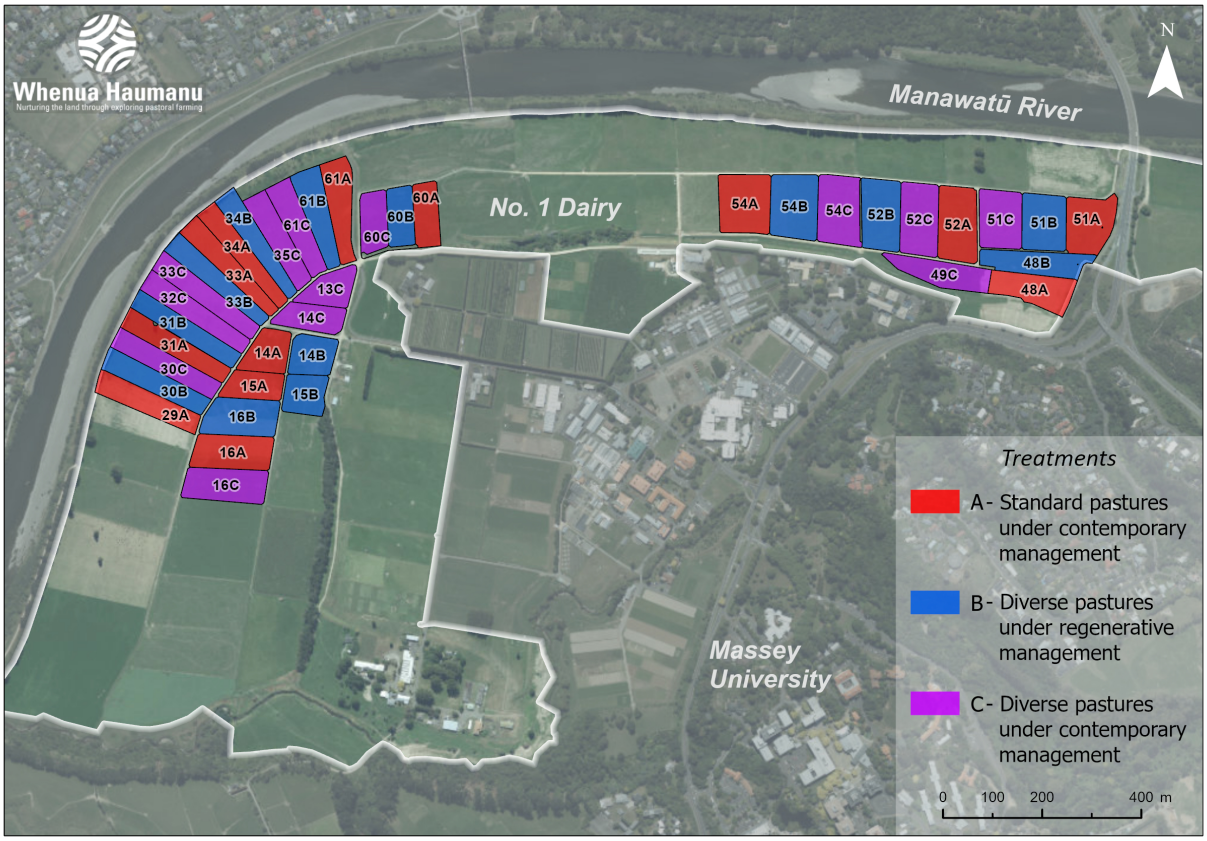

Figure 3.3: Map of the dairy farmlets site showing the different farmlets. Different colours indicate the different farmlets. On each farmlet a separate treatment was applied. Each farmlet was self contained and independent of the other farmlets.

Table 3.6: Soil chemical fertility averaged across each treatment and soil type on the dairy farmlets, measured in May 2024 (0-7.5 cm depth).

Soil series	Treatment	pH	Olsen P (mg/l)	Total P (mg/kg)	Org-S[1] (mg/kg)	Total N (%)	K (me/100g)	CEC[2] (me/100g)	Base[3] (%)	HWEC[4] (mg/kg)
Manawatu	Std-Con	5.9	29.3	734.0	5.3	0.2	0.5	12.0	74.7	1536.3
	Div-Con	5.8	39.0	793.0	5.3	0.3	0.6	13.0	72.3	1803.3
	Div-Reg	6.2	40.3	804.3	4.7	0.2	1.0	12.7	76.7	1728.0
Rangitikei	Std-Con	6.3	33.3	681.0	4.7	0.2	0.4	11.7	82.7	1315.0
	Div-Con	6.3	40.7	750.3	5.0	0.2	0.6	11.3	84.0	1701.3
	Div-Reg	6.2	38.7	701.3	4.7	0.2	0.7	11.3	81.7	1404.0

[1]Org-S = Extractable organic sulphur, [2]CEC = Cation Exchange Capacity, [3]Base = Total base saturation, [4]HWEC = Hot water extractable carbon;

P = Phosphorus, S = Sulphur, K = Potassium, N = Nitrogen, pH measured in water

3.2 Soil biological sampling

Soil samples for analysis in the current study were taken using a standard soil corer to 7.5cm depth. In each paddock sampled, 20 soil cores were taken following a transect diagonally across the paddock, adhering to best practice soil sampling guidelines. In each paddock a single bulked soil sample was taken. When multiple soil types were present in one paddock, sampling was restricted to the area of the paddock containing just the soil type being sampled. A subset of the paddock present in the Whenua Haumanu farmlets were sampled, as shown in Table 3.7. An aluminium plate was placed on the tread of the corer before taking the core to prevent the sole of the boot touching the top of the soil core and contaminating it. A glove was worn on the hand used to remove the soil cores from the corer to prevent contamination of the sample with the skin microbiome. Between paddocks, the aluminium plate and soil corer were wiped down with a cloth and immersed in 70% ethanol for 30 seconds and a fresh glove was put on. The first core taken in the new paddock was discarded to prevent excess ethanol from the corer entering the sample. At the beginning, middle and end of each transect, the soil temperature in the top 10 cm of the soil was measured with a digital soil thermometer. Samples were taken back to the lab and processed immediately (within 3 hours of being sampled), to prepare the soil for further processing. See subsequent sections for details.

As some of the methods used in this study needed to be carried out on fresh soil samples and only a limited number of samples could be processed within day, it was not possible to collect all samples on the same day. Therefore, a subset of the paddocks to be sampled were sampled on consecutive days, ensuring one paddock of each treatment was sampled on each sampling date (Table 3.7). Samples were collected during similar morning hours across the different sampling days. The treatments sampled on the same day were selected to group together paddocks that had the same soil type and that are physically adjacent to each other on the farmlets, and were therefore subject to

Table 3.7: The date that each paddock was sampled, showing that on each sampling date one paddock of each treatment was sampled, with replicate treatments sampled across different dates. The dairy farmlets do not contain the Std-Reg treatment.

		Paddocks sampled				
Farmlet system	Sampling date	Std-Con	Std-Reg	Div-Con	Div-Reg	Soil series
Dairy	19/11/2024	48A	-	49C	48B	Manawatu
	20/11/2024	52A	-	52C	52B	
	21/11/2024	54A	-	54C	54B	
	22/11/2024	31A	-	32C	31B	Rangitikei
	23/11/2024	33A	-	33C	33B	
	24/11/2024	34A	-	35C	34B	
Sheep	09/12/2024	A2	B1	C2	D2	Tokomaru
	11/12/2024	A10	B11	C11	D11	
	16/12/2024	A4	B3	C4	D5	
	17/12/2024	A8	B9	C9	D10	

similar conditions and grazing rotations throughout the year. This allowed the data to be analysed as an unreplicated complete block design, as each block (sampling date) contained one replicate of all possible treatments, with replicate treatments sampled on different days. Therefore, any differences in conditions between sampling dates and location on the farmlets could be accounted for in the statistical analysis. However, on the dairy farmlets, this also resulted in samples on the two different soil types being sampled on separate days, causing soil type to be fully confounded with the sampling date (Table 3.7). On the sheep farmlets, four replicates of each treatment were sampled, while on the dairy farmlets six replicates of each treatment were sampled, with three replicates in each of the two soil types (Table 3.7).

3.3 Typesetting and statistical software

This thesis text was produced using the bookdown v0.43 (Xie, 2015/2025) and knitr v1.50 (Xie, 2025) R packages, with references and citations generated using the rbbt v0.0.0.9000 R package (Dunnington & Wiernik, 2018/2025). Tables were produced using the flextable v0.9.9 (Gohel & Skintzos, 2025) and ftExtra v0.6.4 (Yasumoto, 2024) R packages. Graphs were produced using the ggplot2 v3.5.2 R package (Wickham, 2016). All statistical analysis was performed using R Statistical Software v4.5.0 (R Core Team, 2025), using RStudio v2025.5.0.496 (Posit team, 2025). Data manipulation was performed using the tidyverse v2.0.0 R package (Wickham et al., 2019). For further details of statistical analysis, please see individual chapters.

CHAPTER 4: ABIOTIC CONDITIONS

4.1 Introduction

Abiotic conditions have a strong influence on soil biota. Three major factors are soil pH, soil temperature and soil moisture (Banerjee & van der Heijden, 2023; Siebert et al., 2023). As soil moisture and temperature fluctuate on a day to day time scale, and can quickly change depending on weather and climate, accounting for these factors in soil biology studies is important.

4.2 Methods

4.2.1 Data collection

Daily climate data was measured on both the dairy farmlet (40°22'35.5"S 175°37'15.7"E, 27.74 m above sea level, located on the Manawatu sandy/silty loam) and on the sheep farmlet (40°23'21.1"S 175°36'36.7"E, 56.62 m above sea level, located on the Tokomaru silt loam). Both weather stations used a Campbell Scientific CR1000X Datalogger, OS Version 8.01 (26-09-2024). Rainfall (mm) was measured using a Texas Electronics TR-525W2 200 mm diameter 0.2 mm tipping bucket, air temperature (^{o}C) was measured using a Vaisala HMP60 sensor. Soil temperature (^{o}C) was measured at 150 mm depth using a Campbell Scientific 107L sensor on the dairy farmlets, but was not available for the sheep farmlets. The actual soil temperature, at the time of sampling, was measured for each paddock at three points along the sampling transect, as described in section 3.2.

4.2.2 Soil moisture content

Soil samples were collected as described in section 3.2. Soil moisture content was determined for each sample by placing ~10 g of soil into a pre-weighed glass beaker, recording the exact mass of the soil used. This was then dried at 105 ^{o}C for 24 hours, after which the beaker and soil were weighed again to determine the mass lost as water. From this, the gravimetric water content was calculated as shown in equation 4.1.

$$\text{Gravimetric water content} = \frac{\text{Soil wet mass} + \text{Beaker mass} - \text{Beaker and Soil dry mass}}{\text{Soil wet mass}} \tag{4.1}$$

4.2.3 Statistical analysis

The three soil temperature measures from each paddock were averaged and the model was fitted on the average temperature. There was only one measure of soil moisture for each sample, on which the model was fitted. Statistical analysis was carried out by fitting an analysis of variance (ANOVA) model using the aov() function from the stats v4.5.0 R package (R Core Team, 2025). This compares the means of the treatment levels and determines if there are any significant differences with an F-test. Sampling date, as a factor variable, was included in each model as a blocking variable to account for differences caused by sampling on different days. This also takes into account soil type differences on the dairy farmlets as the different soil types were sampled on different days. Therefore, no soil type variable was included. Treatment was included as a single factor with four levels. The models were fitted separately for the sheep and the dairy farmlets. If the ANOVA indicated that there was a significant difference in treatment levels, a pairwise comparison of the treatment levels was carried out using the TukeyHSD() function from the stats v4.5.0

R package (R Core Team, 2025).

4.3 Results

4.3.1 Climatic conditions

There was consistent rainfall in the month leading up to and during the sampling time for both the sheep and the dairy farmlets (Figure 4.1). Note that rainfall on December 9 occurred early in the morning, before sampling on the sheep farmlets was carried out. Soil and air temperature fluctuated daily, however remained fairly consistent across the whole sampling period (Figures 4.2 and 4.3).

4.3.2 Soil temperature of sampled paddocks

On both the sheep and the dairy farmlets, soil temperature differed significantly between treatments ($F_{3,9}$ = 8.54, p = .005 and $F_{2,10}$ = 5.10, p = .03, for the sheep and the dairy farmlets, respectively). The same trend is observed on both the sheep and dairy farmlets, with both diverse pasture and regenerative management causing additive decreases in the soil temperature (Figures 4.4 and 4.5). Figure 4.4 shows differences in soil temperature at a significance level of p<.1, however only the difference between the Std-Con and the Div-Con did not differ significantly at the p<.05 level (p = .09), with the difference between Div-Reg and both Std-Con and Std-Reg being significant at p<.05 (p = .004 and p = .04, respectively). Diverse pastures had lower soil temperatures than standard pastures, and regenerative management further decreased soil temperature compared to contemporary management. Therefore, the Std-Con treatment had the highest and the Div-Reg treatment the lowest average soil temperature on both the sheep and the dairy farmlets, with the other treatments having intermediate soil temperatures.

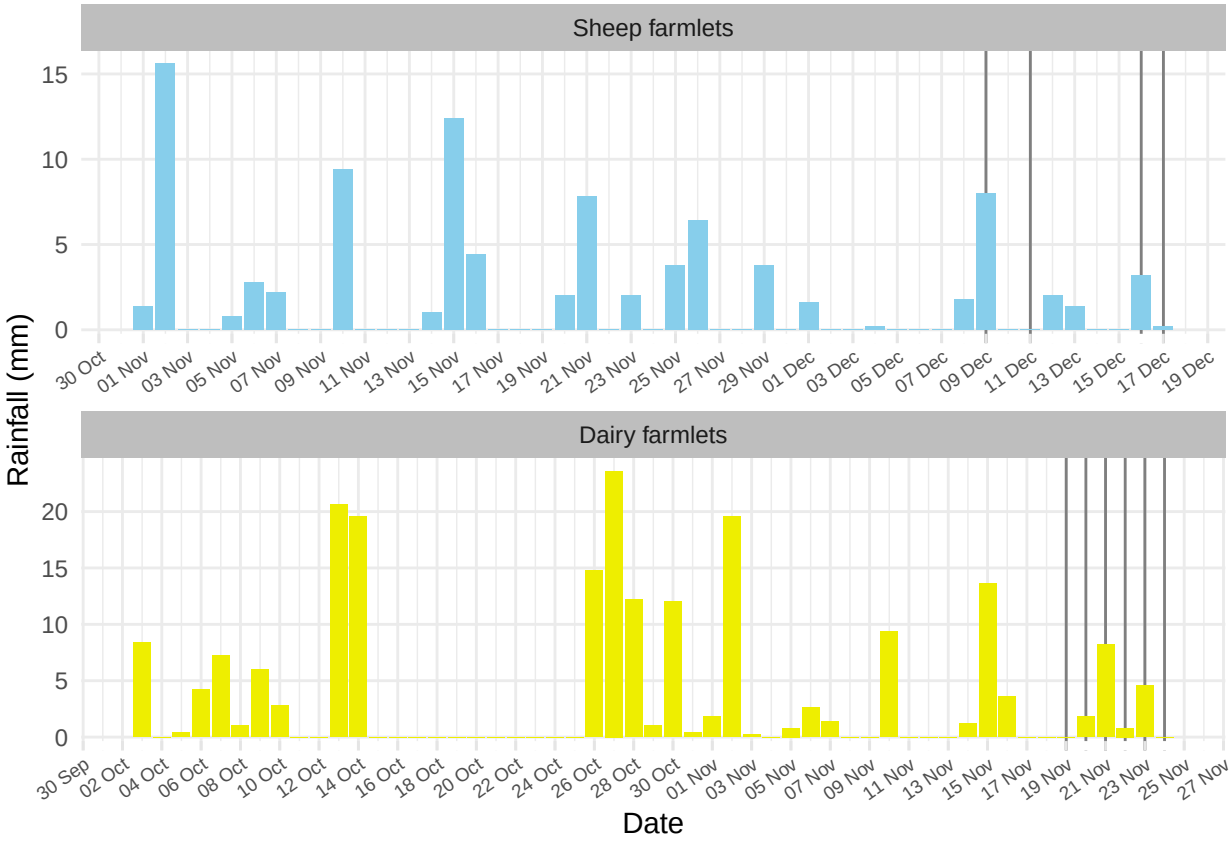

Figure 4.1: Daily rainfall on the sheep and dairy farmlets during the month leading up to the sampling period, in 2024. The vertical lines indicate the days on which soil samples were collected.

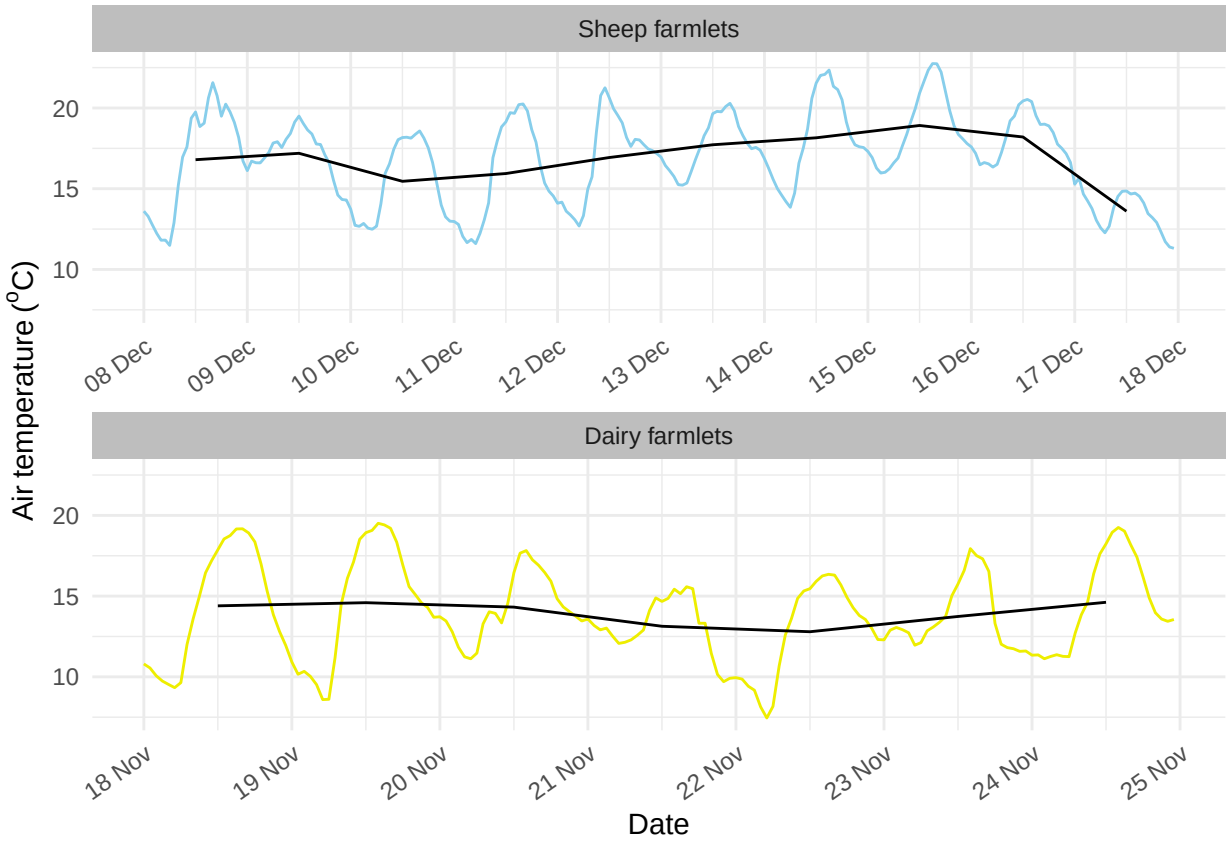

Figure 4.2: Air temperature on the sheep and dairy farmlets during the sampling period, in 2024. The black line shows the daily average air temperature.

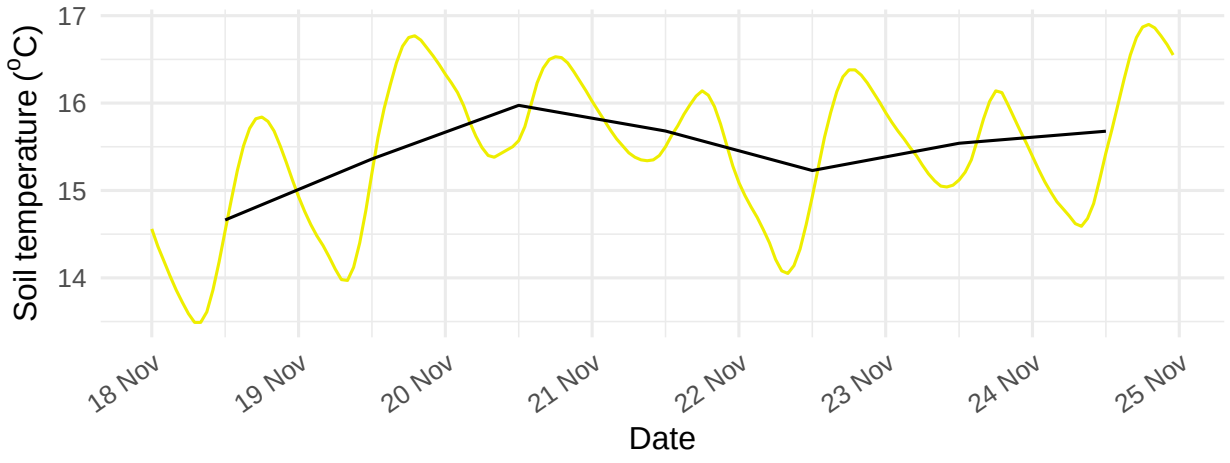

Figure 4.3: Soil temperature to 150 mm depth on the dairy farmlets during the sampling period, in 2024. The black line shows the daily average soil temperature. No daily soil temperature data was available for the sheep farmlets.

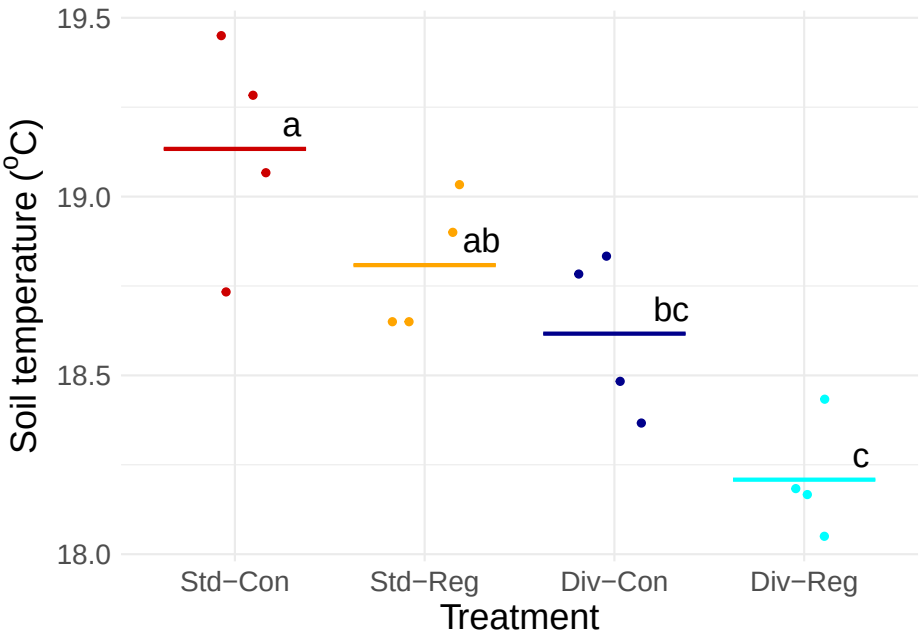

Figure 4.4: Average soil temperature to 100 mm depth at the time of sampling, across the different treatments on the sheep farmlets, after adjusting for differences between sampling day. Letters indicate significant differences at p<.1. Of the differences shown, only the Std-Con and Div-Con did not differ significantly at p<.05.

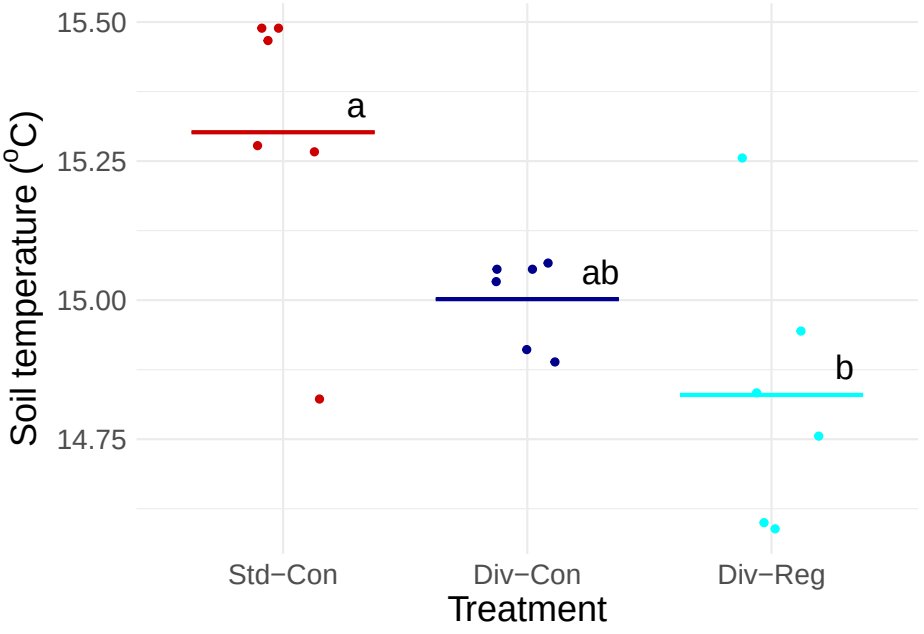

Figure 4.5: Average soil temperature to 100 mm depth at the time of sampling, across the different treatments on the dairy farmlets, after adjusting for differences between sampling day. Letters indicate significant differences at p<.05.

4.3.3 Soil moisture of sampled paddocks

There was no significant difference in soil moisture between treatments on either the sheep or the dairy farmlets ($F_{3,9}$ = 1.24, p = .35 and $F_{2,10}$ = 0.42, p = .67, respectively). However, when considering the soil types on the dairy farmlets separately, the soil moisture between treatments on the Manawatu sandy loam did differ significantly ($F_{2,4}$ = 15.07, p = .01) while on the Rangitikei loamy sand there was no difference between treatments ($F_{2,4}$ = 2.13, p = .23). On the Manawatu sandy loam soil on the dairy farmlets, the Div-Reg had significantly higher soil moisture than the Div-Con treatment (p = .01), and although not significant at p<.05, the Div-Con treatment also had lower soil moisture than the Std-Con treatment (p = .09) (Figure 4.6). Despite there being no difference in soil moisture between treatments on the sheep farmlets, Figure 4.7 shows that the soil moisture did vary between sampling days.

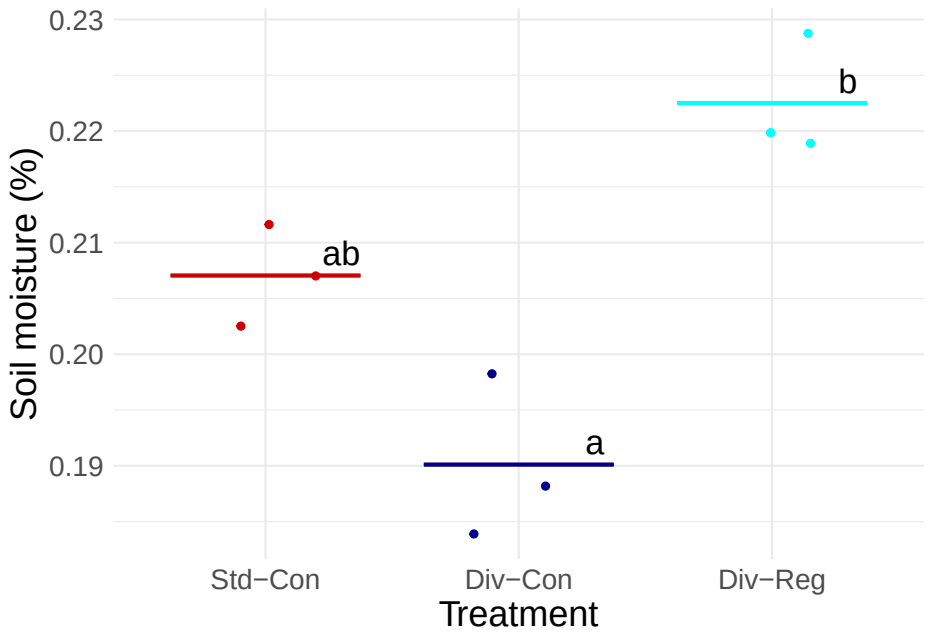

Figure 4.6: Average soil moisture to 75 mm depth at the time of sampling, across the different treatments on the Manawatu fine sandy loam soil on the dairy farmlets, after adjusting for differences between sampling day. Letters indicate significant differences at p<.05.

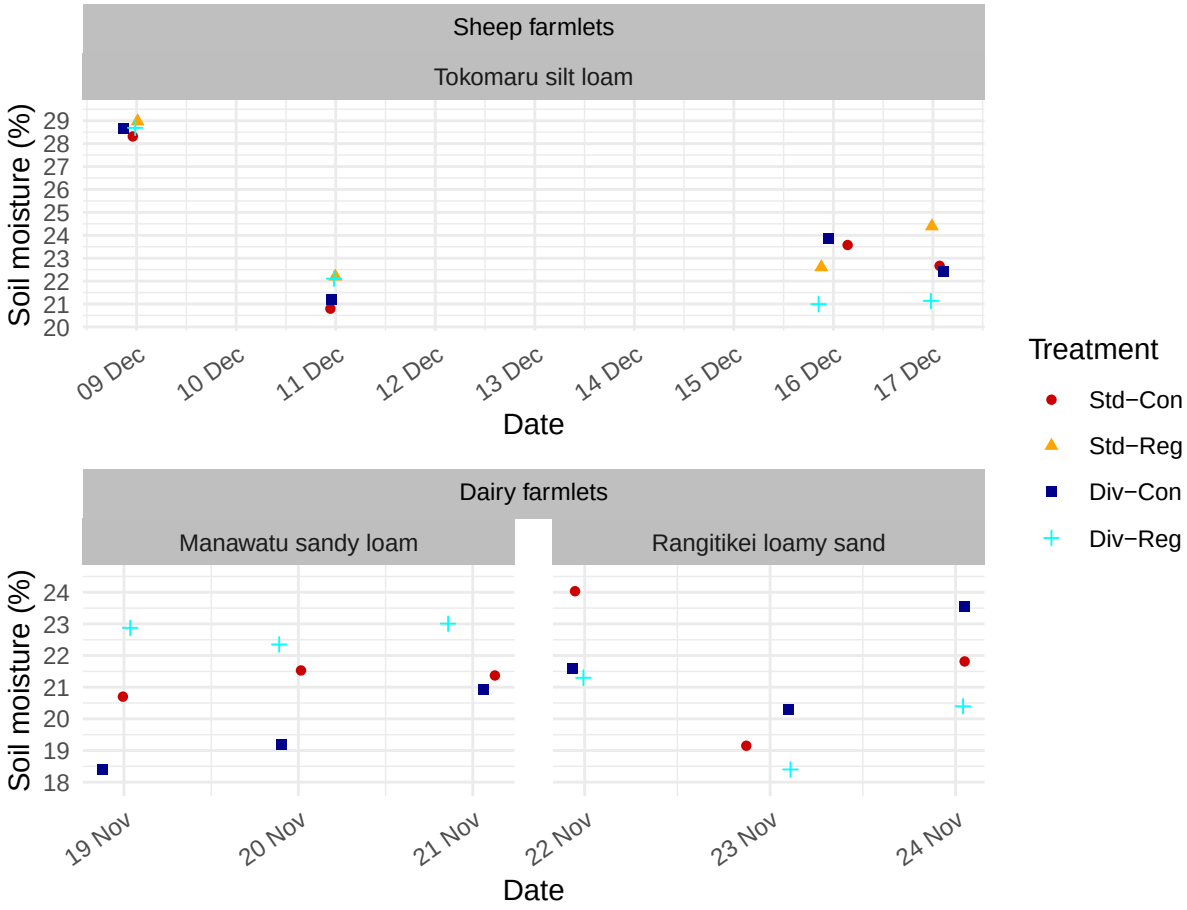

Figure 4.7: Soil moisture of each sample to 75 mm depth across sampling dates for both the sheep and the dairy farmlets, in 2024.

4.4 Discussion

In New Zealand, the wet season occurs in winter (June - September), with the soils drying out as summer approaches in December - March. Therefore, the intention was to collect samples for this study in late winter to early spring (July - September), to avoid the effects that low soil moisture can have on biological activity. However, delays in the arrival of equipment meant that samples could not be collected until November - December. During this time of the year, soil moisture is decreasing heading into summer and is often already low. However, the consistent, seasonally late rainfall that occurred in 2024 during the months leading up to the sampling period meant that soil moisture remained relatively high, allowing late sampling to be carried out with minimal effect of low soil moisture on biological activity.

Average soil and air temperature were relatively constant during the sampling period, with daily fluctuations corresponding to day - night cycles. However, over the long term, temperatures would be increasing from their minimum in July to their maximum in January (Earth Sciences New Zealand, 2023). As all samples were collected during similar morning hours across the sampling days, the daily temperature fluctuations should have minimal effects on the results.

On both the sheep and the dairy farmlets there was a difference in soil temperature between treatments, with the Std-Con treatment having the highest temperature and the Div-Reg having the lowest. The almost linear decrease in soil temperature with treatment seen in Figures 4.4 and 4.5 could be interpreted as an additive effect of reducing soil temperature by both diverse pastures and regenerative management, with the pasture type having a greater effect. Samples were taken in November and December (southern hemisphere late spring to early summer) meaning average soil temperatures are increasing (Earth Sciences New Zealand, 2023). This means that these results could indicate that standard pasture and contemporary management cause the soil to warm up

faster, or conversely that diverse pasture and regenerative management insulate the soil more and thereby buffer it from rapid temperature change. Higher plant density and leaf area in diverse pastures has been correlated to increased soil moisture due to shading from the plants (Lange et al., 2014). It is possible the same mechanism causes lower soil temperature under diverse pasture. Similarly, higher grazing intensity could reduce plant cover which in turn would affect soil abiotic conditions such as soil moisture and temperature (Hu et al., 2015). Therefore, increased soil shading by higher plant density in diverse pasture and lower grazing pressure under regenerative management could be the cause for lower soil temperatures in these treatments. Combined with the fact that the soil temperatures are increasing, this would indicate that these have a greater ability to buffer the soil from temperature changes. However, to confirm this, a study where repeated soil temperature measurements are taken throughout the year would be required, and the data compared to plant density and pasture cover. If diverse pasture and regenerative management do buffer the soil from temperature change, the opposite trend would be expected as the season changes from summer to winter, as the warm soils would cool down slower in these treatments. A short term-experiment, where soil temperature is measured regularly in a 24 hour period could also provide evidence for this hypothesis.

The mechanism proposed to explain differences in soil temperature between treatments implies a similar effect would occur for soil moisture. However, the evidence for this from the results of the current study is less strong. This could be due to the overriding effect rainfall has on soil moisture conditions. For example, the high soil moisture observed in Figure 4.7 on the December 9 on the sheep farmlets corresponds with large amount of rainfall during the night preceding the sampling day, shown in Figure 4.1. On the Manawatu sandy loam soil, the treatments did have a significant effect on soil moisture, with the Div-Reg treatment having the highest soil moisture. This would support with the hypothesis that diverse pasture and regenerative management buffers soil moisture and

temperature due to increased shading of the soil, as soils are expected to be drying out at this time of the year. However, as not all of the treatment combinations are present in the dairy farmlets, this effect cannot be confirmed across both management type and pasture type. The reasons that only the Manawatu sandy loam showed significant differences in soil moisture could be due to differences in soil texture between the soil types. The Manawatu sandy loam has the coarsest soil texture (in contrast with Rangitikei loamy sand and Tokomaru silt loam) and therefore is expected to drain fastest and dry out quicker. This would result in any moisture buffering effects to become apparent sooner on this soil, as the other soils retain water longer and therefore still contain excess water at the time of sampling from the last rainfall event (often less than 2 days - see Figure 4.1). Therefore, it is possible that the treatments do have an effect on soil moisture in these soils, however they have retained sufficient water from the previous rainfall that this is not apparent in the current study. To confirm this, a study that takes repeated measures of soil moisture across the soil types for a few weeks following a rainfall event, or as soils dry out over summer, would be required.

While there is some evidence to suggest that the treatments affect soil moisture and temperature, conclusions drawn from the results of the current study could be an over interpretation of limited data. The effects of the treatments on soil abiotic factors were not a primary objective of this study and this was only measured to account for the potential influence it could have on the soil biota measurements. Therefore, the soil moisture and temperature were not extensively sampled. Further studies with more replications of these measurements and over multiple sampling times would be required to confirm if the treatments have an effect on soil moisture and temperature and, if so, determine the mechanisms.

CHAPTER 5: PHYSIOLOGICAL STATE OF SOIL BACTERIA

5.1 Introduction

Microbes in the soil exist in four physiological states: active, potentially active, dormant and dead (Blagodatskaya & Kuzyakov, 2013). The active pool consists of the small portion of microbes that are metabolising substrates, grow and reproducing (Blagodatskaya & Kuzyakov, 2013). It is this pool of microbes that carry out the microbial driven soil processes and therefore have a strong influence on the functions performed by the soil microbiome. The potentially active state consists of microbes that are not currently active but can quickly (within hours) switch to an active state following substrate input or environmental change (Blagodatskaya & Kuzyakov, 2013). Microbes in this state contribute to microbial driven processes when conditions change, allowing the soil microbial community to adjust quickly to disturbances. Microbes in the dormant state have strongly reduced physiological activity and persist for long periods utilising energy reserves with minimal respiration and endogenous metabolism (Blagodatskaya & Kuzyakov, 2013). This pool of microbes does not contribute to turnover processes. Dead microbes are in an irreversible state of no growth, reproduction or metabolism (Blagodatskaya & Kuzyakov, 2013). These do not actively contribute to biochemical processes but provide a pool of available substrate for other microbes and contribute to soil organic matter (SOM) (Blagodatskaya & Kuzyakov, 2013). As these different physiological states have different functions within the soil, it is important to distinguish the size of these pools when studying the soil microbiome, rather than just investigating total biomass or community composition. The relative size of the different physiological

pools can also inform the state of the soil conditions and the influence different factors have on it. As active microbes carry out the majority of soil processes, this pool is of particular interest, especially in agricultural systems.

The proportion of soil bacteria in different physiological states can be determined using fluorescent microscopy in combination with differential stains (dyes) that cause only specific physiological states to fluoresce (Blagodatskaya & Kuzyakov, 2013; Foght & Aislabie, 2005). By selecting the right combination of stains, the number of bacteria in all four physiological states can be enumerated. Stains that are taken up by all cells or indiscriminately bind to cell components can be used to enumerate total bacteria present (Babiuk & Paul, 1970; Blagodatskaya & Kuzyakov, 2013; Foght & Aislabie, 2005). Stains that bind to intracellular components but are unable to cross intact cell membranes can be used to count membrane destruct and dead cells (Blagodatskaya & Kuzyakov, 2013; Foght & Aislabie, 2005; Luna et al., 2002). Stains that do not fluoresce unless modified by a metabolically active cell, can be used to determine the number of active cells (Blagodatskaya & Kuzyakov, 2013; Créach et al., 2003; Lundgren, 1981). Potentially active cells can also be measured using these stains for active cells by first incubating the sample with nutrients, which will activate the potentially active cells and therefore allow them to be stained (Luna et al., 2002; Maraha et al., 2004). The number of potentially active cells is determined by taking the difference in active stained cells before and after the incubation. Dormant cells cannot be directly stained, however can be inferred by taking the difference of the total cells and the number of bacteria in the other physiological states.

This study investigates the effects that diverse pasture and regenerative agricultural practices implemented in New Zealand's pastoral farming system have on the physiological states of soil bacteria. It aims to determine whether any measurable effects occur in the short term, within two and a half years of transitioning to these practices. Regenerative agriculture is stated to improve soil biological conditions.

Therefore, the hypotheses being tested are that both diverse pasture and regenerative management, after two and a half years under these treatments, cause; 1) higher total number of bacteria, and 2) higher proportion of fluorescein diacetate (FDA) active bacteria. Note that increases in total bacteria or proportion of active bacteria is not necessarily a good indicator of improved soil health, despite the hypotheses presented here. See section 8.5.3 for further discussion on the challenges of defining soil biological health.

5.2 Methods

5.2.1 Soil processing

Soil samples were collected as described in section 3.2. Processing was carried out on the fresh soil samples within 3 hours of the samples being collected. Soil cores were thoroughly crushed by hand while still in the zip lock bag to break up the soil cores and aggregates, sieved to 4 mm then sieved again to 2 mm. The soil was then tipped onto a clean large plastic sheet and homogenised by lifting one corner of the sheet making the soil roll over to the opposite corner, doing the same for each corner of the sheet and repeating the whole process six times. All equipment used was washed with water and wiped down with 70% ethanol between samples.

5.2.2 Stains used for fluorescent microscopy

Fluorescein iso-thiocyanate (FITC) binds to cell proteins, and therefore indiscriminately stains all cells (Babiuk & Paul, 1970; Blagodatskaya & Kuzyakov, 2013). It was used to enumerate total bacteria present (Figure 5.1). Propidium iodide (PI) is unable to cross the membrane of an intact cell (Blagodatskaya & Kuzyakov, 2013; Luna et al., 2002; Maraha

et al., 2004), and therefore was used to stain dead, membrane destruct cells (Figure 5.1). Fluorescein diacetate (FDA) is a non-fluorescent molecule that only fluoresces after being hydrolysed to fluorescein after being taken up by an actively metabolising cell (Lundgren, 1981; Rotman & Papermaster, 1966). Therefore, it was used to stain active bacteria (Figure 5.1). FDA was also used to determine the number of potentially active bacteria following a short incubation with nutrients. The incubation causes potentially active bacteria to become active, and therefore be stained with FDA. The number of potentially active bacteria is the difference between the FDA stained bacteria before and after the incubation with nutrients (Figure 5.1). Although there is no stain to measure dormant bacteria, this can be inferred by taking the difference of the total (FITC stained) cells and the other physiological states (FDA after activation and PI) (Figure 5.1).

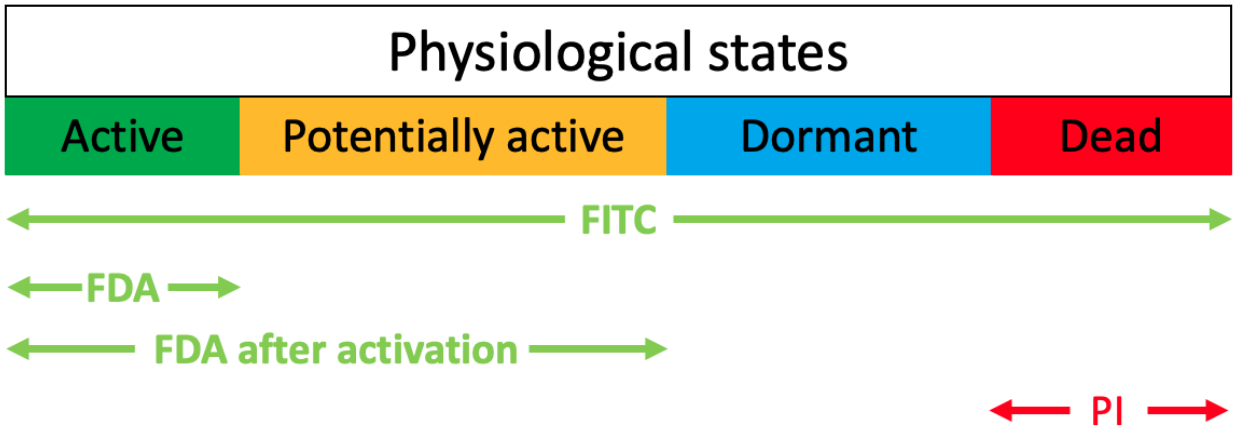

Figure 5.1: The bacteria in different physiological states that are stained and enumerated by the different staining procedures. All four physiological states can be determined by calculating the difference between certain stain counts. FITC = Fluorescein iso-thiocyanate, FDA = Fluorescein diacetate, PI = Propidium iodide.

5.2.3 Extraction and staining of soil bacteria

Soil bacteria were extracted, stained and prepared for microscopy using a modified membrane filtration method (Busse et al., 2009; Hobbie et al., 1977). The stains used differed depending on what was available. Activation of cells for determination

of potentially active cells used the method from Maraha et al. (2004). Extraction buffers, volumes and staining procedure were adapted to optimise staining and minimise background fluorescence. Approximately 1 g of soil was added to a sterile tube to which 9 ml of phosphate buffer (84% 0.1 M K_2HPO_4, 16% 0.1 M KH_2PO_4) was added. For each sample, two replicate extractions were carried out. Samples were placed on an end over end shaker for 10 minutes before being centrifuged for 5 minutes at 500 g.

Activated samples were prepared by adding 5 ml of the supernatant to a sterile tube along with 50 µl of 0.1% yeast extract (Maraha et al., 2004). This was incubated for five hours on an end over end shaker at room temperature. Following the activation, the samples were diluted 10 fold in phosphate buffer and stained and filtered as for the FDA and PI samples detailed below, omitting the PI stain to just use FDA.

All other slides were prepared by diluting the supernatant following centrifugation 10 and 100 fold by serially adding 1 ml of supernatant to 9 ml of phosphate buffer. From the 10 fold dilution, 1 ml was stained for 10 minutes in the dark with 0.5 ml FDA (0.005 mg ml^{-1} phosphate buffer) and 10 µl of PI (0.001 mg ml^{-1} deionised water). From the 100 fold dilution, 1 ml was stained for 30 minutes in the dark with 0.5 ml of FITC (0.4 mg ml^{-1} 10% 0.5 M $NaCO_3$, 45% 0.01 M K_2HPO_4, 45% 0.145 M NaCl (Babiuk & Paul, 1970)). Following staining, 2 ml of phosphate buffer was added to the sample and they were vortexed for 10 seconds before being passed through a 0.2 µm black polycarbonate filter using low vacuum. The FDA and PI stained samples were rinsed twice, first with 10 ml of 5.3% Na_2CO_3 then with 10 ml of 5% NaH_2PO_4. The FITC stained samples were rinsed three times, first with 10 ml of 5.3% Na_2CO_3 then twice with 10 ml of 5% $Na_4P_2O_7$. Following filtration and rinsing, the filters were placed on a glass slide with a drop of glycerol, covered with a coverslip and kept in the dark until imaging with the microscope. Each day a blank sample (containing just phosphate buffer) was prepared along with the samples for each stain, including for the activated samples.

5.2.4 Fluorescent microscopy and image analysis

Slides were observed at 400x magnification with an Olympus BX51 epifluorescent microscope, using the Olympus U-MWIBA2 colour filter (excitation filter 460-490 nm, emission filter 510-550 nm, dichromatic mirror 505 nm) for the FITC and FDA stains and the Olympus U-MWIG2 colour filter (excitation filter 520-550 nm, emission filter 580IF nm, dichromatic mirror 565 nm) for the PI stain. Three passes across the filter were made, taking three images at random points along the transect for the outside two passes and four images for the middle pass. The samples from the last two days of sampling the sheep farmlets were imaged with a Nikon eclipse Ni microscope at 100x magnification, taking just 5 images per slide, as the microscopy facility being used shut unexpectedly and this was the only one available on short notice. The images were analysed using the ImageJ software (version 1.54g, Java version 1.8.0_345) to count the number of bacteria present in each image.

5.2.5 Statistical analysis

Although bacteria extractions were carried out on field moist soils, all counts are reported relative to the equivalent oven dry mass of the soil, determined as described in section 4.2.2. Counts from replicate extractions were averaged, and the statistical analysis carried out on the average values. Statistical models were fitted on the bacteria counts from each of the stains.

An analysis of variance (ANOVA) model was fitted using the aov() function from the stats v4.5.0 R package (R Core Team, 2025), which tests the null hypothesis that the means of the response variable being tested does not differ significantly between treatments, with an F-test. Sampling date was included in each model as a blocking variable to account for differences caused by sampling on different days. This also takes into account soil

type differences on the dairy farmlets as the soil types were sampled on different days. Therefore, no soil type variable was included. Soil moisture and soil temperature were included as covariates. Treatment was included as a single factor with four levels (Std-Con, Std-Reg, Div-Con and Div-Reg). The models were fitted separately for the sheep and the dairy farmlets. If the ANOVA indicated that there was a significant difference in treatment levels, a pairwise comparison of the treatment levels was carried out using the TukeyHSD() function from the stats v4.5.0 R package (R Core Team, 2025), which compares the mean of the response variable being tested for each of the treatment levels and determines which are different based on the Studentized range statistic using Tukey's 'Honest Significant Difference' method. Only the stains that showed a significant difference between treatments are shown in the results section. All results are reported in the format mean ± standard error. Bacteria counts are reported as the count of bacteria per gram of dry soil. Results considered statistically significant at $\alpha < .05$, unless stated otherwise.

5.3 Results

The total number of bacteria was higher on the sheep farmlets than on the dairy farmlets (Table 5.1). On both the sheep and the dairy farmlets, the mean for the activated samples (FDA after activation) was lower than that of non-activated (FDA) samples (Table 5.1), which is unexpected, as the activation processes should increase the number of active, and therefore FDA stained, bacteria. However, comparing the FDA and FDA after activation counts with a paired t-test, indicated there was no significant differences between the two for either the sheep ($t_{15} = 1.41$, $p = .18$) or the dairy ($t_{17} = 1.2$, $p = .25$) farmlets. As the difference in number of bacteria that were FDA stained and FDA stained after activation is the potentially active pool, this indicates that there was no potentially active bacteria present, or a failure of the activation process to activate them. The proportion of active (FDA stained) and dead (PI stained) bacteria on the sheep

Table 5.1: Average number of bacteria in each physiological state, as determined by different staining procedures, on both the sheep and the dairy farmlets. The mean and standard error columns are reported as $x10^7$ bacteria g^{-1} soil. F-statistic and p-value columns show the F-statistic and p-value, respectively, of the ANOVA comparing bacteria count between treatment levels for each stain on both the sheep and the dairy farmlets. The numerator and denominator degrees of freedom for the ANOVA on the sheep farmlets are three and seven, respectively, while on the dairy farmlets they are two and eight, respectively. Significant p-values are in bold.

Farmlet system	Stain*	Physiological state	Mean	Standard error	F-statistic	p-value
Dairy	FDA	Active	5.85	0.53	7.720	**.01**
	FDA after activation	Active + potentially active	5.11	0.39	0.073	.93
	PI	Dead	14.24	0.94	0.447	.65
	FITC	Total	26.87	2.55	1.226	.34
Sheep	FDA	Active	5.63	0.86	0.088	.96
	FDA after activation	Active + potentially active	4.25	0.82	0.452	.72
	PI	Dead	18.65	1.63	5.077	**.04**
	FITC	Total	38.18	4.23	3.826	.07

*FDA = fluorescein diacetate, PI = propidium iodide, FITC = fluorescein isothiocyanate

farmlets was 0.15 and 0.49, while on the dairy farmlets it was 0.22 and 0.53, respectively. As there is no potentially active pool, the proportion of dormant bacteria is the remainder of the active and dead portions. This is 0.36 and 0.25 for the sheep and dairy farmlets, respectively.

On the sheep farmlets, only the number of dead (PI stained) bacteria differed significantly between treatments ($F_{3,7} = 5.08$, $p = .04$). The Div-Con treatment had a significantly higher number of dead bacteria than Div-Reg ($p = .04$) and also had more dead bacteria than both the Std-Con ($p = 0.0989$) and Std-Reg ($p = .07$) treatments with this result significant at $p<.1$ (Figure 5.2). There was no significant difference between the treatments for any

of the other stains on the sheep farmlets (Table 5.1). On the dairy farmlets, only the number of active (FDA stained) bacteria differed significantly between treatments ($F_{2,8}$ = 7.72, p = .01). The Std-Con treatment had significantly higher active bacteria than the Div-Con treatment (p = .01) (Figure 5.3). There was no significant difference between the treatments for any of the other stains on the dairy farmlets (Table 5.1).

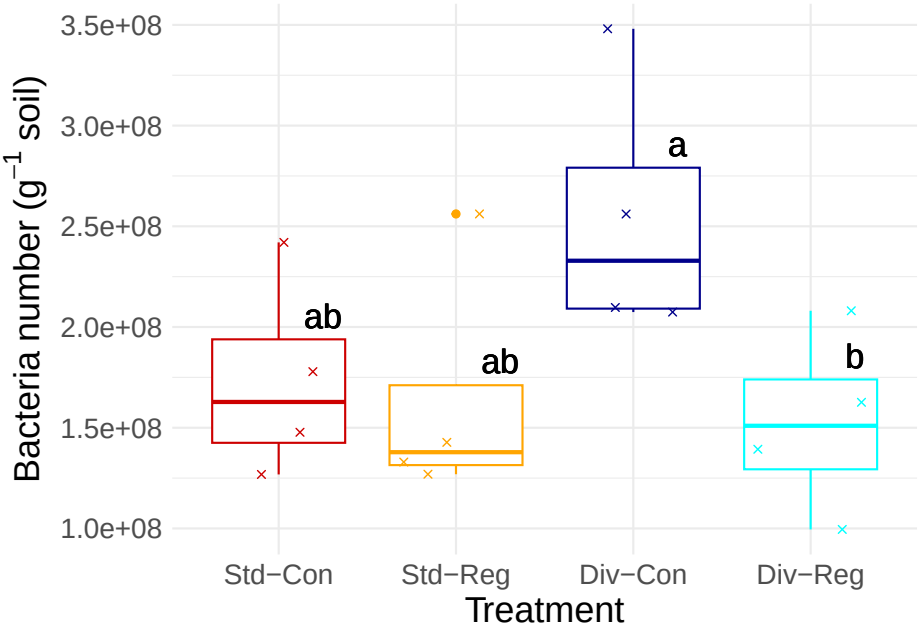

Figure 5.2: Number of dead (propidium iodide stained) bacteria for each treatment on the sheep farmlets. Different letters indicate significant differences between means (p<.05).

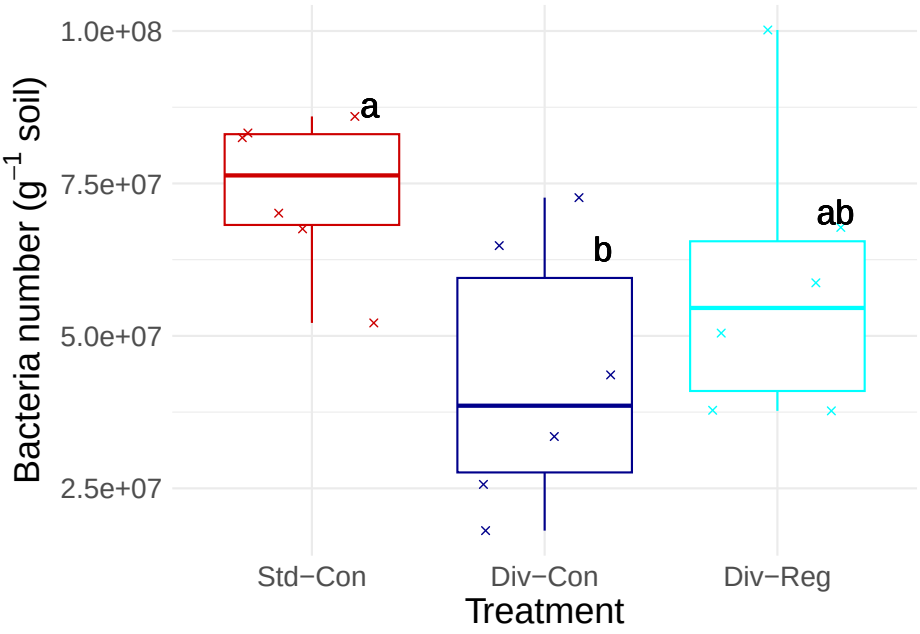

Figure 5.3: Number of metabolically active (fluorescein diacetate stained) bacteria for each treatment on the dairy farmlets. Different letters indicate significant differences between means ($p<.05$).

5.4 Discussion

During the image analysis to count the number of bacteria, the brightness threshold to distinguish between background and stained bacteria was set manually for each image. This was to ensure only stained bacteria were counted, as the background fluorescence differed between images and slides. Therefore, it is possible that the bacteria counts were impacted by the threshold selected, which could bias the results. A negative relationship is expected, as using a higher threshold would reduce the bacteria count. However, Figure A1 in Appendix 1 shows that using higher thresholds did not cause the counts to be reduced, with most counts increasing initially with increasing threshold and then plateauing. This initial rise is caused by the blank slides, which tended to have low counts and low thresholds set. It also indicates that for slides that had few bacteria present, the threshold tended to be set lower. The lack of a negative relationship indicates that bacteria counts were not artificially reduced by using a threshold that is too high. The

plateau indicates that bacteria counts were not influenced by the set threshold. However, it could also mean that the threshold was set such that each image had a similar number of bacteria. Despite this, images from the same slide are likely to have a similar number of bacteria, so a uniform count across images of the same slide is expected. There is also no consistent difference in the threshold set for the different treatments, that would indicate that the results are biased due to differences in the set threshold, except the blanks, which tend to have lower thresholds for the reasons discussed above (Appendix 1, Figure A2).

No difference in the number of FDA stained bacteria was observed before and after a 5 hour incubation with added nutrients in the form of 0.1% yeast extract, which was expected to cause any potentially active bacteria to become active and therefore increase the number of FDA stained bacteria. This could indicate that there were no potentially active bacteria present in the samples. However, this could also indicate that the activation process failed to activate the potentially active bacteria that were present. The activation process followed was as described by Maraha et al. (2004). However, they do not state the temperature at which the incubation should be carried out, therefore in this study the samples were incubated at room temperature. This was deemed appropriate, as soil bacteria live and grow at ambient temperatures. Luna et al. (2002) also carried out their activation of potentially active bacteria in marine sediments at *in situ* temperature, however they used a longer activation time. Other incubation steps described by Maraha et al. (2004) were carried out at 28 ^{O}C and therefore it is possible that their activation process was also carried out at this temperature. As bacteria generally metabolise faster at higher temperatures (Membré et al., 2005; Ratkowsky et al., 1982), this could be a reason for failure to activate in the current study.

The proportion of active bacteria found in the current study is well above what is usually found in soil, with Blagodatskaya & Kuzyakov (2013) reporting that active microorganisms usually only compose between 0.1 to 2% of total microbial biomass, and rarely exceed 5%. The total number of bacteria in the sample was measured by the number of bacteria

stained by FITC. The number of total bacteria on both the sheep and dairy farmlets, shown in Table 5.1, was very low compared to what is usually found in soil samples, with other studies finding over 10 fold more bacteria (Ananyeva et al., 2008; Frostegård & Bååth, 1996; Kennedy, 1995). However, the FITC stained slides in the current study had very high background fluorescence. Therefore, these counts likely underestimate the total bacteria present. This would also be the cause of the overestimation of the proportion of active bacteria, as the lower total count makes the proportion of active bacteria higher. Using the counts for active (FDA stained) bacteria and calculating the expected number of total bacteria, assuming that the active bacteria make up between 1 and 5% of the population, gives total bacteria estimates of between 1.13×10^9 and 5.63×10^9 bacteria g^{-1} soil for the sheep farmlets and between 1.17×10^9 and 5.85×10^9 bacteria g^{-1} soil for the dairy farmlets, which is more in line with what would be expected from the literature. Ananyeva et al. (2008) found up to 7.50×10^9 bacteria g^{-1} in meadow soil and between 1.80×10^9 and 3.45×10^9 bacteria g^{-1} in arable soil. As cultivation tends to reduce soil bacteria (van Groenigen et al., 2010), the actual total bacteria present in the current study, which is on pasture soil, is likely to be higher than the values for arable soil from Ananyeva et al. (2008) and possibly closer to the value for meadow soil. This would mean the actual bacteria numbers are on the upper end of the estimated range calculated above using the FDA counts, and could even be higher than the upper value, which would mean that active bacteria make up less than 1% of the population. Therefore, it is likely that the FITC staining procedure used in the current study is underestimating the amount of total bacteria and that the active bacteria actually make up between <1 and 5% of the total population. This would mean the proportion of dead bacteria is also lower than calculated in the results section. Due to the issues with the FITC staining, it is difficult to determine whether hypothesis 1 is supported, however there is no evidence to suggest that there was a difference in total bacteria count between treatments, and therefore it is likely not supported.

The results indicate that the treatments do have an effect on bacteria physiological state. On the sheep farmlets, the number of dead bacteria was higher in the Div-Con treatment than all other treatments. A higher number of dead bacteria could be indicative of unfavourable conditions for bacterial growth and survival, however it could also indicate higher microbial turnover and a greater contribution of bacterial necromass to the SOM pool (Bradford et al., 2013; Hu et al., 2023). On the dairy farmlets, the number of active bacteria in the Std-Con treatment was higher than in the Div-Con treatment, in contrast with hypothesis 2. Increased bacterial activity can occur due to conditions more favourable to microbial growth and metabolism or increased inputs of nutrients or energy source (Blagodatskaya & Kuzyakov, 2013). Soil temperatures in the Std-Con treatments were also higher than the other treatments (see chapter 4), therefore the increased bacterial activity could be caused due to the higher soil temperatures. The lack of consistent results across treatments and between the sheep and the dairy farmlets makes it difficult to generalise the effects of the different treatments on soil bacteria physiological state.

The total bacteria count was higher on the sheep farmlets than on the dairy farmlets. This could be due to differences in the livestock present and associated farm management practices, however, as the two farmlets are on different soil types, this could also be due to soil type differences. The pallic Tokomaru silt loam soil on the sheep farmlets is more mature than the recent Manawatu sandy loam and raw Rangiktiei loamy sand soils of the dairy farmlets, and therefore has higher organic matter content and total carbon. As soil bacteria is correlated with SOM, this could be the cause for the higher bacteria counts. However, the soil samples from the two farmlet systems are fully confounded with soil type and different sampling dates and therefore these effects cannot be separated to determine which is responsible for the change.

As the physiological state of the soil bacteria changes with drying and storage of the soil sample, fluorescent microscopic analysis needs to be carried out on fresh soil as soon after

sampling as possible (Foght & Aislabie, 2005). The high labour and time requirements for preparing and observing the microscope slides limit the number of samples that can be processed each day. Therefore, samples had to be collected over multiple days. This creates additional variation and complicates the analysis of the results. It also reduces the practicality of using this method to compare a large number of samples in extensive experiments.

The intention was to measure total and active soil fungi by fluorescent microscopy alongside the measures of soil bacteria for the current study. However, an extraction and staining procedure that provided adequate and consistent slides of soil fungal hyphae was not achieved before sampling began, and therefore this analysis could not be carried out. This, along with the limitations of the FITC staining procedure discussed above indicate that a number of problems were encountered in the use of fluorescent microscopy for the current study. Many of the issues experienced with the fluorescent microscopy methods used in the current study stem from my lack of experience with utilising these techniques and attempting to implement methods based on published literature without direct instruction, as well as being largely limited to the stains and equipment available. If properly implemented, fluorescent microscopic analysis of soil microbes has the potential to provide detailed information on the physiological states of both soil bacteria and fungi, as well as details about the size, quantity and biomass of these two microbial groups. Additionally, by utilising automated confocal laser scanning microscopy or flow cytometry ,the throughput of fluorescent microscopy analysis would be increased, making it more applicable to large sample sizes (Bloem et al., 1995; Maraha et al., 2004).

5.5 Conclusion

Due to methodological limitations, neither the total bacteria nor the potentially active bacteria could be accurately enumerated. This also means the relative proportions of

active and dead bacteria and the size of the dormant bacteria pool cannot be determined, as these are calculated based on the number of total bacteria. There were some differences in the total size of the active and dead bacteria pools between treatments, however these were inconsistent across the treatments and between the farmlet systems and therefore generalised conclusions of the effect of diverse pastures and regenerative management on soil bacteria physiological state cannot be drawn. On the sheep farmlet, total bacteria was higher than on the dairy farmlets, which could be due to farmlet system or soil type differences. Overall, there is little evidence from the results of the current study that the treatments cause a difference in the relative proportions or total size of the different bacteria physiological pools.

CHAPTER 6: COMMUNITY COMPOSITION OF SOIL MICROBES

6.1 Introduction

The community composition of the soil microbiome was investigated using phospholipid fatty acid (PLFA) analysis. Phospholipids are the main component of the cell membranes of all living cells, except archaea (Joergensen, 2022; White et al., 1979; Zelles, 1999). As phospholipids break down rapidly after cell death, they do not accumulate in the soil outside of living cells (Joergensen, 2022; Nielsen & Petersen, 2000; White et al., 1979; Zhang et al., 2019). This makes phospholipids a useful indicator of living microbial biomass and they have been used extensively in the study of the soil microbiome (Joergensen, 2022; Parfitt et al., 2010; Zelles et al., 1992).

Phospholipids consist of a glycerol back bone to which a phosphate head group and two fatty acid tails are attached (Gómez-Brandón et al., 2010). The fatty acid tails can differ in composition (length, number and position of double bonds, presence of alkyl- chains, hydroxyl groups or cyclic moieties), with certain fatty acids being present predominantly or exclusively in specific microbial groups (Gómez-Brandón et al., 2010; Joergensen & Wichern, 2008; White et al., 1979). These can be used as indicators, or biomarkers, of the main microbial groups present in the soil (Joergensen, 2022; White et al., 1979; Zelles et al., 1992). Therefore, analysis of the presence and abundance of PLFA extracted from soil can be used to research the microbial community structure (Joergensen, 2022; Zelles, 1999). This provides information on the main microbial groups present, however does not provide higher level resolution of the taxa present (Zelles, 1999). PLFA analysis is sensitive to rapid changes in the soil microbiome in a wide range of soils and provides a

whole community approach to measuring microbial populations at a phenotypic level with respect to super families and domains (Zelles, 1999). The main soil microbial groups that can be distinguished are actinobacteria, gram positive and gram negative bacteria as well as mycorrhizal and saprotrophic fungi (Joergensen, 2022; Lewe et al., 2021). The relative portions of these microbial groups can therefore also be compared, which have variously been used as indicators for the state of the soil microbiome, including the fungi to bacteria ratio and the gram-positive to gram-negative bacteria ratio (Fanin et al., 2019; Wang et al., 2019). A low fungi to bacteria ratio has been shown to indicate disturbed soils with a degraded soil food web, while a high fungi to bacteria ratio indicates a stable soil food web and more closely resembles natural soils (Bardgett & McAlister, 1999; de Vries et al., 2006; Ingham & Slaughter, 2004; Parfitt et al., 2010; Yeates et al., 1997). The gram-positive to gram-negative bacteria ratio has been linked to soil carbon source availability (Fanin et al., 2019).

Neutral lipids are similar to phospholipids, however, they lack the phosphate head group and instead have a third fatty acid attached to the glycerol back bone. These are not found in cell membranes but are energy storage compounds in eukaryotes (Bååth, 2003; Joergensen, 2022; Olsson et al., 1995). They are rarely found in bacteria as they do not store energy as lipids (Bååth, 2003; Olsson et al., 1995). The neutral lipid fatty acid (NLFA) 16:1ω5 is found predominantly in arbuscular mycorrhizal fungi (AMF), as it contains large amounts in its spores and vesicles as energy stores (Bååth, 2003; Olsson et al., 1995). This makes it a useful indicator of AMF in soil (Frostegård et al., 2011; Joergensen, 2022). Although NLFA analysis can provide information on the total biomass of AMF present in the soil (Bååth, 2003; Olsson et al., 1995), it does not indicate the extent to which roots are colonised. Although some studies have found good correlation between NLFA 16:1ω5 concentration and root colonisation (Barceló et al., 2020), it is not always a good predictor of this (Sharma & Buyer, 2015). Direct microscopic examination of roots allows the extent of AMF colonisation of roots to be determined, but is much more labor intensive and prone

to biases caused by user subjectivity and requires higher level of expertise (Kokkoris et al., 2019). Which plant species have AMF associations and differences in the extent of AMF colonisation between species cannot be determined with NLFA analysis. However, NLFA analysis allows direct comparisons with other components of the soil microbiome, as these can be measured from the same sample using the same method, which microscopy does not. Genomic analyses provide a higher level of resolution on the species present than NLFA analysis (Barceló et al., 2020).

Microbial biomass can also be determined using measures of microbial carbon, including hot water extractable carbon and chloroform fumigation (Ghani et al., 2003; Jenkinson & Powlson, 1976b; Vance et al., 1987). Unlike PLFA analysis, these methods do not allow different microbial groups to be distinguished. Genomic analyses can be used to investigate soil microbial community composition, and provides a much higher taxonomic resolution than PLFA analysis (Semenov, 2021). However, genomic analysis is subject to several sources of bias, including; differences in cell lysis and deoxyribonucleic acid (DNA) extraction efficiency between different microbial groups, quality of DNA template, GC-content of template DNA, choice of primers and differences in binding efficiency of selected primers between microbial groups, and choice of polymerase chain reaction (PCR) parameters and polymerases (Lear et al., 2018; Lewe et al., 2021). These create uncertainty and therefore results from genomic analysis may not be representative of the actual community composition in the soil (Lear et al., 2018; Lewe et al., 2021). PLFA analysis is also subject to biases, such as differences in PLFA content of different microbes (Blagodatskaya & Kuzyakov, 2013). DNA can remain in the soil for weeks to years after cell death, with this ‘relic’ DNA having a significant contribution to the environmental DNA collected from soil, potentially impacting results (Carini et al., 2016). In contrast, PLFA has been shown to degrade rapidly, within days, in soil, and therefore provides a more accurate measure of the current living soil microbiome (Zhang et al., 2019). PLFA analysis has been used on a variety of soils in New Zealand. The microbial community composition

under New Zealand pastoral soils has been shown to be influenced by soil fertility, grazing management and irrigation using PLFA analysis (Lambie et al., 2021; Parfitt et al., 2010).

This study investigates the effects that diverse pasture and regenerative agricultural practices implemented in New Zealand's pastoral farming system have on the total microbial biomass and microbial community composition, using PLFA analysis, as well as on the biomass of AMF, using the NLFA biomarker 16:1ω5. It aims to determine whether any measurable effects occur in the short term, within two and a half years of transitioning to these practices. Regenerative agriculture is stated to improve soil biological conditions. Therefore, the hypotheses being tested are that both diverse pasture and regenerative management, after two and a half years under these treatments, cause; 1) higher total microbial PLFA concentration, 2) a measurable change in the relative proportions of biomarker PLFAs, to indicate a change in the composition of the microbial community, and 3) higher concentration of the NLFA biomarker 16:1ω5. Note that increases in total microbial biomass is not necessarily a good indicator of improved soil health, despite the hypotheses presented here. See section 8.5.3 for further discussion on the challenges of defining soil biological health.

6.2 Methods

6.2.1 Soil processing

Soil samples were collected as described in section 3.2. Processing was carried out on the fresh soil samples within 3 hours of the samples being collected. Soil cores were crushed by hand while still in the zip lock bag, sieved to 4 mm then sieved again to 2 mm. The soil was then tipped onto a clean large plastic sheet and mixed by lifting one corner of the sheet making the soil roll over to the opposite corner, doing the same for each corner of the sheet and repeating the whole process six times. About 20 g of mixed soil was

placed in a zip lock bag and frozen until it could be lyophilized, after which it was stored frozen until the lipid extraction and analysis could be carried out. All equipment used was washed with water and wiped down with 70% ethanol between samples.

6.2.2 Chemical extraction

6.2.2.1 Phospholipids

Three replicate extractions of phospholipids, and analysis of their fatty acids, were carried out on each sample, following the method described by Lewe et al. (2021). Chemical extraction and analysis of soil lipids was carried out at Victoria University of Wellington, using the School of Biological Sciences laboratory and equipment. Briefly, lipids were extracted from 0.5 g of lyophilized soil using a two phase solution, spiked with the phospholipid 1,2-dinonadecanoyl-sn-glycero-3-phosphocholine (19:0) as an internal standard. Phospholipids were separated from neutral lipids and glycolipids by solid phase extraction on a silica column. Transesterification was carried out on the phospholipids by alkaline methanolysis to generate fatty acid methyl esters (FAMES).

6.2.2.2 Neutral lipids

The extraction of neutral lipids followed the same method as for phospholipids, except during the separation of lipids by solid phase extraction on silica columns, the eluted neutral lipid fraction was retained. These are also transformed to FAMEs by transesterification.

6.2.3 Analysis of extracted fatty acid methyl esters

FAMEs were analysed by gas chromatography-mass spectrometry (GC–MS), as described by Lewe et al. (2021). Briefly, FAMEs were separated by gas chromatography before being detected by an electron impact mass spectrometer. To control the instrument, GC–MS solutions version 4.44 (Shimadzu) was used, as well as for the qualitative and quantitative analysis of the resulting chromatograms. The FAMES were identified by comparing the retention times and mass spectra to commercially available standards. Calibration curves for these had been calculated to determine their response factor (RF), which was used to calculate its ratio to the RF of the FAME 19:0, which was used as an internal standard, to obtain the relative response factor (RRF). This was used to calculate the concentration of PLFA in the original sample from the chromatogram peak area, thereby accounting for any losses during the extraction process.

Designation of the PLFAs as biomarkers of specific microbial groups was carried out according to the designations in the second set of PLFA biomarkers (PLFA2) for soil samples described by Lewe et al. (2021). Lewe et al. (2021) suggest that using a wide range of biomarkers that encompasses a larger portion of the total PLFA should be used when characterising complex microbial communities, and therefore the soil PLFA2 biomarker set was selected instead of the PLFA1 biomarker set that they also described. PLFAs 18:1ω9c and 18:2ω6 were assigned as fungal biomarkers (Joergensen, 2022; Joergensen & Wichern, 2008; Parfitt et al., 2010). PLFA 16:1ω5 was assigned as an AMF biomarker (Olsson et al., 1995; Parfitt et al., 2010). To calculate fungal to bacterial PLFA ratio, the total concentration of all bacterial biomarkers, as defined by soil PLFA2 in Lewe et al. (2021), was compared to the sum concentration of 18:1ω9c and 18:2ω6 fungal biomarkers. PLFA 16:1ω5 was not included in this as it is debated whether it is found exclusively in fungi and therefore whether it is a reliable fungal biomarker (Bååth, 2003; Frostegård et al., 2011; Joergensen, 2022). Concentration of 16:1ω5 from the

neutral lipid faction was used as a indicator of AMF in the soil (Frostegård et al., 2011; Olsson et al., 1995). Olsson et al. (1995) suggest using the PLFA 20:5 along with 16:1ω5 to measure AMF in soil, however this fatty acid was not measured in the current study. The gram-positive to gram-negative bacteria ratio was calculated by taking the ratio of the sum of all the gram-positive, including Actinobacteria, PLFA biomarker concentrations and the sum of all the gram-negative PLFA biomarker concentrations, as defined by soil PLFA2 in Lewe et al. (2021).

6.2.4 Statistical analysis

The fatty acid concentration from triplicate extractions of the same sample were averaged and all statistical analyses were carried out on the average values. Statistical models were fitted on the total PLFA concentration, the ratio of fungal to bacterial PLFA, the ratio of gram-positive to gram-negative bacterial PLFA and the concentration of NLFA 16:1ω5.

Statistical analysis was conducted by fitting an analysis of variance (ANOVA) model using the aov() function from the stats v4.5.0 R package (R Core Team, 2025), which tests the null hypothesis that the means of the response variable being tested does not differ significantly between treatments, with an F-test. Sampling date was included in each model as a blocking variable to account for differences caused by sampling on different days. This also takes into account soil type differences on the dairy farmlets as different soil types were sampled on different days. Therefore, no soil type variable was included. Soil moisture and soil temperature were included as covariates. Treatment was included as a single factor with four levels (Std-Con, Std-Reg, Div-Con and Div-Reg). The models were fitted separately for the sheep and the dairy farmlets. If the ANOVA indicated that there was a significant difference in treatment levels, a pairwise comparison of the treatment levels was carried out using the TukeyHSD() function from the stats v4.5.0 R package (R Core Team, 2025), which compares the mean of the response variable

being tested for each of the treatment levels and determines which are different based on the Studentized range statistic using Tukey's 'Honest Significant Difference' method. All results are reported as the mean ± standard error. Results considered statistically significant at $\alpha < .05$, unless stated otherwise.

A comparison of the average PLFA concentration in the different soil types on the dairy farmlets was carried out by fitting an analysis of variance model, as above, however the sampling date blocking variable was omitted to allow the soil types, which were sampled on different days, to be compared. This confounding of sampling date with soil type means it is not possible to separate differences in soil type with differences in sampling date.

Multivariate analysis was carried out to compare the proportion of biomarker PLFA for each microbial group in each treatment, using the adonis2() function from the vegan v2.6.10 R package (Oksanen et al., 2025), which uses a permutation test on distance matrices with pseudo F-ratios. Sampling date was included as a blocking variable, however, as adonis2() does not contain the capacity to model covariates, these were not included in this analysis. A unique sample ID for each paddock was also included as a grouping variable to account for the pseudo replication caused by carrying out multiple extractions of the same sample. PCA plots were created using the PCA() function from the FactoMineR v2.11 R package (Lê et al., 2008) and the fviz_pca() function from the factoextra v1.0.7 R package (Kassambara & Mundt, 2020).

6.3 Results

6.3.1 Total PLFA concentration

In total, 20 biomarker PLFAs were detected in all samples across both the sheep and the dairy farmlets (Table 6.1). The number and type of biomarker PLFAs detected did not differ between the sheep and the dairy farmlets. There was no significant difference in the total

Table 6.1: Phospholipid fatty acids that were detected in samples from both the sheep and the dairy farmlets, with the microbial group they were designated to.

Biomarker designation	Fatty acid
Arbuscular mycorrhizal fungi	16:1ω5c
Actinobacteria	10Me16:0
	10Me17:0
	10Me18:0
All bacteria - generic	14:0
	15:0
	16:0
	17:0
	18:0
Fungi	18:1ω9c
	18:2ω6
Gram-negative bacteria	16:1ω7c
	18:1ω7c
	delta17:0
	delta19:0
Gram-positive bacteria	a15:0
	a17:0
	i15:0
	i16:0
	i17:0

PLFA concentration between treatments on either the sheep or the dairy farmlets ($F_{3,7}$ = 1.73, p = .25, and $F_{2,8}$ = 1.50, p = .28, for the sheep and the dairy farmlets, respectively). The average total PLFA concentration on the sheep farmlets (816.66 ± 24.68 nmol g^{-1} soil) was higher than on the dairy farmlets (398.13 ± 15.41 nmol g^{-1} soil). On the dairy farmlets, there was a significant difference in total PLFA between the soil types ($F_{1,14}$ = 8.13, p = .01), with the Manawatu sandy loam soil having a higher total PLFA concentration than the Rangitikei loamy sand (Figure 6.1).

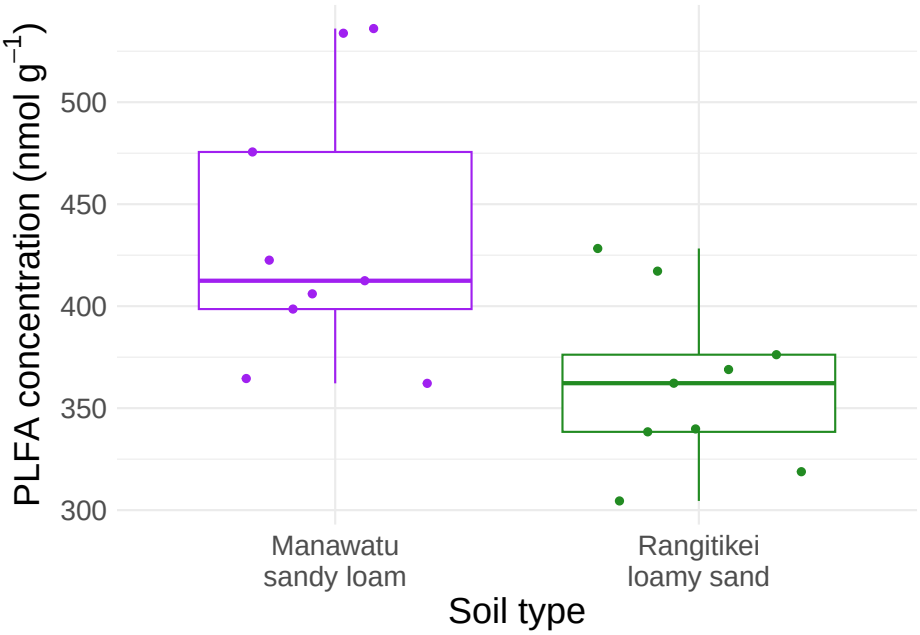

Figure 6.1: Total PLFA concentration by soil type on the dairy farmlets. Levels differed significantly by soil type (p<.05).

6.3.2 Fungal to bacterial PLFA ratio

On the sheep farmlets, there was no significant difference in the fungal to bacterial PLFA ratio between treatments ($F_{3,7}$ = 0.08, p = .97). The mean fungal to bacterial PLFA ratio on the sheep farmlets was 0.14 ± 0.002. On the dairy farmlets, the fungal to bacterial PLFA ratio did differ significantly between treatments ($F_{2,8}$ = 4.59, p = 0.047), with the Div-Reg treatment having a significantly higher proportion of fungi than the Div-Con treatment (p = .04) (Figure 6.2). The mean fungal to bacterial PLFA ratios on the dairy farmlets were 0.16 ± 0.006, 0.15 ± 0.003 and 0.16 ± 0.004 in the Std-Con, Div-Con and Div-Reg treatments, respectively. Although there was no significant differences in the total concentration of bacterial ($F_{2,8}$ = 2.04, p = .19) or fungal ($F_{2,8}$ = 0.01, p = .99) PLFA between treatments, the effect sizes showed that the Div-Reg treatment had both more total fungal PLFA and less total bacterial PLFA than the Div-Con treatment, which caused the difference in fungal to bacterial PLFA ratio.

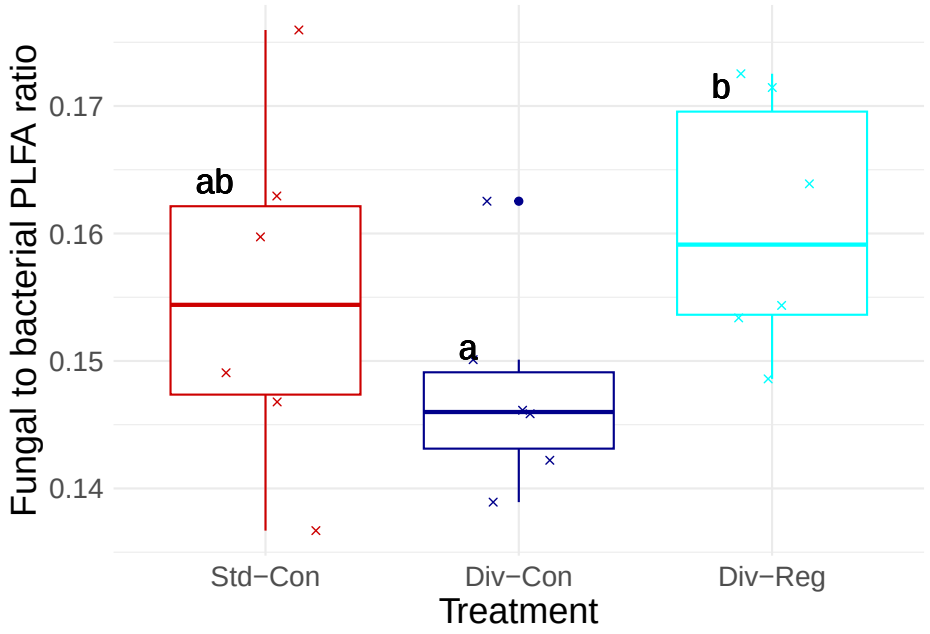

Figure 6.2: Fungal to bacterial PLFA ratio in each treatment on the dairy farmlets. Different letters indicate treatments with significant differences ($p<.05$).

6.3.3 Other measures

There was no significant difference in the ratio of gram-positive to gram-negative bacterial PLFA between treatments on either the sheep or the dairy farmlets ($F_{3,7} = 0.94$, $p = .47$, and $F_{2,8} = 0.86$, $p = .46$, for the sheep and the dairy farmlets, respectively). It was similar across both the sheep and the dairy farmlets, with mean ratios of 0.90 ± 0.01 and 0.96 ± 0.01 for the sheep farmlets and the dairy farmlets, respectively.

There was no significant difference in the amount of AMF, as measured by the NLFA 16:1ω5 concentration, between treatments on either the sheep or the dairy farmlets ($F_{3,7} = 0.04$, $p = .99$, and $F_{2,8} = 2.81$, $p = .12$ for the sheep and the dairy farmlets, respectively). The mean NLFA concentration on the sheep farmlets was more than double (90.46 ± 5.00 nmol g^{-1} soil) than on the dairy farmlets (39.02 ± 3.36 nmol g^{-1} soil).

6.3.4 Community composition

The amount of PLFA in each of the microbial groups was similar across all treatments and the relative amounts were similar between the sheep or the dairy farmlets (Figures 6.3 and 6.4). Multivariate analysis indicated that there were no significant differences in microbial community composition between treatments on either the sheep or the dairy farmlets ($F_{3,9}$ = 1.38, p = .24, and $F_{2,10}$ = 1.50, p = .18, for the sheep and the dairy farmlets, respectively). However, there was a significant difference between soil types on the dairy farmlets ($F_{1,16}$ = 7.09, p <.001) as illustrated in Figure 6.5. There was also a significant differences between the sheep and the dairy farmlets ($F_{1,32}$ = 25.74, p <.001), as shown in Figure 6.6.

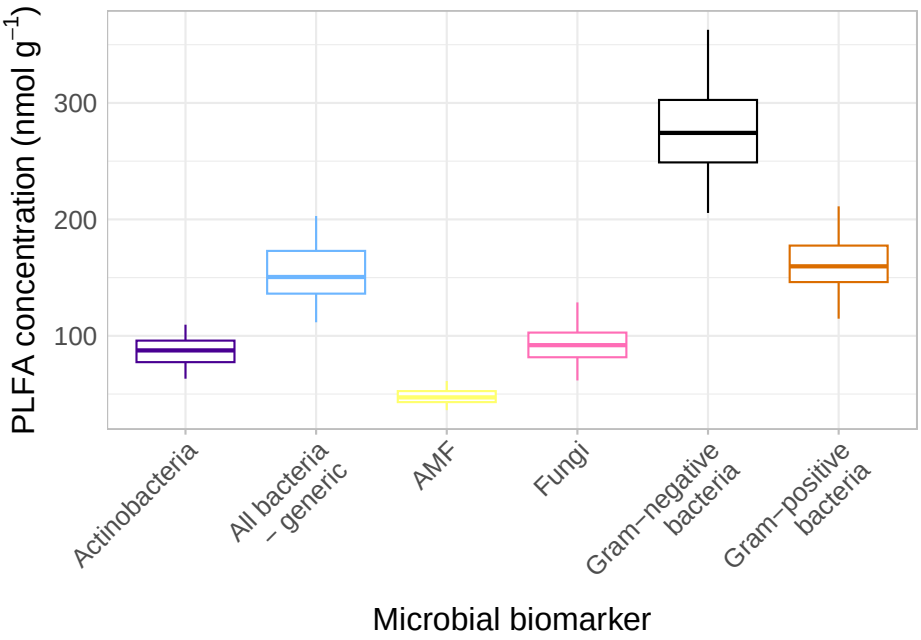

Figure 6.3: Total concentration of all PLFA biomarkers for different microbial groups on the sheep farmlets. ”All bacteria - generic” are non-specific bacterial biomarkers found in all bacteria.

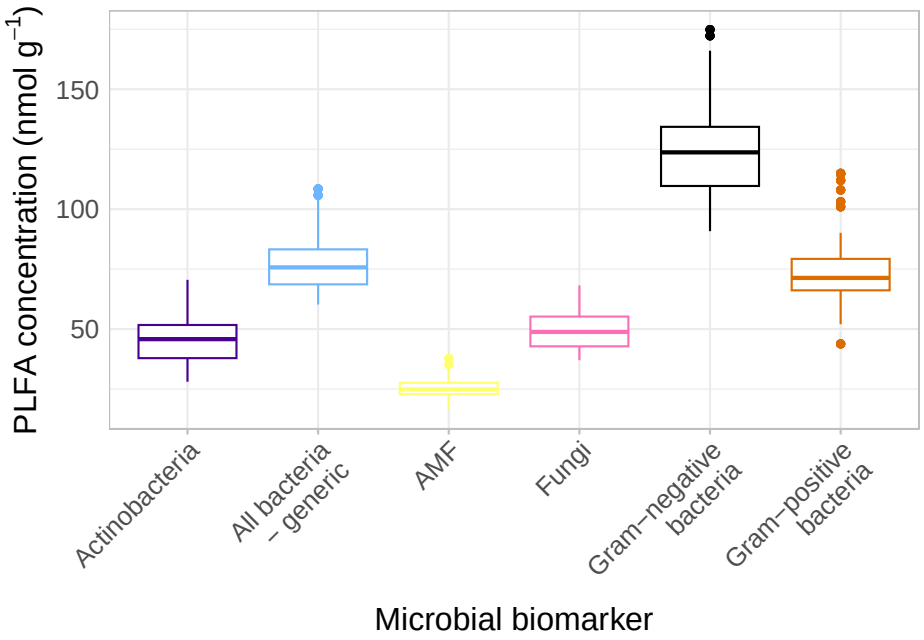

Figure 6.4: Total concentration of PLFA biomarker for different microbial groups on the dairy farmlets. "All bacteria - generic" are non-specific bacterial biomarkers found in all bacteria.

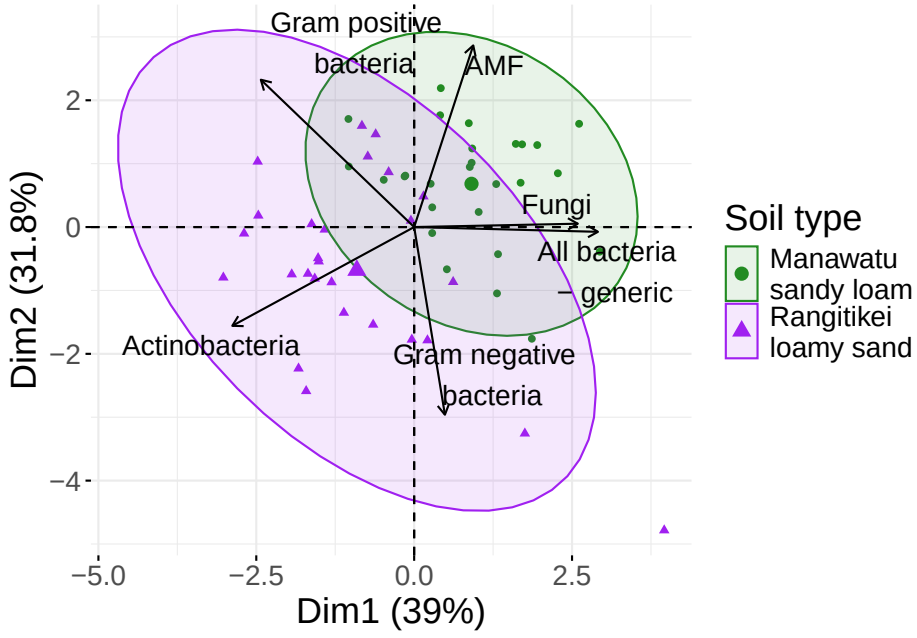

Figure 6.5: Principle component analysis of the PLFA biomarkers for different microbial groups comparing the Manawatu sandy loam and the Rangitikei loamy sand soil types on the dairy farmlets. "All bacteria - generic" are non-specific bacterial biomarkers found in all bacteria. Points are pseudo replicates of each sample.

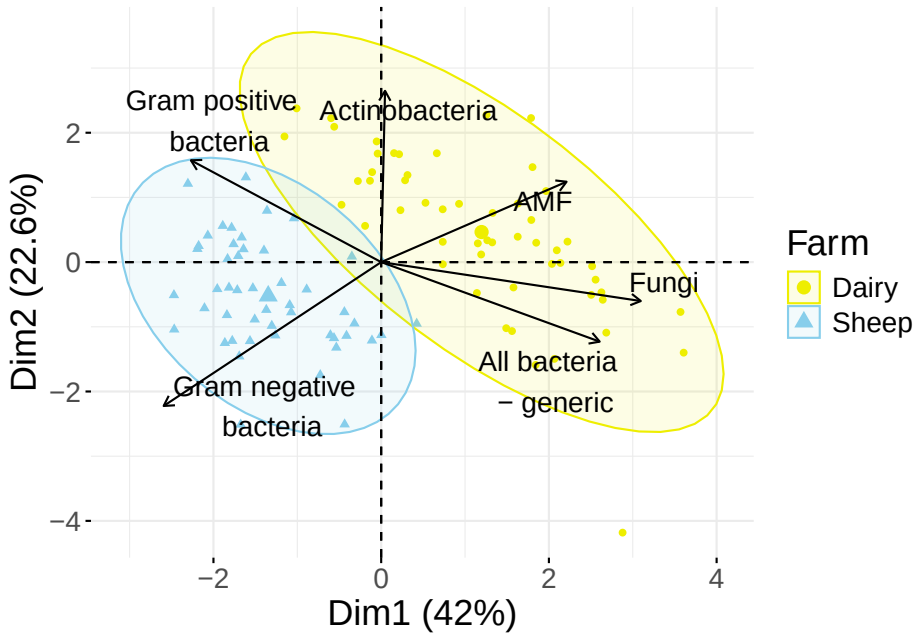

Figure 6.6: Principle component analysis of the PLFA biomarkers for different microbial groups comparing the sheep and the dairy farmlets. ”All bacteria - generic” are non-specific bacterial biomarkers found in all bacteria. Points are pseudo replicates of each sample.

6.4 Discussion

There was no treatment effect on total PLFA concentration, and therefore hypothesis 1 is rejected. In total, 20 PLFA biomarkers were detected in all samples from both the sheep and the dairy farmlets. This is similar to the 21 biomarker PLFAs detected in soil samples by Lewe et al. (2021). However, PLFAs 3OH14:0, 2OH16:0, 19:1ω9c, 18:1ω7t, 16:1ω7t, 16:1ω5t (all gram-negative biomarkers) were not detected in the current study, in contrast to Lewe et al. (2021). Additionally, PLFAs 16:1ω5c (AMF biomarker), 18:1ω9c and 18:2ω6 (fungal biomarkers) were detected in this study but were not used as biomarkers by Lewe et al. (2021).

The average total PLFA was much higher in this study on both the sheep and the dairy farmlets than detected by Lewe et al. (2021), with the dairy farmlets having double and the sheep farmlets having four times higher PLFA concentrations as those reported by

Lewe et al. (2021). However, samples from Lewe et al. (2021) were New Zealand forest soils, compared to agricultural soil in this study. Lewe et al. (2021) also sampled to 10 cm depth and in autumn (March), therefore the lower PLFA concentration could be due to the dilution effect of sampling deeper, as soil biota tends to be concentrated at the soil surface, as well as seasonal differences. Total PLFA concentration was also higher in the current study than the maximum reported by Lambie et al. (2021) from a range of New Zealand soils under intensively grazed pasture, including pallic and recent soils from the Manawatū. Lambie et al. (2021) found a median PLFA concentration of around 250 nmol g^{-1} and a maximum of 400 nmol g^{-1}, however, although they sampled at the same time of the year as the current the study (November), they sampled to 10 cm depth and therefore the dilution effect of sampling deeper would account for at least part of this difference. The sheep farmlets in the current study had two fold higher PLFA concentrations than the maximum found by Lambie et al. (2021), while the dairy farmlets PLFA concentration were comparable to the maximum they found. This is an unusual result as it would suggest that the soils in the current study had a higher microbial biomass.

Within this study, the higher total PLFA concentration on the sheep farmlets would suggest that they had higher microbial biomass than the dairy farmlets. This could be due to differences in the livestock present and associated farm management practices. However, as the sheep farmlets has a different soil type than the dairy farmlets, it could also be a continuation of the difference in soil type observed on the dairy farmlets. Total PLFA has been shown to be positively correlated with soil organic matter (Frostegård et al., 1991). The increase in total PLFA from the raw Rangitikei loamy sand soil to the recent Manawatu sandy loam soil, on the dairy farmlets, to the pallic Tokomaru silt loam soil, on the sheep farmlets, represents a gradient of increasing soil maturity and associated increase in organic matter. This is supported by the total organic carbon stocks measured on the farmlets in 2022 (P Jeyakumar, personal communication, July 28, 2025). Therefore, the difference in total PLFA concentration between the soils on the dairy farmlets and

between the sheep and the dairy farmlets could be due to the soils having more time to develop and build organic matter which supports an abundant microbial population. However, as the sheep and the dairy farmlets are fully confounded with soil type and sampling date, and the soils on the dairy farmlets are fully confounded with sampling date, the soil type and farmlet system effect cannot be separated to determine which is responsible for the change, and the possibility that the differences are due to sampling date cannot be excluded. Despite this, it is unlikely that soil microbial biomass doubled over a period of two weeks. Stevenson et al. (2014) found that soil PLFA concentration in pasture soils in the Waikato, New Zealand, only increased by up to 100 nmol g^{-1} over a three month period transitioning from winter to spring. Similarly, Bardgett et al. (1999) found that soil PLFA increased by less than 20 nmol g^{-1} over three months when transitioning from winter to spring in the United Kingdom. These findings support the suggestion that soil type and stock type are likely responsible for differences in total PLFA concentration.

In contrast to hypothesis 3, there was no treatment effect on NLFA 16:1ω5 concentration (which is used as an indicator of AMF), on either the sheep of the dairy farmlets. The NLFA concentration was over double on the sheep farmlets (90.46 ± 5.00 nmol g^{-1}) compared to the dairy farmlets (39.02 ± 3.36 nmol g^{-1}). This indicates that AMF biomass was higher on the sheep farmlets and, as for total PLFA, this could be due the either soil type or stock type and associated farmlets practices. Again, it is likely due to the more mature Tokomaru silt loam soil, with higher organic matter content, on the sheep farmlets compared to the recent and raw soils on the dairy farmlets, which have had less time to establish abundant microbial communities (Ohtonen et al., 1999). However, it is not possible to separate differences in farmlet system effects and soil effects as these are fully confounded. Comparisons between the sheep and the dairy farmlets need to be interpreted with caution due to confounding with a sampling date.

A treatment effect was detected for the fungal to bacterial PLFA ratio on the dairy farmlets with the regeneratively managed treatment (Div-Reg) having a higher ratio. A high fungi

to bacteria ratio has been shown to indicate a stable soil food web that more closely resembles natural soils (Bardgett & McAlister, 1999; de Vries et al., 2006; Ingham & Slaughter, 2004; Parfitt et al., 2010; Yeates et al., 1997). Whether this is caused by regenerative management cannot be determined as the treatments on the dairy farmlets are not fully crossed, and therefore this is the only regenerative treatment on the farmlets. This effect was not measured under regenerative management on the sheep farmlets, suggesting a need for caution in concluding that regenerative management caused the increased fungal to bacterial PLFA ratio. The average fungal to bacterial PLFA ratio on the sheep farmlets was lower than on the dairy farmlets. On both the sheep and the dairy farmlets, the fungal to bacterial PLFA ratio was higher than that found by Bardgett et al. (1997) on sheep grazed pastures. However, in that study, a smaller subset of biomarker PLFAs was used, and therefore a direct comparison cannot be made.

The gram-positive to gram-negative bacterial PLFA ratio did not differ between treatments and was similar between the sheep and the dairy farmlets. It was also similar to that found by Lambie et al. (2021) on non-irrigated pastures in a wide range of New Zealand soils. The fungal to bacterial PLFA ratio and the gram-positive to gram negative bacterial PLFA ratio cannot be interpreted as biomass ratios. Although PLFA is directly correlated with microbial biomass, the PLFA content of different microbial groups differs (Blagodatskaya & Kuzyakov, 2013). For example, fungi have less PLFA per unit biomass than bacteria, as fungal hyphae have a lower surface area to volume ratio compared to smaller bacterial cells, and gram-negative bacteria have double the PLFA content of gram positive bacteria due to the additional phospholipids in the outer membrane (Blagodatskaya & Kuzyakov, 2013). To determine biomass ratios, the PLFA concentration would need to be converted to biomass. However these calculations rely on set conversion ratios and assumptions, which are biased by differences in PLFA content of cultured microbes vs starving soil microbes and by differing biomarker designations, and have therefore not been widely adopted (Joergensen, 2022)

Multivariate analysis comparing all the microbial groups indicated that there was no treatment differences in community composition on either the sheep or the dairy farmlets, in contrast with hypothesis 2. However, on the dairy farmlets there was a difference in community composition between the soil types, and there was also a difference when comparing the sheep to the dairy farmlets. The difference between the sheep and the dairy farmlets could be a continuation of the soil type difference observed on the dairy farmlets, as the sheep farmlets also has a different soil type, or it could be caused by the different livestock and associated management practices on the sheep and the dairy farmlets. There is no way to determine which of these factors caused this effect as they are fully confounded. Bossio et al. (1998) found that the microbial community differed under both different management type and soil type, however determined that soil type had a greater effect, and was the most important factor influencing microbial community composition, a finding supported by Zelles et al. (1992). Therefore, it is likely that the difference in microbial community structure between the sheep and the dairy farmlets is caused by the different soil types.

A major limitation in interpreting the results from this study is the confounding of soil type with sampling date and farmlet system. The study was designed to test the difference between treatments, with soil type being a control variable rather than an independent variable, as soil type effects are not of primary interest. However, given that it has a large influence on the results, it would have been interesting to be able to conclusively evaluate the effect of soil type. This could have been done on the dairy farmlets by sampling across the two soil types each day, thereby including different soil types within the blocks. This was considered when setting up the sampling design, however was ultimately unable to be done due to limitations in the number of samples that could be processed each day. The confounding between soil types between the sheep and the dairy farmlets is not possible to ameliorate as the long term farmlet trials were established in different locations and could not be crossed with soil type for practical reasons.

There is currently no consensus on the correct designation of biomarker PLFAs. They are known to have imperfect specificity for any one microbial group, meaning no biomarker PLFA will be found exclusively in the group it is designated to (Joergensen, 2022; Zelles, 1999). However, the use of certain biomarkers is disputed, with conflicting opinions. While the use of NLFA 16:1ω5 as an indicator of AMF is well established, using this same fatty acid from the phospholipid fraction as an indicator of AMF is not as clear. Some authors argue that it is a good indicator of AMF, and should be included as a fungal biomarker (Joergensen, 2022; Joergensen & Wichern, 2008; Olsson et al., 1995), while others state that it is also found in various bacteria and cannot be used as a fungal biomarker when bacterial biomass is high (Bååth, 2003; Frostegård et al., 2011). Similarly, straight chain fatty acids have variously been attributed to bacteria only (Lewe et al., 2021), fungal only (Bååth, 2003) or all microbial groups (both bacteria and fungi) (Joergensen, 2022). Certain fungal biomarkers are also found in plants and can therefore influence the results if a large amount of plant material is included in the sample (Joergensen, 2022). This can complicate the interpretation of results and, depending on which biomarker designation is used, can affect the conclusions drawn.

6.5 Conclusion

There is limited evidence to suggest that implementing diverse pasture and regenerative management can in the short term change the microbial biomass or community composition. Neither total microbial biomass (total PLFA concentration), AMF biomass (NLFA 16:1ω5 concentration), gram-positive to gram-negative bacterial PLFA ratio, or microbial community composition differed between treatments. Diverse pastures and regenerative management increased the fungal to bacterial PLFA ratio on the dairy farmlets. However, as this effect was not seen on the sheep farmlets, this cannot be generalised to a treatment effect. Consistently higher total microbial biomass (both

total PLFA concentration and NLFA 16:1ω5 concentration) were observed on the sheep farmlets than the dairy farmlets as well as differences in the microbial community composition. Total microbial biomass (total PLFA concentration) and microbial community composition also differed between the soil types on the dairy farmlets, with the Manawatu sandy loam having a higher total PLFA concentration. The higher microbial biomass and differences in microbial community composition correspond with increasing soil maturity and soil organic carbon. However, these effects cannot be separated from confounding sampling date and farmlets system effects. Therefore, there is minimal evidence that diverse pasture and regenerative management caused a change in the microbial community after two and a half years, however, differences potentially caused by soil type were observed and have important implications on correctly controlling for this in studies on soil biology.

CHAPTER 7: NEMATODE ABUNDANCE AND TROPHIC GROUPS

7.1 Introduction

Nematodes are among the most abundant multicellular organisms in soil and include genera at most trophic levels within the soil food web (Bongers, 1990; Yeates, 2003). They fall into eight trophic groups, of which five are commonly used, including bacterivores, fungivores, herbivores, predators and omnivores (Kennedy, 1995; Ritz & Trudgill, 1999; Yeates et al., 1993). This makes nematodes an important component of the soil food web by providing a link between the soil microflora and larger soil organisms. This crucial position in the soil food chain makes nematodes a good indicator of the state of the soil food web (Bongers, 1990; Yeates, 2003). Nematodes carry out many functions in the soil that benefit pasture growth, including cycling nutrients from microbes to make them plant available, controlling bacterial population, regulating the abundance of harmful opportunist species and providing a food source for larger soil fauna (Ferris, 2010a; Yeates, 2003). However, some soil nematodes are herbivorous, also often referred to as plant parasitic, and are therefore detrimental to plant growth, while others are internal parasites of ruminant livestock, making them important agricultural pests (Playford & Besier, 2025; Yeates et al., 1993, 2008).

Soil nematode communities are influenced by abiotic soil conditions. Soil moisture affects the abundance of nematodes, while temperature has a larger influence on diversity (Renčo et al., 2010). Nematodes are also known to be affected by many agricultural practices. Increased pasture diversity has been shown to improve nematode diversity and food web complexity (Eisenhauer et al., 2011). Conversely, intensive agricultural practices, such as

intensive grazing and application of nitrogen (N) fertilisers, have been linked to reduced nematode diversity, shifting communities to simpler food webs with higher trophic levels absent, and shifting to herbivorous dominated communities that are detrimental to plant growth (Eisenhauer et al., 2011; Herren et al., 2020; Hu et al., 2015; Lazarova et al., 2021; Schon et al., 2010). This negatively impacts the ecosystem services provided by a well-structured nematode community. Ferris et al. (2012) described a healthy nematode community as one that contains nematodes with different feeding behaviours and life strategies, including the whole range of trophic levels from fast-growing and fast-breeding bacterivorous nematodes, to low fecundity and long generation predaceous nematodes.

Family and species level identification of soil nematodes allows complex nematode indices to be applied which provide a lot of information about the biological health of the soil and the state of the soil food web (Bongers, 1990; Du Preez et al., 2022; Ferris et al., 2001; Ferris et al., 2012). However, this requires sufficient expertise to identify nematodes to this level. Therefore, in the current study, nematodes were identified to trophic group level and used to determine if there is any difference in community composition between treatments.

For nematodes, the primary consumers consists of the trophic group herbivores, fungivores and bacterivores. Herbivores feed directly on vascular plants and are therefore often referred to as plant parasitic nematodes (Yeates et al., 1993). Fungivorous nematodes feed on hyphae and fungal spores of both saprophytic and mycorrhizal fungi while bacterivores feed on soil bacteria (Yeates et al., 1993). Predatory nematodes feed on invertebrates including protozoa, rotifers, enchytraeids and other nematodes (Yeates et al., 1993). Omnivorous nematodes feed on a wide range of foods, including components from all other trophic groups, except the herbivore trophic group (Yeates et al., 1993).

This study investigates the effects that diverse pasture and regenerative agricultural practices implemented in New Zealand's pastoral farming system have on the abundance

and trophic group composition of soil nematodes. It aims to determine whether any measurable effects occur in the short term, within two and a half years of transitioning to these practices. Regenerative agriculture is stated to improve soil biological conditions. Therefore, the hypotheses being tested are that both diverse pastures and regenerative management, after two and a half years under these treatments, cause; 1) higher total nematode abundance, and 2) the nematode community to shift towards a more complex soil food web, as indicated by a higher proportion of omnivorous and predatory nematodes, and an increase in the relative importance of the fungal energy channel, as indicated by an increase in the proportion of fungivorous nematodes. Note that an increase in nematode abundance in and of itself is not necessarily an indicator of improved soil biological conditions, and only if a well-structured nematode community is present, as described in hypothesis 2, could this be interpreted as an improvement in soil health. See section 8.5.3 for further discussion on the challenges of defining soil biological health.

7.2 Methods

7.2.1 Soil processing

Soil samples were collected as described in section 3.2. Processing was carried out on the fresh soil samples within 3 hours of the samples being collected. Soil cores were thoroughly crushed by hand while still in the zip lock bag to break up the soil cores and aggregates. The soil was then tipped onto a clean large plastic sheet and homogenised by lifting one corner of the sheet making the soil roll over to the opposite corner, doing the same for each corner of the sheet and repeating the whole process six times. All equipment used was washed with water and wiped down with 70% ethanol between samples.

7.2.2 Nematode extraction

Nematodes were extracted using a modification of the Baermann funnel method described by van Bezooijen (2006). A filter holder was created by placing a ~10 cm^2 piece of industrial milk filter between a 76 mm and 62 mm rubber union cone seal, as shown in Figure 7.1. A glass funnel was set up on a clamp stand with a rubber hose attached and a clamp to seal the hose, as shown in Figure 7.2. The glass funnel was filled three quarters of the way up with reverse osmosis water, ensuring no air bubbles were present in the rubber tube or neck of the funnel. Approximately 25 g of fresh soil was placed in the filter holder and uniformly distributed. The filter holder with soil was then placed on top of the funnel (Figure 7.2). The funnel was carefully topped up with reverse osmosis water so that the base of the soil in the filter holder was in contact with the water, but the soil was not completely submerged. The top of the filter holder and funnel were covered with plastic-wrap to prevent evaporative water loss and prevent cross contamination of samples. Samples were left for 48 hours. They were checked after 12 hours to ensure the water level in the funnel was sufficient and got topped up if necessary. After 48 hours a ~10 ml sample was collected from the funnel by briefly opening the clamp on the rubber hose. Between samples, all equipment was rinsed with reverse osmosis water, sprayed with 70% ethanol and left for 30 seconds then rinsed again with reverse osmosis water to remove the ethanol. For each sample, three replicate extractions were carried out.

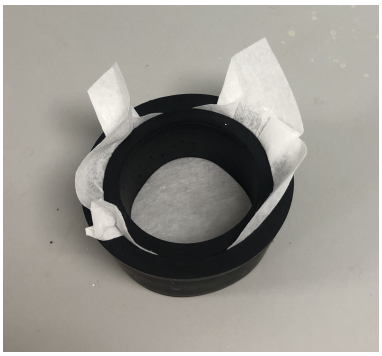

Figure 7.1: Filter holder setup for nematode extraction.

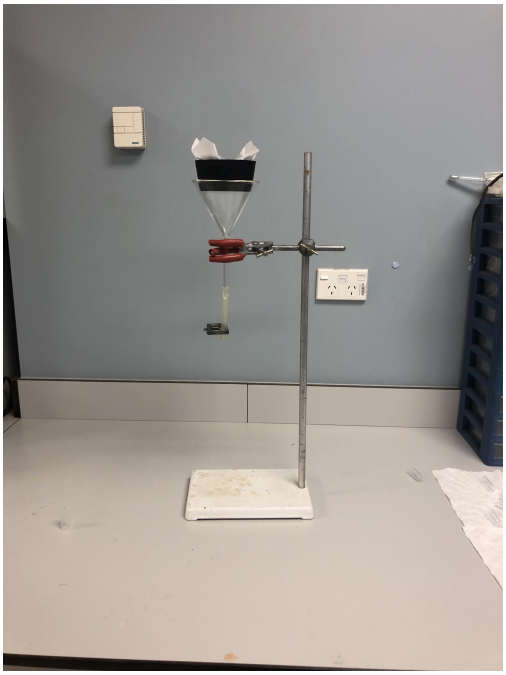

Figure 7.2: Baermann funnel setup for extracting nematodes.

7.2.3 Nematode preservation

Preservation of the nematodes was carried out the same day the nematode extracts were collected. Nematodes were heat-killed by holding the container with the nematode sample in boiling water for 1-2 minutes. To preserve the nematodes, ~10 ml of 10% formalin was then added to the sample. The preserved samples were refrigerated at 4 ^{o}C until they could be analysed.

7.2.4 Nematode abundance

Refrigerated samples were allowed to settle overnight. They were carefully removed from the refrigerator to ensure the nematodes, which had settled to the bottom, were not disturbed. Samples were then reduced to ~10 ml by carefully pipetting excess liquid off the top of the sample. The formalin was diluted by adding ~40 ml of reverse osmosis water, as total counts are carried out over an open dish. Samples were again allowed to

settle for 4 hours, or overnight, in the refrigerator before being reduced to ~10 ml again, as described above. They were then poured onto a Doncaster counting dish (Doncaster, 1962) and the container was rinsed two times into the dish with a small amount of reverse osmosis water to ensure all the nematodes were removed. The dish with the sample was left for 10 to 15 minutes to allow the nematodes to settle to the bottom. All of the nematodes in the second, fourth and eighth rings of the dish were counted using an Olympus SZX12 dissecting microscope at 25 times magnification. After counting, the sample was poured into a 100ml beaker and the dish rinsed three times with water to ensure all the nematodes were removed from the dish. Replicate extraction of the same sample were pooled together after counting to ensure sufficient nematodes were present in the sample for nematode identification.

7.2.5 Re-preservation and concentration of nematodes

As the formalin in the samples was diluted for carrying out the total counts, its concentration was too low to keep the nematodes preserved (formalin concentration should be no lower than 4% (van Bezooijen, 2006.)). To carry out nematode identification, the concentration of nematodes in the sample must be sufficiently high that more than 100 nematodes are transferred to a slide in 100 µl of sample, as at least 100 nematodes need to be identified. After carrying out total counts and pooling the replicate extractions, the samples were allowed to settle for 3 hours and reduced to ~5 ml by pipetting excess liquid off the top without disturbing the nematodes settled on the bottom. They were then poured into 15 ml falcon tube, rinsing the container into the falcon tube 3 times and allowed to settle overnight in the refrigerator. Following this, they were reduced to ~2 ml by carefully pipetting the excess liquid off the top and then 2 ml of 10% formalin was added. Samples were stored in the refrigerator until identification could be carried out.

7.2.6 Nematode identification

Re-preserved samples were allowed to settle overnight before being reduced to ~1 ml by pipetting excess liquid off the top. A large (50 by 76 mm) glass slide was prepared by stamping a square of wax on it using a wire loop that was heated in a Bunsen burner flame and briefly placed on the solid wax. The sample was thoroughly mixed before placing 100 µl of the sample in the center of the wax square on the slide using a wide mouth pipette tip. A large (45 by 50 mm) coverslip was placed over the wax square containing the sample. The slide was then placed on a slide warmer at 70 ^{o}C for just long enough to allow the wax to melt and form an air tight seal. At least 100 nematodes on each slide were identified using an Olympus BX51 compound microscope at 400x magnification, with phase contrast. The trophic group of each nematode was identified based on the head shape and internal structures. Trophic groups recorded were herbivorous, bacterivorous, fungivorous, omnivorous or predatory.

7.2.7 Statistical analysis

Although the nematode extractions were carried out on field moist soils, all counts are reported relative to the equivalent oven dry mass of the soil, determined as described in section 4.2.2. The counts from triplicate extractions of the same sample were averaged and the statistical analysis was carried out on the average values. The number of nematodes in different tropic groups was converted to the proportion of the total in that sample, as an arbitrary number of nematodes were identified which was not always consistent across samples. Statistical analysis was carried out on these proportions.

Statistical analysis was conducted by fitting an analysis of variance (ANOVA) model using the aov() function from the stats v4.5.0 R package (R Core Team, 2025), which tests the null hypothesis that the means of the response variable being tested does not differ

significantly between treatments, with an F-test. Sampling date was included in each model as a blocking variable, to account for differences caused by sampling on different days. This also takes into account soil type differences on the dairy farmlets as the soil types were sampled on different days. Therefore, no soil type variable was included. Soil moisture and soil temperature were included as covariates. Treatment was included as a single factor with four levels (Std-Con, Std-Reg, Div-Con and Div-Reg). The models were fitted separately for the sheep and the dairy farmlets. If the ANOVA indicated that there was a significant difference in treatment means, a pairwise comparison of the treatment levels was carried out using the TukeyHSD() function from the stats v4.5.0 R package (R Core Team, 2025), which compares the mean of the response variable being tested for each of the treatment levels and determines which are different based on the Studentized range statistic using Tukey's 'Honest Significant Difference' method. All results are reported in the format mean ± standard error. All counts reported are the number of nematodes per m^2 of soil to a depth of 7.5cm. Results considered statistically significant at $\alpha < .05$, unless stated otherwise.

A comparison of the average nematode counts in the different soil types on the dairy farmlets was carried out by fitting an analysis of variance model, as above, however the sampling date blocking variable was omitted to allow the soil types, which were sampled on different days, to be compared. This confounding of sampling date with soil type means it is not possible separate differences in soil type with sampling date.

Multivariate analysis was carried out on the proportion of nematodes in each trophic group to compare the nematode community structure in each treatment, using the adonis2() function from the vegan v2.6.10 R package (Oksanen et al., 2025), which uses a permutation test on distance matrices with pseudo F-ratios. Sampling date was included as a blocking variable, however, as adonis2() does not contain the capacity to model covariates, these were not included in this analysis. PCA plots were created using the PCA() function from the FactoMineR v2.11 R package (Lê et al., 2008) and the

fviz_pca() function from the factoextra v1.0.7 R package (Kassambara & Mundt, 2020).

7.3 Results

7.3.1 Nematode abundance

The sheep farmlets had 2.4 times higher nematode abundance across all treatments than the dairy farmlets, with a mean of $5.54 \times 10^6 \pm 3.96 \times 10^5$ m^{-2}, while the dairy farmlets had a mean of $2.29 \times 10^6 \pm 2.4 \times 10^5$ m^{-2}.

On the sheep farmlets, the ANOVA showed that there was a significant difference in nematode abundance between treatments ($F_{3,7} = 4.66$, $p = .04$). However, a pairwise comparison indicated that none of the treatment levels differed significantly at $p<.05$ (Table 7.1). Comparing the effect sizes shows that treatments under different management differed by an order of magnitude more than treatments under the same management, with the contemporary treatments (Std-Con and Div-Con) containing between $16.6x10^5$ and $16.6x10^5$ m^{-2} more nematodes than the regenerative treatments (Std-Reg and Div-Reg), while treatments within the same management type only differed in nematode number by around $3.0x10^5$ m^{-2} (Table 7.1, Figure 7.3). The contrasting results of the F-test and the pairwise comparison is likely due to the adjustment of p-values to account for multiple tests in the pairwise comparison. Therefore, it is likely that differences between treatments detected by the F-test are as a result of differences in management type, indicating that there is some evidence that nematodes differ in abundance under different management.

Table 7.1: Average difference in nematode abundance between treatments on the sheep farmlets, with adjusted p-values from a Tukey's Honest Significant Difference test. Highlighted rows are treatments that have the same management type while unhighlighted rows have contrasting management types.

Pairwise comparison	Difference ($x10^5$ m^{-2})	Adjusted p-value
Std-Reg:Std-Con	-13.5	0.132
Div-Con:Std-Con	3.1	0.929
Div-Reg:Std-Con	-10.5	0.277
Div-Con:Std-Reg	16.6	0.060
Div-Reg:Std-Reg	3.0	0.935
Div-Reg:Div-Con	-13.6	0.129

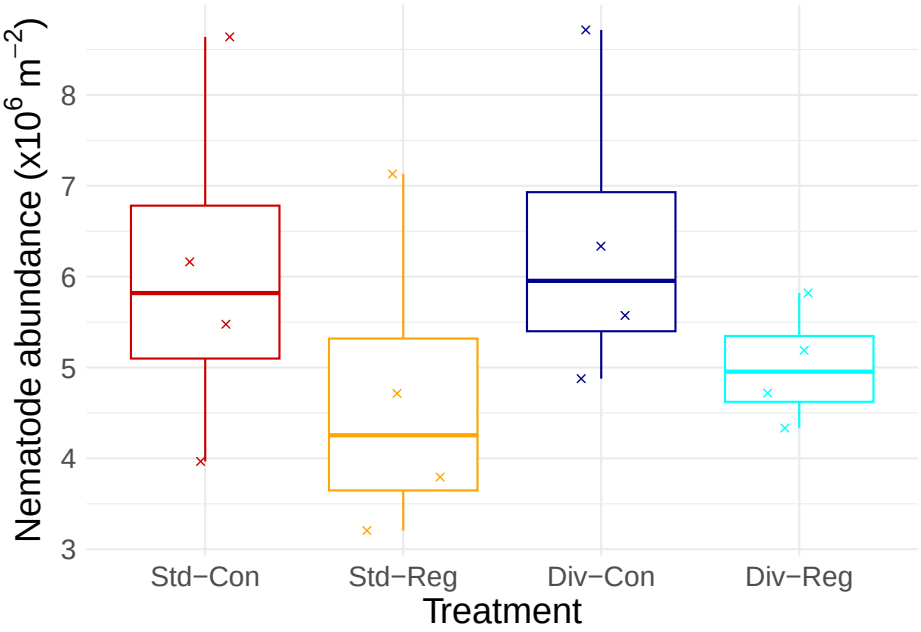

Figure 7.3: Soil nematode abundance measured to 7.5 cm depth in each treatment on the sheep farmlets. Differences were not significant at p<.05.

There was no significant differences in nematode abundance between treatments on the dairy farmlets ($F_{2,8}$ = 0.26, p = .78). However, there was a significant difference in nematode abundance between soil types on the dairy farmlets ($F_{1,14}$ = 13.26, p = .003), with the Rangitikei loamy sand soil having a higher mean nematode abundance of $2.91 \times 10^6 \pm 2.36 \times 10^5$ m^{-2} compared to the Manawatu sandy loam soil which had a mean abundance of $1.66 \times 10^6 \pm 3.03 \times 10^5$ m^{-2} (Figure 7.4).

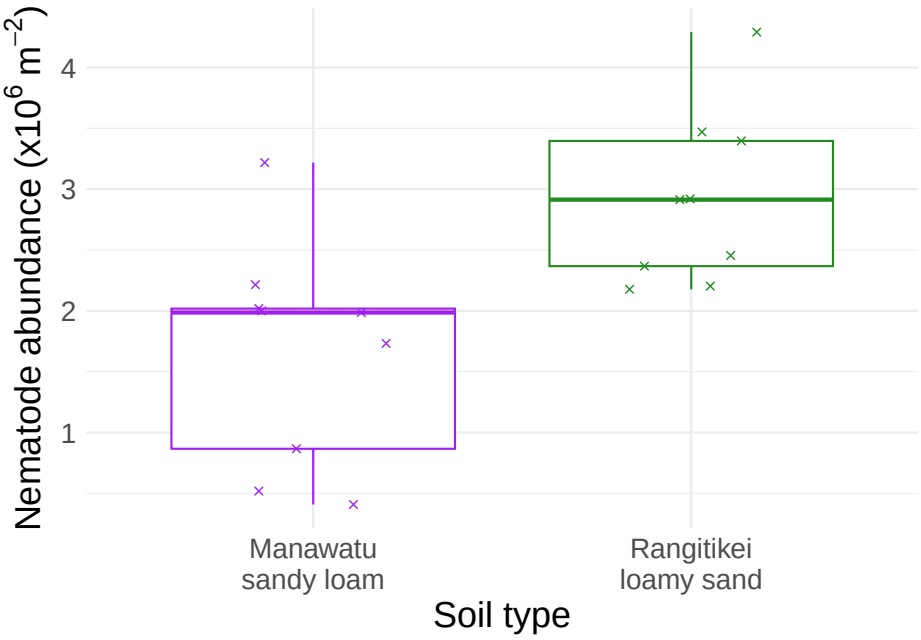

Figure 7.4: Soil nematode abundance measured to 7.5 cm depth in the Manawatu sandy loam and the Rangitikei loamy sand soil types on the dairy farmlets. The difference in mean abundance between the two soil types was significant (p<.05).

7.3.2 Nematode community composition

On both the sheep and the dairy farmlets, the dominant nematode trophic groups were bacterivorous and herbivorous, with low numbers of fungivorous, omnivorous and predatory nematodes identified (Figures 7.5 and 7.6).

On the sheep farmlets, the proportion of nematodes in each trophic group did not significantly differ between treatments. On average, bacterivorous nematodes made

up 43.5±3.6% of the sample, while herbivorous nematodes made up 53.3±3.4% on the sheep farmlets. Omnivorous nematodes made up 2.5±0.5% of the sample and the other trophic groups made up less than 1% on the sheep farmlets.

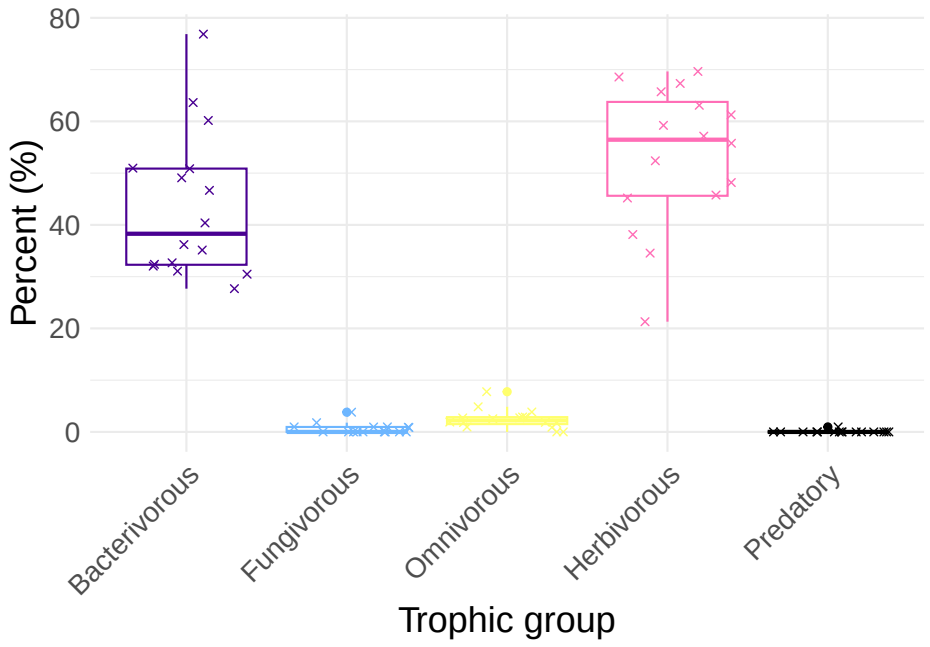

Figure 7.5: Percentage of soil nematodes in each trophic group on the sheep farmlets.

On the dairy farmlets, there were marginally insignificant differences in the proportion of bacterivorous nematodes measured between treatments ($F_{2,8}$ = 4.14, p = 0.058). The pairwise comparison showed that the Div-Reg treatment had a significantly higher proportion of bacterivores than the Std-Con treatment (p = 0.0497), with mean proportions of 63.6±5.1% and 52.0±4.5%, respectively. Although only significant at p<.1, the proportion of herbivorous nematodes had the opposite effect, with the Div-Reg treatment having a lower proportion of herbivorous nematodes than the Std-Con treatment (p = .08). This indicates that the increase in proportion of bacterivores in the Div-Reg treatment is likely offset by a decrease in proportion of herbivores. However, this was not significant at p<.05. On average, across all treatments, the dairy farmlets had a higher proportion of bacterivorous nematodes (57.4±2.8%) and lower proportion of herbivorous nematodes (36.8±2.6%) compared to the sheep farmlets.

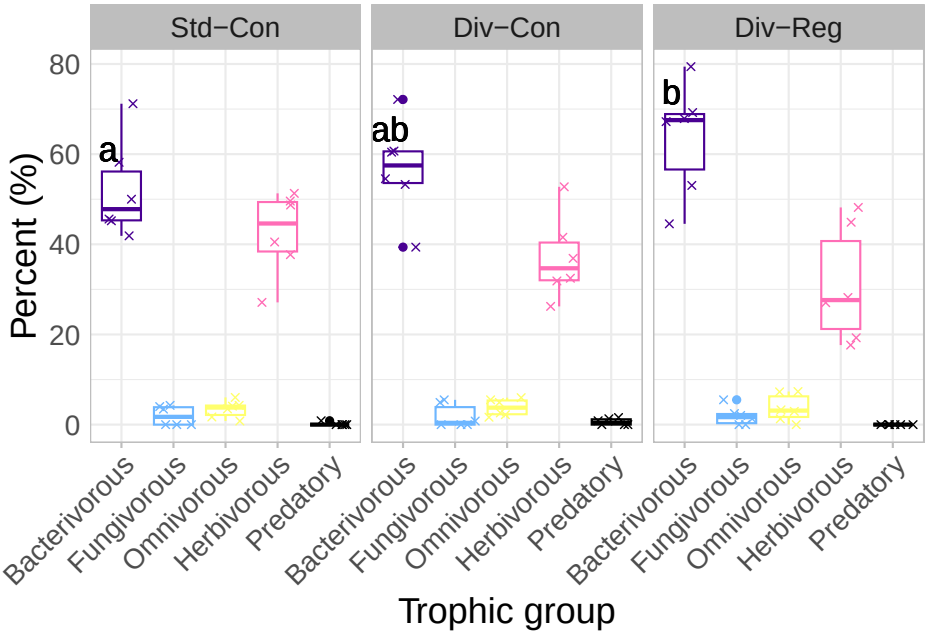

Figure 7.6: Percentage of soil nematodes in each trophic group within each treatment on the dairy farmlets. Letters indicate significant difference in proportion of bacterivorous nematodes between treatments (p<.05).

Multivariate analysis indicated that none of the treatments differed from each other on either the sheep or the dairy farmlets ($F_{3,12}$ = 0.32, p = .83, and $F_{2,15}$ = 1.53, p = .24, for the sheep and the dairy farmlets, respectively). There was also no significant difference in community composition between the soil types on the dairy farmlets ($F_{1,16}$ = 0.34, p = .63). There was a significant difference in the multivariate analysis ($F_{1,32}$ = 11.83, p = .001) indicating that the community composition of nematodes differed between the sheep and the dairy farmlets (Figure 7.7). This supports the finding that the sheep farmlets had a higher proportion of herbivorous nematodes, while the dairy farmlets had more bacterivorous nematodes, as the main axis of the PCA in Figure 7.7 is defined by increasing herbivorous nematodes and decreasing bacterivorous nematodes in the direction of the sheep farmlets.

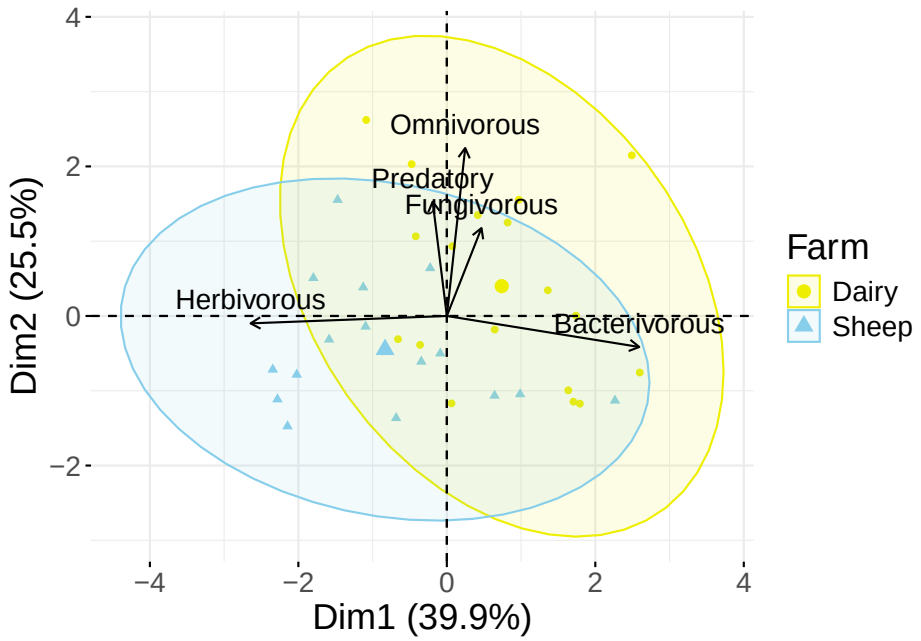

Figure 7.7: Principle component analysis of the nematode community composition, based on trophic groups, comparing the sheep and the dairy farmlets.

7.4 Discussion

Nematode abundances in this study have been reported as the number of nematode m^{-2} of soil to a depth of 7.5cm. However, many previous studies report nematode abundances to a depth of 10cm. Therefore, the average abundances are discussed here again after recalculating to 10cm depth to allow comparison. However, it is noted that since samples were only taken to a depth of 7.5cm, these values could be an overestimation of the actual number of nematodes, as nematode numbers decrease with depth (Renčo et al., 2010; Schon et al., 2010; van Bezooijen, 2006; Yeates et al., 1999, 2008). The mean nematode abundance adjusted to a depth of 10cm depth on the sheep farmlets was $7.39 \times 10^6 \pm 5.28 \times 10^5$ m^{-2} while on the dairy farmlets it was $3.05 \times 10^6 \pm 3.2 \times 10^5$ m^{-2}. This is within the range of 10^5 to 13×10^6 m^{-2} found by Yeates (1984) across a range of New Zealand soils. However, the nematode abundance on the sheep farmlets is much higher than the maximum found by Yeates (1978) on the same soil type (Tokomaru silt loam) but

under dairy pasture. The poorly drained Tokomaru silt loam soils are highly susceptible to pugging damage under intensive dairy grazing (Howes, 2019; Singleton & Addison, 1999), possibly explaining this lower nematode abundance in these previous studies. The abundances measured in the current study are also much higher than what was found in New Zealand by Parfitt et al. (2010) under long term sheep pasture on acidic orthic brown soils, in the Manawatu, and by Schon et al. (2010) under dairy pasture grazing in Taranaki on allophanic soils. The nematode abundance measured on the dairy farmlets in the current study was in the upper end of the range reported by these previous studies. Some of this can be attributed to the possible overestimation due to sampling depth, as discussed above, however even when comparing the average abundance without recalculating to a 10 cm depth, the nematode abundance measured from the sheep farmlets in the current study is well above these reported ranges, being more comparable to the maximum found by Steel et al. (2018) during composting. Samples in the current study were extracted over 48 hours using a Bauermann funnel method, while all of the previous studies cited here used the Whitehead & Hemming (1965) tray method, or modification thereof, and extracted for 24 hours. Different extraction methods cannot be directly compared, and lack of standardisation is an issue within nematode studies (McSorley & Frederick, 2004; van Bezooijen, 2006). The longer extraction time of the samples in the current study could be the cause for the elevated total nematode counts, as McSorley & Frederick (2004) showed that increasing the extraction time increased the number of nematodes extracted. Similarly, Bell & Watson (2001) found that increasing extraction time from 24 to 48 hours increased the number of nematodes extracted from around 60% of the total extracted in 144 hours to around 80%. Therefore, studies that used a 24 hour extraction time may have missed a large portion of the nematodes. Any differences in extraction efficiency of the different extraction methods are difficult to infer.

In contrast with hypothesis 1, the current results indicate that treatments under regenerative management (Std-Reg and Div-Reg) have lower nematode abundance

than the treatments under contemporary management (Std-Con and Div-Con) on the sheep farmlets. A number of other studies on pastoral farms in New Zealand have found that reducing grazing intensity causes nematode abundance to decrease (Parfitt et al., 2010; Schon et al., 2010), with similar trends observed in other parts of the world (Bardgett et al., 1997; Hu et al., 2015). As the regenerative management in this study is defined by increased stock rotation length and increased post grazing residuals of pastures compared to contemporary management, thereby reducing grazing pressure despite having similar stocking rates, the results in this study support these findings. The reason for higher nematode numbers under more intensive grazing has been attributed to increased food for bacterivorous nematodes through increased manure inputs from grazing herbivores and increased food for herbivorous nematodes through increased quality and quantity of root exudates, higher root mortality and increased plant growth due to the grazing (Bardgett et al., 1997; Hu et al., 2015; Schon et al., 2010). In a study of long term pasture under sheep grazing, Parfitt et al. (2010) found low stock numbers and low soil fertility reduced nematode abundance and attributed this to lower net primary production causing reduced below ground inputs. This may explain the differences in nematode abundance between management types in the current study, as it is also on a sheep grazed pasture, with lower N fertiliser inputs occurring along with reduced grazing intensity on the regenerative treatment. The response of nematode abundance to inorganic N fertiliser application is complex, with both positive and negative relationships observed (Atira & Kakouli-Duarte, 2025; Parfitt et al., 2012; Sarathchandra et al., 2001). Decreases in nematode abundance with increasing N fertiliser applications have been associated with unfavourable changes in soil chemical properties and pH, while increases in nematode abundance due to N fertiliser applications have been linked with increased primary productivity of plants and microbial growth, resulting in an increase in nematode food availability (Atira & Kakouli-Duarte, 2025). Therefore, higher nematode density under contemporary management is likely caused by increased grazing pressure resulting

in higher nutrient return to the soil through animal excreta, and increases in below ground inputs from plant by stimulating plant growth and increased excretion of root exudates and root turnover. The higher N inputs on the contemporary managed treatments are likely to cause an increase in net primary production, which would contribute to this. As the majority of the nematodes are herbivorous, a reduction in nematode numbers is likely to be beneficial to pasture production, as this reduction is not offset by an increase in the proportion of herbivorous nematodes.

Hu et al. (2015) suggested that reduced plant cover under higher grazing intensity changes soil abiotic factors which can influence soil nematodes. Evidence of this is seen in this study, as soil temperatures were significantly higher on the contemporary treatment than on the regenerative treatment (see chapter 4). Nematode response to temperature and seasonal dynamics are difficult to determine. Renčo et al. (2010) suggested that soil moisture is more important in determining nematode abundances than soil temperature. Yeates (1981) found no clear seasonal trend in nematode abundances, however suggests cold soil temperatures can limit numbers, while Yeates (1978) found that on the Tokomaru silt loam soil, soil nematode count decreased in summer, with negative correlations with temperature, suggesting abundances decrease under dry soil conditions. In contrast, Renčo et al. (2010) found that nematode numbers increased with temperature, although they received consistent rainfall during their study. Bardgett et al. (1997) found higher nematode numbers in summer than winter. Therefore, it is likely that nematode density increases with temperature, however clear seasonal patterns linked to temperature often do not occur due to the overriding effect of moisture limitation in summer. As soils had not begun to dry out when samples for the current study were collected, due to late season rainfall (see chapter 4), it is possible that nematode numbers in the contemporary treatment had increased faster in response to the higher temperatures observed, without being moisture limited, and this caused the difference in nematode numbers. If this is the case, it is possible that the differences observed in the current study are a transient effect,

with nematode abundances in the regenerative treatments reaching similar levels as observed in the contemporary treatments as soils continue to warm up towards summer, or abundances in all treatments decreasing as moisture becomes limiting in summer. Repeated sampling throughout the year would be needed to determine whether this is the case.

Bacterivorous and herbivorous nematodes were the dominant trophic groups on both the sheep and the dairy farmlets, together making up over 90% of all nematodes. This is indicative of disturbed basal food webs that are lacking the higher trophic groups (omnivores and predators) of more complex food webs. Many studies indicate that intensive grazing shifts the nematode community towards simpler food webs dominated by bacterial and plant feeders and lacking the higher trophic groups, as well as reducing the populations of fungivorous nematodes (Hu et al., 2015; Lazarova et al., 2021; Schon et al., 2010). This is attributed to increased manure inputs from grazing herbivores increasing bacterial nematode food sources, while stimulation of root growth and turnover increases herbivorous nematode food sources (Bardgett et al., 1997; Hu et al., 2015; Schon et al., 2010). However, this could also indicate that intensive grazing makes the plants more susceptible to attack by herbiovorous nematodes, as more energy and resources are used for regrowth, leaving less for defences. N application has also been linked to increased herbivorous nematodes and reduced fungivorous and predatory nematodes (Herren et al., 2020). No treatment effect was observed on the sheep farmlets to indicate that reduced grazing pressure and N application caused a shift in the community composition away from this basal state, as is suggested in previous studies. On the dairy farmlets, there is a higher proportion of bacterivorous nematodes at the expense of the herbivorous nematodes in the Div-Reg treatment. This could indicate the start of a shift away from the herbivore-dominated nematode communities found in the other treatments, however as not all the treatment combinations are present on the dairy farmlets it is not possible to determine if this effect is caused by the management or

the pasture type. Furthermore, the same effect is not observed on the sheep farmlets, which contains all the treatment combinations, indicating this may not be a consistent effect. There are also a number of studies that suggest diverse pastures increase nematode diversity and promote complex food webs, with higher nematode trophic levels (Eisenhauer et al., 2011; Lazarova et al., 2021). However, this effect was not measured in the current study.

Although there is some evidence from the dairy farmlets of a shift in the nematode community composition away from a basal state, hypothesis 2 is not supported by the current study. The lack of response in nematode community composition to either pasture diversity or grazing intensity, despite evidence in the literature that they have an influence, could be due to a number of reasons. Firstly, this study was, at the time of sampling, only running for two and a half years. Many studies indicate that changes in nematode community composition occur slowly over many years (Eisenhauer et al., 2011; Parfitt et al., 2010; Yeates et al., 1999). Eisenhauer et al. (2011) found that nematode communities took 5 years to shift to a more structured and complex food web when changing from low to high plant diversity pastures. Therefore, it is possible that no treatment effect is observed because insufficient time has passed since establishing the study for a change in the nematode community to occur. This study is part of an ongoing seven-year trial, in which nematode analysis will continue to be carried out, which will contribute to determining whether this is the case. Secondly, identifying nematodes to just trophic group level, rather than genera or species level, could obscure any trends of increasing diversity due to the low resolution of trophic level designation. Identifying the nematodes to a higher level of specificity, or by including metagenomic analysis of nematode DNA, could improve the resolution of data. Thirdly, my limited experience in identifying nematodes could mean that the nematodes were not accurately assigned to the correct trophic group, which could further obscure any trends present. Finally, for the pasture diversity effect specifically, the lack of difference between the levels of pasture

type could also be due to the relatively low number of pasture species present in the diverse treatment and the small difference in number of species between the standard and diverse treatments. The study by Eisenhauer et al. (2011) which found an effect of diverse pasture on nematode community composition had treatments ranging from monocultures (one plant species) to sixty different plant species. In contrast, in the current study, the diverse treatment had seven different pasture species remaining (representing the three functional groups of grasses legumes and herbs) in the sward at the time of sampling, while the standard pasture had five (primarily grasses and legumes), of which two were self sown and present at low percentages (see Table 3.1).

Nematode abundance differed between the sheep and the dairy farmlets, as well as between the two soil types on the dairy farmlets. As the sheep farmlets has a different soil type to the dairy farmlets, it is likely that differences between the sheep and the dairy farmlets are due to soil type rather than farm system. Nematode abundance increased as soil texture grain sizes decreased; Manawatu sandy loam (dairy farmlets) had the lowest, Rangitikei loamy sand (dairy farmlets) had higher and Tokomaru silt loam (sheep farmlets) had the highest nematode abundance. In contrast Bell & Watson (2001) found more nematodes in the coarser textured silt loam soil than the clay loam. Eisenhauer et al. (2011) indicated that differences in soil type affected the nematode communities in their study. Differences in soil organic matter could also have an influence, with the Tokomaru sandy loam soil on the sheep farmlets having higher soil carbon than the soils on the dairy farmlets (P Jeyakumar, personal communication, July 28, 2025). However, as the farmlet system and soil types are fully confounded, there is no way to separate the effects of these. Similarly, within the dairy farmlets, soil type is fully confounded with the sampling date and therefore it is not possible to determine if the difference is due to soil type or caused by differences in sampling date, therefore these results should be interpreted with caution. Further study would be needed to confirm whether there is a soil type effect. Nematode community composition also differed between the sheep and

the dairy farmlets, as indicated by the multivariate analysis. Again, this effect could be due to soil type or to stock type and farm management. Grain size has been shown to influence nematode communities in marine sediments, with increasing grain size (courser texture) increasing nematode diversity (Heip & Decraemer, 1974). This indicates that it is possible the difference in community composition between the sheep and the dairy farmlets is caused by soil type.

7.5 Conclusion

Across all treatments on both the sheep and the dairy farmlets, the nematode community composition indicates a basal, degraded soil food web dominated by herbivorous and bacterivorous nematodes. The results indicate that there is some evidence that regenerative agricultural management and diverse pasture have an impact on soil nematodes after two and a half years of implementation. On the sheep farmlets, regenerative management caused total nematode abundance to be reduced, while on the dairy farmlet, diverse pasture and regenerative management shifted the nematode trophic group composition to reduce herbivorous nematodes and increase bacterivorous nematodes. These changes are possibly due to differences in the grazing management between treatments. However, as consistent differences between the treatments and the sheep and the dairy farmlets were not observed, generalised conclusions of the effects of the treatments cannot be drawn. Differences in both nematode abundance and community composition were observed between the soil types and the farmlet systems. This could be due to differences in soil texture, soil carbon or stock type and associated management practices. Therefore, there is some evidence to suggest that regenerative management caused a change in the nematode community within two and a half years. The finding of differences potentially caused by soil type have important implications on correctly controlling for this in studies on soil biology.

CHAPTER 8: GENERAL DISCUSSION

The current study investigated how various components of soil biota are influenced by diverse pastures and regenerative agricultural management when implemented in New Zealand's pastoral system. It attempted to determine whether the regenerative agriculture (RA) objective of improving soil health is achieved. The results from the analysis of soil bacteria physiological states using fluorescent microscopy did not indicate any consistent differences in the active or dead bacteria pools between treatments. Phospholipid fatty acid (PLFA) analysis indicated that there was an increase in fungal to bacterial PLFA ratio in the diverse pasture under regenerative management treatment on the dairy farmlets, despite no corresponding difference on the sheep farmlets. The largest and most consistent differences in total PLFA, total 16:1ω5 neutral lipid fatty acid (NLFA) and community composition were between the soil types on the dairy farmlets and between the two farmlet systems. This was also the case for nematode abundance and trophic group composition, however, there is some evidence that the nematode abundances were higher under contemporary management on the sheep farmlets and that the trophic group composition shifted towards bacterivore dominated in the diverse pasture under contemporary management on the dairy farmlets. The analysis of the soil abiotic conditions showed that soil moisture and temperature were being influenced by the treatments, on both the sheep and the dairy farmlets, with both diverse pasture and regenerative management causing soil temperatures to be lower and some evidence from the dairy farmlets that these treatments also cause soil moisture to be higher.

The small effect sizes between treatments seen in this study could be due to the fact that the treatments are all under the same long term pastoral agriculture system. New Zealand's pastoral soils are infrequently cultivated and are therefore relatively stable systems (Schipper et al., 2017). Much more dramatic contrasts in soil biota have been

observed between intensive arable cropping systems and low input extensive pasture systems or even natural environments (Kabir, 2005; Lazarova et al., 2021; Tomazelli et al., 2023; Zelles et al., 1992). Overall, the microbial community appears to be dominated by the bacterial decomposition pathway and the bacterivorous and herbivorous energy channels.

8.1 Composition of microflora and microfauna communities

The results indicate a prevalence of the bacterial decomposition pathway in all soils considered in the current study. The fungal to bacterial PLFA ratio was low (0.14 ± 0.002 (fungal PLFA:bacterial PLFA around 1:7.1) for the sheep farmlets and 0.15 ± 0.003 (fungal PLFA:bacterial PLFA around 1:6.7) for the dairy farmlets). This is lower than has been found in other pasture soils (0.34 - 0.5) (Bardgett & McAlister, 1999; de Vries et al., 2006), and forest soils (0.3 - 0.8) (Liang et al., 2008; Ohtonen et al., 1999) but higher than under conventionally tilled soils (0.07) (Mathew et al., 2012). However, direct comparisons between studies is difficult due to differences in methods used, biomarker designations and conversion factors, with de Vries et al. (2006) questioning whether comparisons are appropriate. The total number of fungivorous nematodes was small (>1% of the total population on the sheep farmlets and >2% of the total population on the dairy farmlets) relative to the number of bacterivorous nematodes. The high number of herbivorous and bacterivorous nematodes indicated that these were the dominant energy channels present in the studied soils, with only a minor contribution from the fungal energy channel. The fungal to bacterial PLFA ratio was higher in the Div-Reg treatment on the dairy farmlets. However, this did not correspond with an increase in fungivorous nematodes, with bacterivorous nematodes actually being higher in this treatment than in the other treatments on the dairy farmlets. This appears to conflict with the result of increasing fungal to bacterial PLFA ratio. However, the proportion of fungivorous

nematodes of the total population was very low, especially compared to bacterivorous nematodes, so it is possible that this small change in food source availability was insufficient to cause a noticeable increase in fungivorous nematodes. The increase in bacterivorous nematodes is likely driven by some factor other than the change in food source availability. As herbivorous and bacterivorous were the dominant trophic groups present, a shift in their dynamics is more likely to be the cause of change in proportion of bacterivorous nematodes. This is discussed further below. As fungi make up only a small portion of the microbial community in the studied soils, as indicated by the PLFA analysis, this change in fungi to bacteria ratio likely had negligible effect on food source availability for bacterivores. Bacterivorous nematodes were lowest in the Std-Con treatment on the dairy farmlets, which also had the highest number of active bacteria. This could indicate that higher predation pressure by bacterivorous nematodes is the cause for reduced active bacteria (Ingham et al., 1985; Yeates, 2003). The prevalence of bacterivorous and herbivorous energy channels, the lack of nematodes of higher trophic groups and the low fungal to bacterial PLFA ratio, indicate that the soil food webs across all treatments are in a disturbed, basal state (de Vries et al., 2006; Ferris et al., 2001; Ferris, 2010b; Lazarova et al., 2021; Parfitt et al., 2010; Yeates et al., 1997).

8.2 Abundance of microflora and microfauna

There was no difference in soil microbial biomass between treatments on either the sheep or the dairy farmlets, as measured by total PLFA concentration and by fluorescent microscopy. However, total nematode abundance did differ, with both treatments under contemporary management having higher total nematode abundances than either treatment under regenerative management on the sheep farmlets. Higher nematode abundances under more intensive grazing management (higher stocking rate and grazing duration) has been attributed to an increase in food availability for nematodes, particularly

for bacterivorous nematodes through increased manure inputs and for herbivorous nematodes through physiological responses of the plant roots (Bardgett et al., 1997; Hu et al., 2015; Schon et al., 2010). Bardgett et al. (1997) found that PLFA concentration, along with nematode abundances, were lower under ungrazed pastures compared to pastures grazed by sheep. In contrast, Parfitt et al. (2010) observed no change in PLFA concentration under different grazing management despite a change in nematode abundance. This is consistent with the current study, where nematode abundances differed between grazing managements, but PLFA concentration did not. Therefore, the explanation that higher food availability for bacterivores is the cause for increasing nematode abundance in the current study is not supported. This suggests that changes in the herbivorous nematodes' food source might be the dominant driver of nematode abundance increase. However, if this was the case then a change in the relative proportion of bacterivorous and herbivorous nematodes between treatments would be expected. This was not observed on the sheep farmlet, however, it was observed on the dairy farmlet, where bacterivorous nematode numbers were lower in the Std-Con treatment, and although not significantly different, herbivorous nematode numbers were higher. This aligns with what is expected if an increase food source for herbivorous nematodes and corresponding increase in their numbers is the cause of increasing total nematode abundance. Nitrogen application on the contemporary managed treatments would also increase the food source for herbivorous nematodes, by increasing plant growth, and has been shown to increase herbivorous nematode abundance (Herren et al., 2020). However, as these two responses (the change in total abundance with management and the change in relative abundance of bacterivorous and herbivorous nematodes) were not observed in the same samples, or even on the same farmlet system, these are tentative explanations and may be unjustified extrapolations. The microscopy analysis indicated that on the sheep farmlet there was a higher number of dead bacteria in the Div-Con treatment, were an increase in nematode abundance also

occurred. However, as this was not observed across both contemporary management treatments on the sheep farmlet, which had higher nematode abundances, this does not provide a convincing explanation for this change.

8.3 Soil type and farmlet system differences

The most consistent and distinct differences in soil biota in this study were between different soil types and between the sheep and the dairy farmlets. The sheep farmlets had higher total microbial biomass (total PLFA concentration, total NLFA 16:1ω5 concentration and total bacteria) and higher nematode abundances than the dairy farmlets. Effects of soil type varied on the dairy farmlets, with the Manawatu sandy loam having higher total PLFA but lower nematode abundance than the Rangitikei loamy sand. This could be due to PLFA concentration being more strongly correlated with soil organic matter (SOM) (Frostegård et al., 1991), which is higher in the Manawatu sandy loam soil, while a greater effect of soil texture, which is coarser in the Manawatu sandy loam soil, on nematodes abundance (Eisenhauer et al., 2011; Heip & Decraemer, 1974). While it is possible that the difference between the sheep and the dairy farmlets is caused by differences in stock type and associated management, it is probable that differences in soil type had a large influence. The Tokomaru silt loam soil is more mature, with greater weathering and higher total soil carbon and therefore SOM, as well as a finer texture. This means organic matter has had more time to accumulate and biological activity to develop, resulting in the consistently higher measures of total soil biota (Ohtonen et al., 1999). The sheep and the dairy farmlets both differed in the composition of nematode tropic groups and the community structure of microbes, with the latter also differing between the soil types on the dairy farmlets. Although differences between soil types or between the farmlet systems were not of primary interest in this study, they are worth noting as they have important implications on accounting for differences in soil type in soil biology studies and

the importance of accurate soil descriptions. Highly significant differences were observed between the Manawatu sandy loam and Rangitikei loamy sand soils, which are very similar, being physically adjacent and formed from the same parent material by the same process, and influenced by the same soil forming factors. This highlights the importance of accurately mapping soils and taking soil type into account when designing and setting up field experiments, as even relatively small changes in soil conditions can affect the results. Despite this, any conclusions drawn from comparing the farmlet systems, and comparing the soil types on the dairy farmlet, must be interpreted with care, as these are fully confounded with the stock type and associated management practices as well as with sampling date. Therefore, any differences observed cannot conclusively be attributed to either sampling date, soil type or farmlet management system. As differences in soil type were not of primary interest in this study, it was deemed acceptable to confound the soil types with sampling date in favour of blocking the treatments together. Although time limitations of the fluorescent microscopy method meant samples from both soil types on the dairy farmlets could not be processed on the same day, it would have been interesting to be able to definitively compare them, or at least explicitly account for their influence.

8.4 Abiotic factors

Treatments under diverse pasture and regenerative management has significantly lower soil temperatures across both the sheep and the dairy farmlets in the current study which was conducted in late spring. Although less clear, there is evidence that soil moisture followed the opposite trend, being higher under diverse pasture and regenerative management. Differences in soil moisture are likely being obscured by the overriding effect that rainfall has on soil moisture, which would account for the inconsistent results. However, as soil moisture loss is largely driven by evaporation, which is dependent on temperature, soil moisture and temperature are closely linked and it is likely that soil

moisture will follow a similar pattern as soil temperature, especially over summer when the overriding effect of consistent rainfall will no longer obscure any potential changes.

Both diverse pasture and regenerative management caused additively lower soil temperature across both the sheep and the dairy farmlets, with evidence from the dairy farmlets that these treatments also caused higher soil moisture. The most intriguing explanation for this is that the diverse pastures and regenerative management practices buffer the soil from moisture and temperature changes (Huang et al., 2024; Odriozola et al., 2014; Savva et al., 2010). This cannot be confirmed with the results of the current study, as only a single time point was sampled. Multiple repeated measures across a gradient of climatic conditions, such as seasonal changes when soils warm up in spring and cool down in autumn, would be required to confirm that the reason for the differences in soil abiotic conditions observed in the current study is due to buffering of the soil by the different treatments. Measuring soil moisture and temperature change over a 24 hour day to night cycle could also provide evidence for the buffering effect, although it is likely that this would cause a smaller and less noticeable effect than seasonal changes. If buffering of the soil abiotic conditions is occurring as a result of the different treatments, this would have significant implications for farm management practices. Maintaining lower soil temperatures and higher soil moisture over summer would benefit pasture growth, soil biota and improve drought tolerance (Curtin et al., 2012; Heinze et al., 2016; Lee et al., 2013). Similarly, warmer soils during winter will extend the pasture growth season and time for soil biota activity (Baars & Waller, 1979; Curtin et al., 2012; Hutchinson et al., 2000; Lee et al., 2013; Yao et al., 2011). In general, buffering of rapid temperature changes would create more stable conditions for all soil biota, including the plants. Altogether, this would have broader implications on climate change resilient agricultural systems.

8.5 Limitations

8.5.1 Limitations due to sampling date

Soil samples for this study were collected over multiple days, as described in section 3.2, due to the limited number of samples that could be processed for fluorescent microscopy each day, and the desire to have all methods carried out on the exact same soil sample rather than sampling the same paddock multiple times for the different analyses. This was to ensure the results of the different analyses could be directly compared. As fluorescent microscopy needs to be carried out on fresh soil (Foght & Aislabie, 2005), samples could not all be taken at once and stored while the first samples were being processed. Therefore, fresh samples were collected each day and processed for all three analyses on the same day. Although samples for PLFA and nematode analysis could have been collected and processed in one day, these were also collected across multiple days to allow direct comparison with the microscopy results. However, this complicates the analysis of the results, as sampling on different days creates additional variation that needs to be accounted for. This also caused the soil types on the dairy farmlets to be confounded with sampling date which hinders comparisons between soil types.

8.5.2 Limitations of fluorescein iso-thiocyanate (FITC) staining

The high background fluorescence present in the fluorescein iso-thiocyanate (FITC) stained slides of soil bacteria prepared for the current study interfered with the image analysis and likely caused the resulting bacterial counts to be under estimated. Comparing the microscopic counts to the microbial PLFA concentration confirmed that this was probable. Due to the lack of consensus on the correct conversion factors needed to calculate microbial biomass from PLFA and microscopy results (Buesing,

2005; Frostegård et al., 1991; Fry, 1990; Joergensen, 2022; Norland, 1993), these results have been reported as PLFA concentrations and bacterial counts per gram of soil in this thesis, without conversion. Also, in order to convert bacteria counts to bacterial biomass, most conversions use biovolume calculated from cell counts and dimension measures (Buesing, 2005; Fry, 1990; Norland, 1993). As the latter was not measured in this study, this conversion was not possible. However, this makes comparison of the results from the PLFA analysis and the fluorescent microscopy analysis methods difficult. Frostegård & Bååth (1996) describe a conversion factor for calculating bacteria counts directly from soil bacterial PLFA concentration. This is used here to estimate the total bacterial count based on the PLFA concentration. Although Frostegård & Bååth (1996) provide an average PLFA content per cell that they recommend using, they also state that different conversion factors may be required for different soils, reporting that the PLFA content per cell was affected by soil pH. Therefore, to calculate bacteria counts from soil PLFA in this thesis, the PLFA content per cell of soil 9 described by Frostegård & Bååth (1996) was used; this was a grassland soil with a pH of 6.3, making it the most similar to the soils examined in the current study. The bacteria count was calculated using the total concentration of only the subset of PLFAs that correspond with those used by Frostegård & Bååth (1996). Frostegård & Bååth (1996) also used the PLFAs 16:1ω9 and 16:1ω7t, which were not detected in the current study and therefore not included in the calculation. This gave estimated counts of 3.71×10^{10} and 1.67×10^{10} bacteria g^{-1} soil for the sheep and the dairy farmlets, respectively. However, Frostegård & Bååth (1996) also state that possibly only 10 - 20% of total soil PLFA is derived from living biomass (Kowalenko & McKercher, 1971). Accounting for this in the calculations gives bacteria counts of between 3.71×10^{9} and 7.43×10^{9} bacteria g^{-1} soil for the sheep farmlets and between 1.67×10^{9} and 3.34×10^{9} bacteria g^{-1} soil for the dairy farmlets. These estimates are similar to the expected bacteria number in agricultural soils (Ananyeva et al., 2008; Frostegård & Bååth, 1996; Kennedy, 1995), and correspond

well with the estimates of total bacteria calculated using the fluorescein diacetate (FDA) stained bacteria in section 5.4 of this thesis. Therefore, even the most conservative estimates of bacteria numbers using the PLFA analysis results are almost 10-fold greater than what was found using fluorescent microscopy. This supports the proposition that the staining and microscopy procedure used in this study for measuring total bacteria with FITC underestimated total bacteria, and the further implications this has on the proportion of active and dead bacteria (section 5.4).

8.5.3 Limitations in interpretation of results

This study investigated whether the objective of RA to improve soil health and enhance soil biota is achieved. However, “soil health” is difficult to define and measure quantitatively (Powlson, 2020). By extension, “enhancing soil biota” is ambiguous. Although soil biology and soil microbiology are well established fields of research, there is still no gold standard of the optimum level for many of the measures of soil biota in agriculture, and the extreme complexity of biological interactions in the soil make defining them even more difficult. This makes it difficult to assess whether certain farm management practice improve soil biota, despite determining whether a change in soil biota occurs being fairly straight forward. Therefore, care must be taken to not simply interpret any change observed to conform with preconceived expectation or desirable outcomes.

Further research is required to determine the optimum level for various measures of soil biota. Simply taking a more is better approach is not necessarily justified. For example, while a certain level of microbial activity is desirable, high microbial activity would result in higher SOM mineralisation (Fontaine & Barot, 2005; Kuzyakov et al., 2000) and is also indicative of disturbed soil conditions, such as tillage, which has been shown to be negative to soil health (Crotty, 2020; Hendrix et al., 1986). High levels of certain soil fauna could have negative consequences. More herbivorous nematodes are likely detrimental

for plant growth (Mercer et al., 2008; Yeates, 2006), although this is an anthropocentric interpretation, as higher herbivorous nematodes would presumably increase energy and nutrient transfer into the soil food web, which would be beneficial for soil biota and SOM storage despite being negative for the growth of plants that are deemed desirable (Ferris, 2010a). More dead bacteria could be interpreted as being due to either unfavourable conditions for soil microbes or higher contribution of bacterial necromass to SOM and increased microbial turnover and nutrient cycling (Hu et al., 2023; Lavallee et al., 2020; Underwood et al., 2024). Therefore, due to the lack of well defined optimums for soil biota measurements, care must be taken when interpreting the results to not simply attribute any change observed to conform with expectations, as this becomes a self-fulfilling prophecy.

8.5.4 Limitations of timescale

While the results of the current study indicate there is some evidence that regenerative management and diverse pastures influence soil biota, they are inconsistent across treatments and between the sheep and dairy farmlets, and the effect sizes were small. Interestingly, there was evidence that the treatments affected the soil abiotic conditions, which are expected to have an impact on soil biota and also directly influence pasture growth. While it is possible that this indicates that regenerative management and diverse pastures may not be impacting soil biota, the lack of treatment effects could also be due to the relatively short time since the treatments were implemented. Although individual components of the soil biological system have a high turnover and can respond quickly to changes, the system as a whole tends to take a long time to respond to changes (Lin, 2011). Therefore, it is possible that the treatment effects will become more pronounced as the study progresses, or that flow on effects are only observed once other components of the system have had time to adapt. As the research project on which this study was based will continue after this study, it will be possible to investigate whether this is the

case. However, even the total time of this project (7 years) is relatively short in the greater context of soil processes, which can take decades to change and often benefit from compounding long term effects (Lin, 2011).

8.5.5 Limitations of methods used

Methodological limitations potentially hindered the ability to detect differences, if present. Soil samples in this study were collected to a depth of 7.5 cm, as this is the standard in New Zealand for soil chemical analysis and also due to the equipment available. However, as many previous studies that conducted PLFA and nematode analysis sampled to 10cm (Lambie et al., 2021; Lewe et al., 2021; Parfitt et al., 2010; Schon et al., 2010; Yeates, 1978, 1984), this made direct comparison of results difficult. Therefore, it would have been beneficial to have sampled to 10cm for the current study, to facilitate comparisons with previous research. PLFA analysis is a relatively coarse measure of microbial community composition, and therefore potentially obscuring any community composition changes, with methods involving genomics and analysis of nucleic acids providing much higher resolution of the species diversity present. Despite this, analyses of nucleic acids also have inherit limitations, including a high potential for biases, issues with obtaining high quality template nucleic acid and a large amount of data that can be difficult to interpret (Lear et al., 2018; Lewe et al., 2021; Semenov, 2021). Lewe et al. (2021) proposes using PLFA analysis to calibrate metabarcoding data which has the potential to help overcome some of these limitations. Issues with the staining procedure used for fluorescent microscopy, as discussed above, limited what could be inferred from these data. Respiration analysis, including substrate induced respiration and selective respiratory inhibition can also be used to provide information on the physiological states of soil microbes, which are less labour intensive than direct microscopy and have the potential to be higher throughput, however, specialised equipment is required (Blagodatskaya &

Kuzyakov, 2013; Mingorance & Peña, 2016). Additionally, metatranscriptomics can be used to study active soil microbes, and provides additional information on the functions the active microbes carry out, however, suffers from the same limitations as described above for genomics (Poursalavati et al., 2023; Semenov, 2021). My lack of experience and expertise in working with and identifying nematodes could also have affected the quality of the data obtained for analysis of the nematode community. Differences in the duration and method used to extract the nematodes between the current study and previous studies on nematodes (Parfitt et al., 2010; Schon et al., 2010; Yeates, 1978, 1984) restricts direct comparison of nematode abundances with these studies. Using an extraction method and time consistent with previous studies would have better facilitated comparisons. Although nematode analysis has been used in the current study to infer the state of the soil food web, actually measuring the individual components of the soil fauna that makes up the higher trophic groups would have provided a more detailed overview, however this would require much more expertise and labour. Also, soil protists are an important component of the soil microflora which have been overlooked in the current study, due to time and methodology constraints, however would provide important insights on the soil biota. Even across all three methods used in the current study, only a small component of the total soil biota was investigated. The soil food web is diverse and complex and cannot easily be summarised by a few measures. Therefore, overarching conclusions of the influence of RA on soil biota cannot be drawn from the current study, rather the current research will contribute to the understanding of how this particular component of soil biota is impacted by RA.

8.6 Conclusion

Although there is some evidence to suggest RA has an impact on soil biota, the effect sizes were small and the differences observed were inconsistent across treatments and

between the two farmlet systems. Overall, across all treatments and farmlet systems analysed in the current study, the results indicate that the soil food web is in degraded, basal state. Treatment effects on soil abiotic factors known to have an influence on soil biota were detected and warrant further research. The largest and most consistent differences in the soil biota detected were between soil types and between the farmlet systems which has important implications on correctly taking these factors into account in soil biology studies. Methodological limitations hinder the interpretation of the results in current study. The lack of response observed could be due to the relatively stable, long term pastoral system on which the treatments were imposed and the short time since treatments were established meaning differences may become apparent as the research program progresses. Although there is evidence to indicate that the soil biota is influenced by diverse pastures and regenerative management, continued monitoring and refinement of the methods used to measure these changes is required to confirm this.

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APPENDIX 1: THRESHOLDS

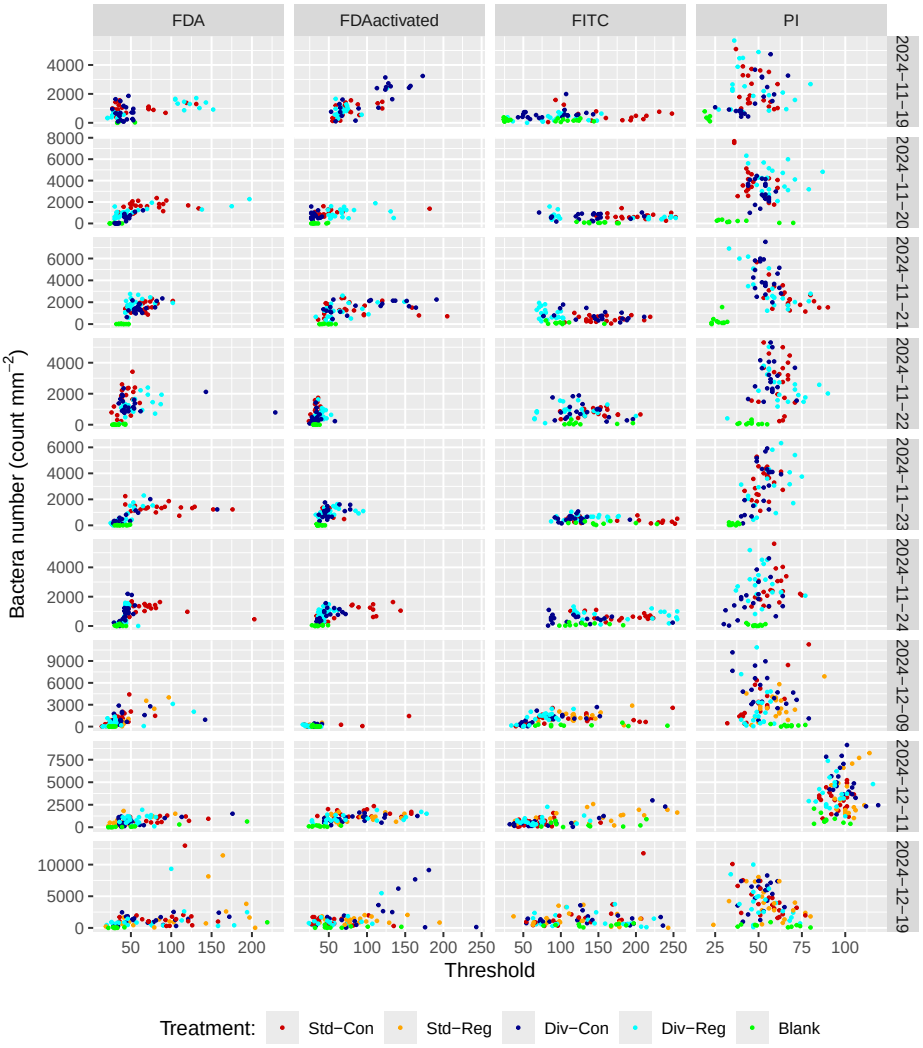

Figure A1: Bacteria count on each epifluorescent microscopy slide by treatment against the threshold set to separate stained bacteria from the background during image analysis, on each day slides were observed. FITC = Fluorescein iso-thiocyanate, FDA = Fluorescein diacetate, FDA activated = FDA staining after activation with nutirents, PI = Propidium iodide.

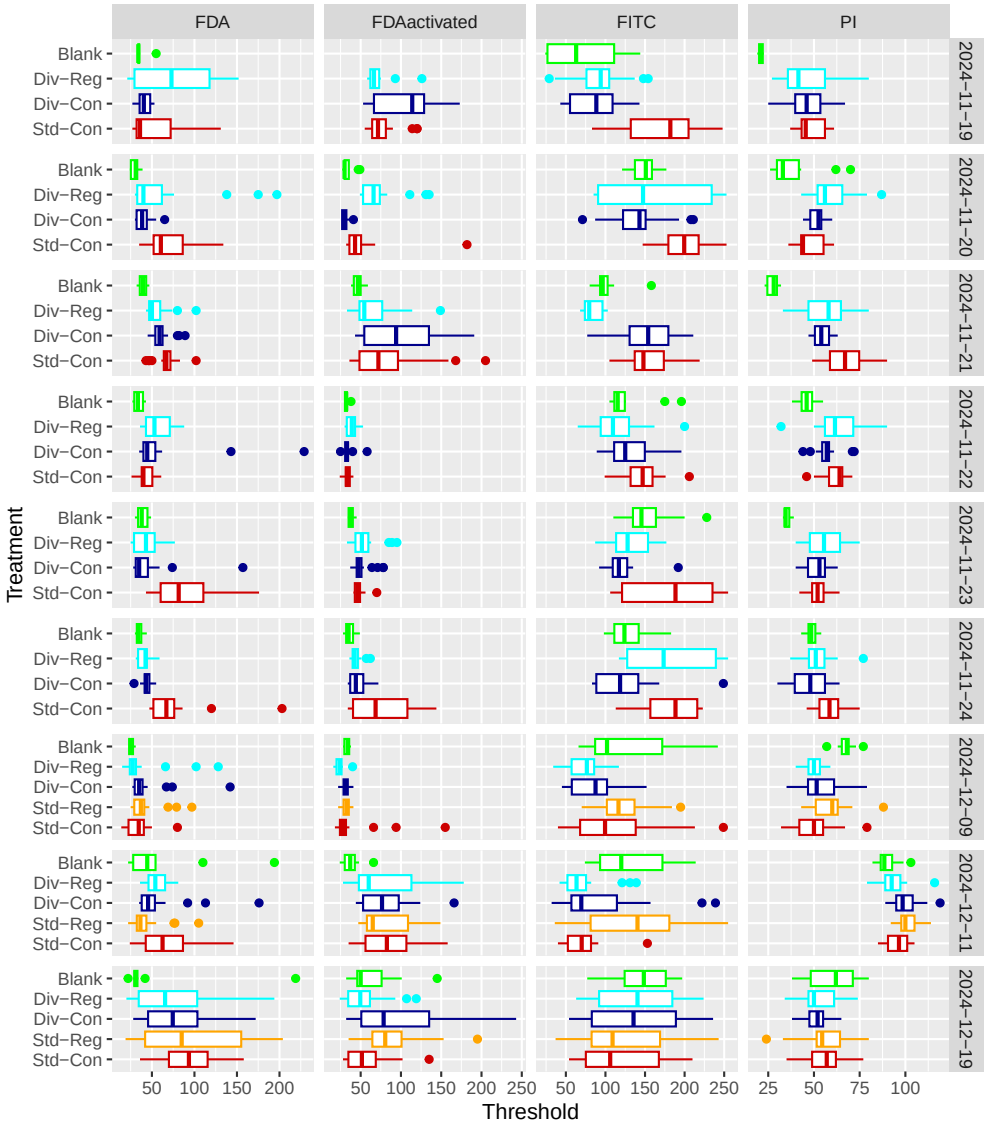

Figure A2: The thresholds set for each image during image analysis by treatment, on each day the epifluorescent microscopy slides were observed and across the different stains used, to determine if there are any consistent differences in the threshold used between the treatments that could bias the results. FITC = Fluorescein iso-thiocyanate, FDA = Fluorescein diacetate, FDA activated = FDA staining after activation with nutirents, PI = Propidium iodide.