Degradation of Dieldrin in the Rhizosphere

A thesis submitted in total fulfilment for the degree of

Master of Science

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Table of Contents

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List of Figures

List of Tables

List of Abbreviations

ANOSIM	Analysis of Similarities
ANOVA	Analysis of Variance
DDD	Dichlorodiphenyldichloroethane
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic Acid
E	Efficiency of qPCR Assay
ECD	Electron Capture Detector
ECH	1, 2 – Epoxycyclohexane
ESV	Exact Sequence Variants
GC	Gas Chromatography
NMDS	Non-Metric Multidimensional Scaling
OTU	Operational Taxonomic Units
PCR	Polymerase Chain Reaction
POP	Persistent Organic Pollutant
RO	Reverse Osmosis
ROS	Reactive Oxygen Species
Tukey HSD	Tukey Honestly Significant Difference
UV	Ultraviolet

Statement of Authorship

Except where reference is made in the text of the thesis, this thesis contains no material published elsewhere or extracted in whole or in part from a thesis accepted for the award of any other degree or diploma. No other person's work has been used without due acknowledgment in the main text of the thesis. This thesis has not been submitted for the award of any degree or diploma in any other tertiary institution.

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Summary

Pesticides pose unique threats to a soils biological health by disrupting the soils organic components. Arguably the most detrimental of the pesticides are the organochlorines. Dieldrin is a member of the organochlorine family whose use as a pesticide peaked during the 1950's, however, due to its insolubility in water, its hydrophobic tendencies allow it to persist in the soil. Rhizoremediation could be a potential method to remove dieldrin from the soil, however this process is largely dependent on the type of plant and its root exudates. The aim of this study was to investigate the ability of grasses and legumes to degrade dieldrin in the rhizosphere. Key findings from this study are reported in the form of a research article, with additional findings that supplement these results in a separate chapter. Soil was obtained from a farm in Emerald, Australia, whom had a history of dieldrin use during the 1960's. One soil only control and eleven species of plants were selected: Lucerne, White Clover, Sub Clover, Annual Ryegrass, Phalaris, Yellow Serradella, Cock's-Foot, Tall Fescue, White Lupin, Wheat & Wheat (- Citrate). Dieldrin concentrations were found to be significantly lower (0.2 µg/g) in only the white lupin treatment compared to a soil only control. Eleven species of fungi were significantly enriched in the white lupin rhizosphere ($p < 0.05$), but neither the abundance of microbes ($p = 0.64$) nor microbial diversity ($p = 0.4$) correlated with dieldrin soil concentrations. Two of the differentially abundant microorganisms (*Penicillium* and *Trichoderma*) have previously been found to degrade dieldrin, while the other two (*Saitozyma* and *Umbelopsis)* demonstrate a history of bioremediation. In addition to the differentially abundant species, there was one indicator species present for both bacteria (Unidentified) and fungi (*Cortinarius*). These results indicate that the presence of a rhizosphere did not influence the degradation of dieldrin in the soil. Rather, the white lupin rhizosphere selected for unique microorganisms which drove dieldrin degradation. This study highlights that in-depth analysis at lower taxonomic levels is required to uncover novel dieldrin degraders.

Chapter 1: An Introduction to Dieldrin

Exponential utilisation of anthropogenic processes in the last century has corresponded to an increase in environmental pollution. Pollutants include a variety of molecules such as industrial by-products produced from refineries and factories, or pesticides applied to agricultural farmland. The health of a soil can be adversely affected by the application of pesticides, as they have the potential to alter the soil's organic components, disrupt biological processes and reduce optimal soil function (Zhang *et al.*, 2015). Arguably, the most detrimental class of pesticides are organochlorines (Heath & Vandekar, 1964).

Organochlorines are a highly effective family of pesticides utilised internationally during the mid-late 1900's. However, due to concerns over negative effects on human health (Robinson & Hunter, 1966), usage of organochlorines is now illegal. Dieldrin, an organochlorine pesticide, was commonly used in Australia during the 1950s and continues to pose a significant threat to human health due to its persistence in the soil. The ability of dieldrin to persist in the soil is due to its chemical composition, as its hydrophobic nature forces dieldrin to bind to organic matter rather than dissolve in the water and leach through the soil (Ru *et al.*, 2007). Although the impact of dieldrin on human health has been investigated, limited information exists on dieldrin's remediation from the environment.

Bioremediation has been proposed as a potential method to remediate dieldrin from the soil as it is cost effective and less invasive than other remedial approaches (Li *et al.*, 2016), one such method is rhizoremediation. Rhizoremediation utilises microorganisms associated with a plant's rhizosphere, the 1-2 mm area of soil surrounding the plant roots, to degrade contaminants from the soil. Not all plants have the same capacity for rhizoremediation. This process is largely dependent on root exudates (Bais *et al.*, 2006), the molecules secreted from the roots of a plant that stimulate microbial associations. The presence of organic acid, carbohydrate and amino acid exudates in the soil increase the degradation of organic hydrocarbons due to the increase and diversity of microbial activity (Lu, Sun & Zhu, 2017). Although the secretion of root exudates increase the degradation of soil contaminations, there is yet to be a study investigating the rhizoremediation of dieldrin.

1.1 Dieldrin: A Persistent Organic Pollutant

Dieldrin was developed in the 1940's to compete with dichlorodiphenyltrichloroethane (DDT), another member of the organochlorine pesticide family. Dieldrin dominated the commercial pesticide market internationally from the 1950-70's before its rapid decline in the 1980's due to concerns over its impact on human health (Robinson & Hunter, 1966).

Dieldrin is also a concern for environmental health, as it is a persistent organic pollutant (POP), an organic compound not readily degraded via photolytic, biological, or chemical processes (Li *et al.*, 2016). This results in the accumulation of dieldrin in the soil, where these quantities of dieldrin can permeate humans via ingestion or inhalation. Ingestion of dieldrin induces neurotoxicity and carcinogenicity in preclinical models such as zebrafish and mice (Sarty, Cowie & Martyniuk, 2017; Bachowski *et al.*, 1998).

To prevent further environmental contamination by dieldrin, the United Nations Stockholm Convention on POPs established an international treaty in 2001 prohibiting the production and use of organochlorines (United-Nations, 2001). However, due to a lack of appropriate legislation and market regulations, there are undeveloped nations who still utilise dieldrin (Jayaraj, Megha & Sreedev, 2016). As dieldrin is still used and can persist in the soil for decades, it is important to identify how it can be remediated from the environment.

1.2 The Impact of Dieldrin on Human Health

Dieldrin impacts multiple vertebrate systems, accumulating in different parts of the body. In a mouse model it caused oxidative damage to neurons via inhibition to aldehyde dehydrogenase and reactive oxygen species (ROS) production (Mao *et al.*, 2007; Díaz-Barriga *et al.*, 2015). Similar damage to neurons has been found in zebrafish where exposure to dieldrin caused mitochondrial aberrations and lipid peroxidation (Sava *et al.*, 2007; Fitzmaurice *et al.*, 2014). These neurotoxic effects have been replicated in largemouth bass where dieldrin alters proliferation, differentiation, and oxidative phosphorylation of proteins responsible for neurodegenerative diseases (Martyniuk *et al.*, 2010). Correlations between neurodegenerative diseases and dieldrin exposure have also been demonstrated in humans, where high levels of

dieldrin have been found in blood samples of Alzheimer's and Parkinson's disease patients (Chhillar *et al.*, 2013; Singh *et al.*, 2013). Although a correlation exists between dieldrin exposure and neurotoxicity, a causative relationship is yet to be defined.

In addition to neurological toxicity, a correlation between dieldrin and carcinogenicity is well established in animal models. Exposure to dieldrin in food sources was linked to liver cancer in mice (Bachowski *et al.*, 1998), whereas dieldrin contaminated water correlated to cancer in the gills of zebrafish (Sarty, Cowie & Martyniuk, 2017). Correlations between carcinogenic properties in dieldrin have been observed in human studies. Prostate cancer is linked to dieldrin exposure, and farmers with a history of dieldrin use were significantly associated with an increased incidence of an aggressive form of prostate cancer (Koutros *et al.*, 2013). These results are reflected in the blood samples of prostate cancer patients with a 20% higher concentration of serum dieldrin compared to controls with no history of exposure (Kumar *et al.*, 2010; Samtani, Sharma & Garg, 2018). Similar results are seen in women, where high concentrations of serum dieldrin are linked to an increased incidence of ovarian cancer (Wrobel *et al.*, 2015). It is suggested that this is due to a disruption in the endocrine system stimulating the ovaries into developing tumours (Samtani, Sharma & Garg, 2018). The observed neoplastic changes from dieldrin contamination replicate carcinogenic properties identified in other chemicals (Sarty, Cowie & Martyniuk, 2017), however there is insufficient evidence to state the aetiology of this process.

1.3 Sorption of Dieldrin in Soil

Many factors influence adsorption rates, or how quickly chemicals bind to the soil. One such factor is water solubility, as a water insoluble chemical will preferentially bind to organic molecules in the soil rather than absorb into water molecules (Ru *et al.*, 2007). Water solubility can be determined by its octanol-water partition coefficient (log (K_{ow})), where dieldrin has a high value ranging between 4.3 – 5.48 (Ru *et al.*, 2007). A high log (K_{ow}) reflects its tendency to persist in particulate organic matter and display hydrophobic properties. Although dieldrin was phased out by the end of the 1970's, it is still present in the soil due to its resistance to degradation by microbes. However, it will not remain at its initial concentration. The

concentration of soil bound dieldrin will decrease over time due to its half-life, a period which takes a chemical to decrease its concentration by half its initial value.

Dieldrin's half-life is largely debated, with studies reporting a range from 5 to 20 years (Martijn, Bakker & Schreuder, 1993; Talekar *et al.*, 1977). It is thought that moisture and temperature affect a chemical's half-life, with chlorinated hydrocarbons degrading faster in higher temperature and moisture conditions (Komprda *et al.*, 2013). Comparatively, soil pH also influences chemical degradation where chlorinated hydrocarbons, like dieldrin, persist longer in acidic soils compared to alkaline soils (Rhodes, 2014). Although temperature and moisture conditions can increase chemical degradation, a faster and more efficient alternatives are required.

1.4 Microorganisms Capable of Dieldrin Degradation

Microorganisms that can metabolise dieldrin have been found to increase the chemical degradation rate, in comparison to its expected half-life (Pan *et al.*, 2017). Fungi are believed to be the dominant microbes contributing to dieldrin degradation in the soil (Xiao & Kondo, 2013). This degradation is facilitated by their mycelium, a vegetative appendage which exudes powerful acids and extracellular enzymes capable of decomposing complex organic compounds (Rhodes, 2014). The ability of fungi to degrade dieldrin is dependent on soil conditions, such as the availability and quantity of organic nitrogen and carbon content. In a low nitrogen environment *Pleurotus ostreatus* degraded dieldrin by 3% (Purnomo *et al.*, 2017), similar results were found in low carbon soils where *Trichoderma koningii* degraded dieldrin by just 3% (Bixby, Boush & Matsumura, 1971). In contrast, *Mucor racemosus* was able to degrade dieldrin by 95.8% in a nitrogen and carbon rich environment (Kataoka *et al.*, 2010).

Although not as efficient as fungi, bacterial degraders of dieldrin have been isolated since the 1960's. To complete the degradation process, co-metabolites are required by bacterial enzymes to degrade dieldrin. It is not uncommon for complex organic molecule degraders to require co-metabolites or additional nutrients to maximise their degradation activity (Wang *et al.*, 2010). *Burkholderia* and *Cupravidus* were able to degrade dieldrin by 49% and 38% respectively (Matsumoto *et al.*, 2008), but only in the presence of a co-metabolite, 1,2-epoxycyclohexane (ECH).

1.5 Dieldrin Degradation Pathway

Microbial degraders of dieldrin have been studied since the late 1960s, however, the complete metabolic pathways for degradation is still unclear. It is known that there are three major metabolites of dieldrin, which are the same for both bacteria and fungi: trans-aldrin diol, photodieldrin and ketoaldrin (Matsumura & Boush, 1967; Turner, Glotfelty & Taylor, 1977; Patil, Matsumura & Boush, 1970). The most frequently reported metabolite in the literature is trans-aldrin diol, reported as a major metabolite of both bacteria (*Bacillus*, *Pseudomonas* and *Aerobacter*) and fungi (*Mucor racemosus*, *Penicillium miczynskii* and *Trichoderma viride*) (Matsumoto *et al.*, 2008). Degradation of dieldrin is catalysed by epoxide hydrolases, where it performs aerobic oxidative degradation to transform dieldrin to trans-aldrin diol (Meijer & DePierre, 1988). Although the epoxide degradation process is well understood, it remains unclear which enzymes in degraders of dieldrin are responsible for this process. The degradation of dieldrin resulting in photodieldrin is due to one of two processes: dieldrin is subject to UV and altered via photoconversion, or dieldrin is metabolised by marine microorganisms creating photodieldrin as a by-product (Turner, Glotfelty & Taylor, 1977). Ketoaldrin as a metabolite has been less reported than the others, but *Trichoderma viride* forms this as its degradation pathway (Patil, Matsumura & Boush, 1970). It is suggested that this process occurs through an oxidative system (Patil, Matsumura & Boush, 1970).

1.6 Remediation of Dieldrin-Contaminated Soil

Research investigating the remediation of dieldrin is scarce, however, there is one study that investigated the use of phytoremediation to remove dieldrin from the soil. A screen of 32 agricultural plants found *Cucurbitaceae* to be the most effective at dieldrin removal (Otani, Seike & Sakata, 2007). This association is due to the ability of cucurbits to exude low molecular weight organic acids capable of dissolving hydrophobic pollutants sorbed to the soil (Otani, Seike & Sakata, 2007). Although phytoremediation is a promising tool for bioremediation, the dieldrin is translocated within the biosphere and is not degraded.

Rhizoremediation is a useful tool for bioremediation as it utilises the microorganisms surrounding the plant roots to degrade pollutants. Although there is currently no study

investigating the rhizoremediation of dieldrin, the literature suggests that rhizoremediation of organochlorines is possible. The rhizosphere of Jatropha, a flowering plant from the *Euphorbiaceae* family, was found to degrade the organochlorine lindane, by 72% - 89% (dependent on initial concentrations and trial length) (Abhilash *et al.*, 2013). However, not all plants have the same potential for rhizoremediation, the main driver of which are their root exudates.

1.7 Root Exudates Influence Rhizoremediation Potential

Root exudates are low molecular weight organic acids that are secreted from the roots of the plant and alter microbial activity in the soil (Zhou *et al.,* 2020). Although the function of most root exudates is unknown, it is well characterized that these low molecular weight compounds play important roles in biological processes such as: phytohormone precursors, cell signalling and metabolic responses (Zhou *et al.,* 2020).

Root exudates can also be associated with structures present in the roots, such as root nodules created by legumes. Li *et al*. (2016) found increased root nodulation expression is positively correlated with root exudate concentrations. Root exudates also can be utilised by microorganisms as an energy source. Vale et al. (2005) found a positive correlation between the concentration of root exudates and microbial activity in the rhizosphere. The ability of root exudates to stimulate the microbial activity in the rhizosphere could be advantageous when trying to recruit microorganisms capable of dieldrin degradation. Thus, legumes and the root exudates derived from their root nodules were selected for this study. Legumes, and grasses, are potentially appropriate solutions for the remediation of contaminated soils over large areas due to their size and cost.

1.8 Aims & Hypothesis

This study aimed to investigate the rhizoremediation potential of grasses and legumes in dieldrin-contaminated soil and to assess changes in the bacterial and fungal communities during dieldrin degradation. It was hypothesized that the presence of the rhizosphere would result in increased rates of dieldrin degradation due to the stimulation of the microbial community. In particular, the legume rhizosphere is expected to exhibit an enhanced ability to degrade dieldrin compared to the grass rhizosphere due to the increased secretion of root exudates.

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Chapter 2: Remediation of Dieldrin in the Rhizosphere

This chapter discusses the ability of the rhizosphere in grasses and legumes to degrade dieldrin, and the correlations between microbial abundance and diversity to dieldrin degradation. It also investigates the changes occurring at the lowest taxonomic levels and how they can potentially have the greatest effect in the soil.

The study presented in Chapter 2 is in the form of a research article, as its current version will be considered for publication in the May 2021 special issue of the Agronomy journal.

Dieldrin degradation in the rhizosphere is impacted by individual microorganisms rather than the whole community

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Abstract

1. Dieldrin is a synthetic organochlorine pesticide that persists in the soil and negatively affects agricultural systems. Dieldrin is also strongly correlated to displaying carcinogenic and neurotoxic properties in rodent and zebrafish models. Despite its ability to persist in the soil and the negative impact it has on the environment and mammalian health, dieldrin's bioremediation from the soil is poorly understood.

2. Dieldrin-contaminated soil was used to grow five species of grasses (*Lolium rigidum*, *Fescuta arundinacea*, *Dactylis glomerate*, *Phalaris aquatica* and *Ornithopus compressus*), four species of legumes (*Trifolium repens*, *Trifolium subterraneum*, *Lupinus albus* and *Medicago sativa*), two lines of isogenic wheat (*Triticum aestivum* and *Triticum aestivum* (aTaMATE1B)) and one soil only control. The concentrations of dieldrin in the rhizosphere were assessed to investigate any correlations between microbial abundance and diversity, determined by qPCR and 16S/ITS rRNA profiling.

3. Dieldrin concentrations were found to be significantly lower (0.2 µg/g) in only the white lupin treatment compared to a soil only control. Eleven species of fungi were significantly enriched in the white lupin rhizosphere ($p < 0.05$), but neither the microbial abundance ($p = 0.64$) nor microbial diversity ($p = 0.4$) correlated with dieldrin soil concentration. Two of the differentially abundant microorganisms (*Penicillium* and *Trichoderma*) have previously been found to degrade dieldrin, and another two (*Saitozyma* and *Umbelopsis)* exhibit a history of bioremediation. In addition to the differentially abundant species, there was one indicator species present for both bacteria (Unidentified) and fungi (*Cortinarius*).

4. *Synthesis and applications.* These results indicate that the presence of a rhizosphere did not influence the degradation of dieldrin in the soil. Rather, the white lupin rhizosphere selected for specific microorganisms which likely drove dieldrin degradation. This study highlights that in-depth analysis at lower taxonomic levels is required to uncover novel dieldrin degraders.

KEYWORDS

Bioremediation, Dieldrin, Differential Abundance, Microbial Abundance, Microbial Ecology, Microbial Diversity, Pesticide, Rhizosphere.

1 Introduction

Anthropogenic processes over the last century have corresponded with a rise in environmental pollution. Persistent organic pollutants (POPs) pose a particular threat as they are not readily degraded via photolytic, biological or chemical processes (Sankoh, Whittle, Semple, Jones & Sweetman, 2016). POPs can reduce the productivity, profitability and sustainability of farmland soils by altering organic components (Ritter, Solomon & Forget, 2007). Dieldrin is a POP that was heavily utilized as a pesticide between the 1950's and the 1980's. The Stockholm Convention on POPs established an international treaty in 2001 prohibiting the production and use of dieldrin (Ritter, Solomon & Forget, 2007). Although dieldrin production was banned twenty years ago its physiochemical properties allow it to persist in the soil, reducing the use of agricultural land.

Dieldrin can accumulate in meat through livestock ingestion of contaminated plant material or inhalation from aerosolized soil particles (Jorgenson, 2001). The consumption of contaminated livestock meat is harmful to humans, thus contamination level of livestock and meat products are tightly regulated by import restrictions (Government of Western Australia, 2019). Correlations have been reported between dieldrin concentrations in blood samples of humans and both Alzheimer's and Parkinson's Disease, as well as oxidative damage to neurons in rodents (Mao et al., 2007; Singh et al., 2013; Chhillar et al., 2013). Dieldrin also displays carcinogenic properties by triggering cancer in the gills of *Danio rerio* (Zebrafish) after ingestion (Sarty, Cowie & Martyniuk, 2017), as well as aggressive forms of prostate cancer (Koutros et al., 2013).

Cost effective and environmentally friendly options are required for the removal of dieldrin from soils, current mechanical removal of contaminated soil is an expensive and destructive process (Cagnetta, Huang & Yu, 2018). Microbial remediation is a sustainable alternative that utilizes microorganisms to degrade soil contaminants. Several bacterial species within the phyla

Proteobacteria and Actinobacteria (Bandala, Andres-Octaviano, Pastrana & Torres, 2006; Jagnow & Haider, 1972), as well as fungi within the Basidiomycota and Ascomycota are known to metabolize dieldrin under laboratory conditions (Purnomo, Nawfa, Martak, Shimizu & Kamei, 2017; Xiao & Kondo, 2013). Dieldrin degradation is also linked to the composition of organic matter in soils, with higher rates reported for soils that encompass a higher microbial-C to total-C ratio (Krohn et al., 2019). The microbial-C in bulk soil can be increased up to 80% when a rhizosphere is present (Joergensen, 2000). The presence of microorganisms capable of dieldrin degradation coupled with the ability to increase microbial-C, make the rhizosphere a promising bioremediation tool.

Rhizoremediation has the potential to increase dieldrin degradation in agricultural soil by stimulating microbial activity in the rhizosphere (van Dillewijn et al., 2007). Rhizoremediation has been proven successful *in situ,* where over a 60-day period the presence of a *Zea mays* (maize) rhizosphere degraded 96% of the organochlorine 2,4,6-trinitrotouene from the soil (van Dillewijn et al., 2007). However, not all rhizosphere communities have the same capacity for rhizoremediation due to differing root exudate profiles (Ryu et al., 2020). Legumes may have an increased ability for rhizoremediation due to the formation of root nodules, a diverse root exudate profile, and an increase in nitrogen providing enhanced activity in the rhizosphere (Ryu et al., 2020). Despite the potential of rhizoremediation, there is yet to be a study investigating its use in dieldrin-contaminated soil.

The aim of this study was to investigate the rhizoremediation potential of grasses and legumes in dieldrin-contaminated soil and to assess changes in the bacteria and fungi communities during dieldrin degradation. It was hypothesized that the presence of the rhizosphere would result in increased rates of dieldrin degradation due to the stimulation of the microbial community. In particular, the rhizosphere of legumes is expected to pertain an enhanced ability to degrade dieldrin compared to the grass rhizosphere due to the increased secretion of root exudates.

2 Materials and Methods

2.1 Sampling of dieldrin-contaminated soil: Soil was obtained in April of 2019 from a farm in Emerald, Australia with a history of dieldrin use. The sampled farmland utilized dieldrin during the 1960's as a pesticide to protect potato crops from insects, before discontinuing use in 1970. The concentration of dieldrin in the soil (1.25 ng/g) has restricted the farmer from growing agricultural products by the National Organochlorine Residue Management Program by the Department of Agriculture, Australia. The farmland has since been converted to a pasture containing clover, perennial and annual grasses with the occasional grazing of cattle. A representative sample of the paddock was obtained by extracting soil cores at 50 sampling points evenly distributed across the paddock. The surface layer of soil was scraped away before the soil core was extracted and the upper layer of soil (0 – 10 cm) was collected. At each sampling point, 3 soil cores were randomly selected within a 1×1 m area. The extracted soil was transported to La Trobe University (Plenty Rd & Kingsbury Dr, Bundoora, Victoria, Australia). Large organic material was removed from the soil via a 2 mm sieve. The soil was homogenized during the sieving process by manual mixing. The homogenized soil was transported to AgriBio (5 Ring Rd, Bundoora, Victoria, Australia) where it was air-dried. The resulting soil was utilized in subsequent rhizosphere experiments and soil analysis.

2.2 Rhizosphere experiment: Plastic pots (6.35 cm diameter) were lined with a fitted plastic bag to prevent leaching and filled with 900 g of air-dried soil. At the beginning of the growth period, week 4 and week 8, each replicate was fertilized with 20 ml of chemical fertilizer (77.22 mg/kg N as ($CO(NH_2)_2$), 162 mg/kg P as (KH_2PO_4), 162 mg/kg Ca as ($CaCl{\cdot}2H_2O$), 162 mg/kg K as (K_2SO_4), 45 mg/kg Mg as ($MgSO_4{\cdot}7H_2O$), 13.5 mg/kg Mn as ($MnSO_4{\cdot}H_2O$), 8.1 mg/kg Zn as ($ZnSO_4{\cdot}7H_2O$), 5.4 Cu as ($CuSO_4{\cdot}5H_2O$), 0.36 mg/kg Co as ($CoCl_2{\cdot}6H_2O$), 0.36 mg/kg Mo as ($Na_2MoO_4{\cdot}2H_2O$) and 4.95 mg/kg Fe as (FeEDTA)).

Five species of grasses, four species of legumes and two isogenic lines of wheat (Table 1) were selected to examine their potential for rhizoremediation. There are two isogenic lines of wheat (*Triticum aestivum*); one capable of exuding the citrate root exudate (Wheat) and the other line in which the gene for citrate root exudation was genetically eliminated (Wheat – Citrate) (Xu, Wang & Tang, 2019). There were four replicates for each of the twelve treatments (including the soil

only control) to a total of 48 replicates. Prior to germination, the white clover, sub clover and lucerne seeds were scarified with fine sandpaper until a white powder formed. This increased their germination rate by reducing the thickness of their seed coat. All the seeds were then germinated by soaking in 10 ml of sterile milli-Q water for 1.5 h, with the water being changed every 20 min. The seeds were then submerged in 2 ml of sterile milli-Q water on filter paper inside a petri dish. The petri dishes were wrapped in aluminum foil and placed in the dark at room temperature (25°C) until seeds germinated.

Table 1. List of plant species (including control) with number of seeds added per pot.

Plant Species	Common Name	Plant Type	Number of Seedlings (no./pot)
-	Soil Only Control	Control	-
Lolium rigidum	Annual Ryegrass	Grass	7
Fescuta arundinacea	Tall Fescue	Grass	7
Dactylis glomerata	Cocks Foot	Grass	7
Phalaris aquatica	Phalaris	Grass	7
Ornithopus compressus	Yellow Serradella	Grass	7
Trifolium repens	White Clover	Legume	7
Trifolium subterraneum	Sub Clover	Legume	8
Lupinus albus	White Lupin	Legume	3
Medicago sativa	Lucerne	Legume	5
Triticum aestivum	Wheat	Grass	3
Triticum aestivum (aTaMATE1B)	Wheat (- Citrate)	Grass	3

Prior to sowing seeds, an initial soil sample was obtained by randomly sampling 3×1 g of soil from each of the pots and pooled together to examine the change in dieldrin concentrations over the growth period. Germinated seeds were sown at 1.5 cm depth and seedlings were thinned to the

designated number per pot (Table 1). Different numbers of seedlings were planted in each treatment to achieve a similar final biomass. Plants were watered twice daily (morning and afternoon) to 80% total water holding capacity for 10 weeks (Wu, He, Wang & Xu, 2010). Plants were grown at 25°C with 14 hours of light and 10 hours of dark and new positions within the growth cabinets assigned to the pots once per week to ensure uniform growth conditions.

Ten weeks after sowing, the rhizosphere soil was harvested from the plants. The roots were massaged to remove the bulk soil. Once the inner roots were exposed, the roots of the plant were placed in a plastic bag and shaken to remove the rhizosphere soil. Although harvested in week 10, white lupin began to deteriorate in week 5 of the growth period, with all replicates of the white lupin treatment displaying signs of stunted growth. The leaves of the replicates began to discolor, turning pale and began to wilt, and all the replicates were deceased at harvest.

2.3 Dieldrin analysis: Approximately 10 g of airdried rhizosphere soil was finely ground using a ball mill (Retsch MM400, Germany). Ground soil (1 g ± 0.002) was then weighed into a 50 ml centrifuge tube, spiked with 40 µl dichlorodiphenyldichloroethane (DDD) (50 µg/ml) as an internal standard and then extracted three times each with 4 ml acetone:hexane (1:1) by vortexing for 10 s followed by side-to-side shaking at 225 rpm for 30 min. Extracts were centrifuged at 2500 rpm for 2 min, decanted into amber glass bottles and stored at 4˚C in the dark for later analysis. Soil extract (1 ml) was blown down to just dryness using nitrogen, then dissolved in 1 mL hexane before a Florisil clean up. The Florisil (500 mg/3 ml) cartridge was first conditioned with 3 ml hexane, then loaded with 1 ml soil extract in hexane, followed by eluting with 5 ml of acetone:hexane (1:9). Eluent was evaporated and redissolved in 1 ml hexane for analysis using a dual column GC/ECD (Varian CP-3800; DB-5MS UI 15 m x 0.32 mm, 0.25 µm; DB-1701 15 m x 0.32 mm, 0.25 µm) equipped with nickel 63 electron capture detector (ECD) and autosampler. A total of 2 µl was injected and separated into two columns. The temperature of injector and detector was held at 280 ˚C and 320 ˚C, respectively. Analytes were measured at a column temperature of 210 ˚C, a flow rate of 2.3 ml/min and a run time 20 min. A calibration curve was created by injecting three levels of dieldrin standards (0.47, 0.094 and 0.0094 µg/ml) with DDD as an internal standard (0.17 µg/ml) at a detection limit of 0.1 µg/g. No background levels of DDD were detected previously in the soil. The ratio of the peak areas of dieldrin and DDD was used for quantification.

2.4 DNA extraction of rhizosphere soil: DNA was extracted from the soils using the Qiagen 'PowerSoil DNA Isolation Kit' and following the provided protocol. In brief, DNA was extracted by bead beating 0.25 g of soil. Non-DNA cell material was lysed by a series of washes and centrifugations with the resulting cleaned DNA stored in 10 mM Tris and frozen at -20 ˚C for downstream applications.

2.5 Next generation sequencing of rhizosphere soil: The V4 515F-806R primers were selected for bacteria (Thompson et al., 2017), and primers FITS7 and ITS4 were selected to investigate fungal community structure (Egidi et al., 2019). Both the bacterial and fungal sequencing libraries were prepared by following the Illumina '16S Metagenomic Sequencing Library Preparation' protocol, and the 'Fungal Metagenomic Sequencing Demonstrated Protocol', respectively. Sequencing was performed on the Illumina MiSeq System (Illumina, San Diego, USA).

2.6 Bioinformatic analysis: QIIME2 (2019.1) software was utilized for the analysis of microbiome data in this study (Bolyen E, 2019). Initially, forward and reverse reads were paired together. The resulting paired sequences were denoised using DADA2 (Callahan et al., 2016), a pipeline which detects and corrects Illumina sequenced amplicon errors. This software also trims the paired sequence to remove both the forward (52 bp) and reverse (54 bp) primers. Utilizing DADA2, the following ends of the sequences were removed via truncation; 290 – 300 bp in the forward read and 230 – 300 bp in the reverse read. The denoised sequences are then screened against a classifier to cluster and assign OTUs at a 99% confidence threshold, a UNITE classifier (version 7) was assigned to fungi (Nilsson RH, 2018), and a SILVA classifier (version 132) was used for bacteria (Quast et al., 2012).

2.7 Quantitative polymerase chain reaction (qPCR) to determine microbial abundances: To compare microbial abundance between treatments, bacterial and fungal gene copies were quantified using qPCR. Primer pairs for the bacterial qPCR were selected to target the 16S rRNA genes and included 1114f (5' - CGGCAACGAGCGCAACCC) and 1275r (5' - CCATTGTAGCACGTGTGTAGC) (Wood, Zhang, Mathews, Tang & Franks, 2016). The fungal primer pairs were selected to target the ITS region with ITS1f (5' -

TCCGTAGGTGAACCTGCGG) and 5.8Sr (5' - TCCGTAGGTGAACCTGCGG) (Morrison et al., 2016). qPCR reaction mixes were adapted from Krohn et al. (2019). The fluorescence was read after 40 cycles of 10 s at 94 ˚C and 30 s at 60 ˚C for bacteria and 30 s at 95 ˚C, 30 s at 55 ˚C and 30 s at 72 ˚C for fungi. The amplified products of the bacterial and fungal primers were compared to a standard of *Escherichia coli* and *Mucor racemosus* respectively. PCR amplifications and fluorescent reading was conducted on the CFX Connect Real-Time PCR Detection System (BioRad, California, USA).

2.8 Statistical analysis: All figures and their corresponding statistics were created in R (R Core Team, 2019). Statistical significance was indicated at $p < 0.05$. Analysis of variance (ANOVA) was used to analyze the significant differences between treatments, with a Tukey HSD test used for subsequent pairwise comparisons. The multiple regression model was conducted by using the 'lm' function within the R console. Differential expression analysis (DESeq) was used to assess quantitative changes in ESV relative abundance between treatments using the *DESeq2* package (Love, Huber & Anders, 2014). Using the *indicspecies* package, an indicator species analysis examined genera of microorganisms that were unique to each treatments (Cáceres & Legendre, 2009).

3 Results

3.1 Dieldrin concentrations in the rhizosphere: Statistically significant differences in the concentrations of dieldrin in soils associated with grasses, legumes and an unplanted control were identified (ANOVA, $F_{(12,36)} = 2.51$, $p = 0.016$). However, pairwise testing indicated that only one of the eleven treatments, white lupin, had significantly lower dieldrin concentrations compared to the unplanted control (Figure 1). There was no effect in dieldrin concentrations over the 10-week growth period when comparing the initial bulked soil samples (pre-sowing) vs the soil only control. Although the pre-sowing samples were lower (1.33 µg/g) than the soil only control (1.42 µg/g), there was no statistically significant differences found ($p = 0.86$).

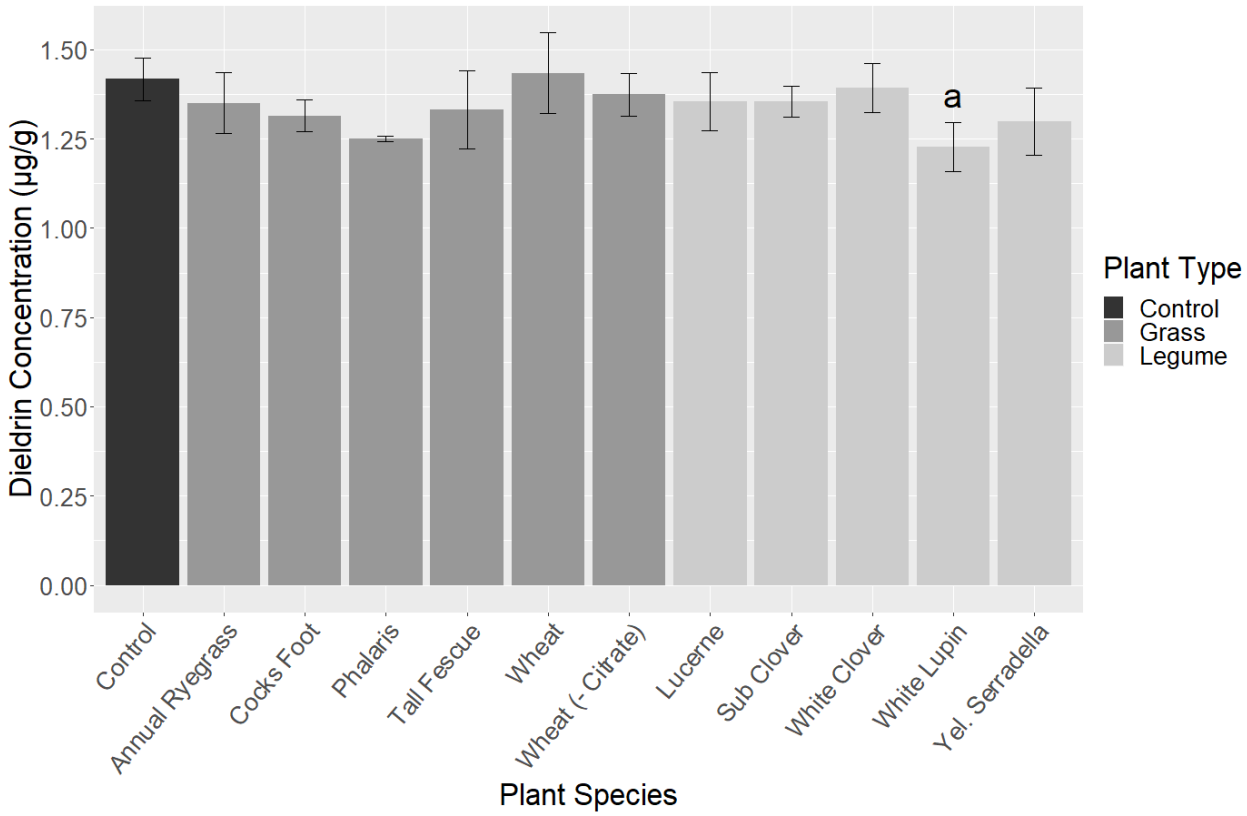

Figure 1. Dieldrin concentration in rhizosphere soil of different species of plants (mean ± standard error) after 10 weeks of growth (n = 4). Letters indicate significant ($p < 0.05$) difference from the unplanted control.

3.2 Decline in plant health: In week 5 of the 10-week growth period, all replicates of the white lupin treatment began to display signs of stunted growth. The leaves of the replicates began to discolor, turning pale and began to wilt. Excluding the lucerne treatment, all legumes had a lower mean root weight compared to the grasses (Figure 2), with no significant differences present. Although the growth of white lupin deteriorated from week 5, there was sufficient roots (0.6 g) remaining in week 10 for the harvest of rhizosphere soil.

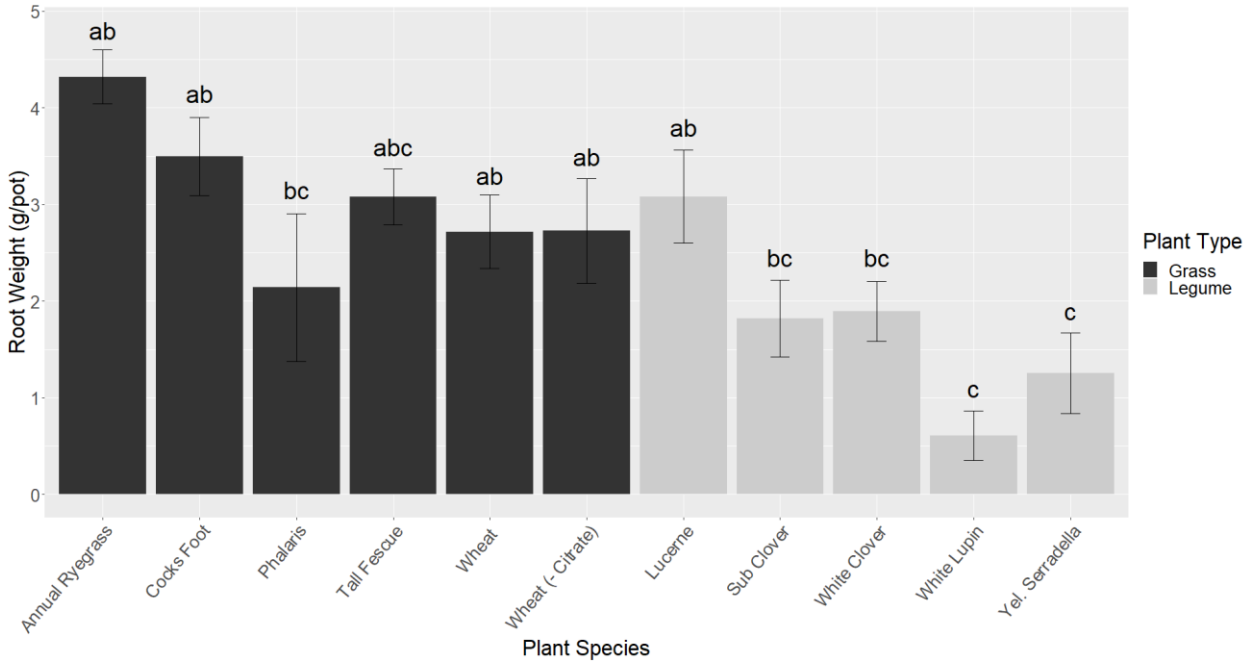

Figure 2. Root dry weight of different species of plants (mean ± standard error) after 10 weeks of growth (n = 4). Letters indicate significant ($p < 0.05$) difference.

3.3 Relationship between microbial abundance and diversity to dieldrin degradation: To determine the influence of microbial abundance on the dieldrin concentrations in the soil, a multiple regression analysis was conducted. The corresponding regression model is defined by the below equation. However, the regression model, $F_{(2,46)} = 0.44$, $p = 0.64$, $R^2 = 0.01$, states that these trends are not statistically significant.

$$Dieldrin = 1.61 - (0.06 \times Bacterial\ abundance) - (0.02 \times Fungal\ abundance)$$

A second multiple regression analysis was used to analyze the relationship between bacterial and fungal species diversity, and the concentration of dieldrin. The below equation defines the estimated model coefficient equation. The regression model ($F_{(2,46)} = 0.9$, $p = 0.4$, $R^2 = 0.03$) resulted in no correlation nor statistical significance.

$$Dieldrin = 0.74 + (0.1 \times Bacterial\ diversity) + (0.03 \times Fungal\ diversity)$$

3.4 Taxonomic changes present in the white lupin rhizosphere:

To disentangle which taxa were associated with the significant reduction in dieldrin concentrations in the white lupin rhizosphere, differential abundance testing was conducted between the white lupin treatment and control communities at the genus level. Direction of change indicates whether the abundance of fungi increased or decreased relative to the control. There were 18 different genera of fungi that were differentially expressed in the white lupin treatment, 11 of which were significantly increased (Table 2).

Table 2. Differential abundance for fungal genera between white lupin and the control.

Fungal genera	Mean abundance	Direction of change	*p*-value
Saitozyma	647.1	↑	0.001
Unidentified (OTU 511)	442.8	↓	0.001
Penicillium	151.6	↑	0.001
Clonostachys	1170.3	↑	0.001
Roussoella	624.6	↑	0.001
Mortierella	10670.1	↓	0.001
Thanatephorus	2888.2	↑	0.001
Rhizophagus	297.9	↓	0.001

Rasamsonia	82.5	↓	0.001
Pseudophialophora	36.5	↓	0.001
Trichoderma	509.5	↑	0.003
Umbelopsis	155.8	↑	0.001
Metarhizium	1124.8	↑	0.02
Microdochium	7.11	↑	0.03
Unidentified (OTU 377)	11.6	↑	0.04
Scutellinia	193	↓	0.04
Ilyonectria	111	↓	0.04
Terramyces	301.4	↑	0.04

3.5 Indicator species present in white lupin treatment: An indicator species analysis was used to identify bacterial and fungal indicator OTUs for white lupin. The analysis revealed a strong correlation for indicator species in both bacteria and fungi, an unidentified microorganism (OTU 539) and *Cortinarius* spp. (Table 3).

Table 3. Indicator species analysis of white lupin.

Kingdom	Genus	R	*p*-value
Bacteria	Unidentified (OTU 539)	0.8	0.02
Fungi	Cortinarius	0.866	0.025

4 Discussion

4.1 Dieldrin degradation in the white lupin rhizosphere

The degradation potential of a chemical is largely reliant on its physiochemical properties (Rissato et al., 2015). Dieldrin's hydrophobicity and increased tendency to persist in particulate organic matter explains the lack of degradation evident in this study. However, the presence of the white lupin rhizosphere overcame these barriers and promoted the degradation of dieldrin by 0.2 µg/g (13%) compared to the soil only control. It has previously been suggested that legumes, such as white lupin, are able to recruit microbial degraders to their rhizosphere due to a diverse root exudate profile (Lesuffleur & Cliquet, 2010 and Walker, Bais, Grotewold & Vivanco, 2003). In addition, low molecular weight organic acids present in root exudates can increase the bioavailability of organochlorines by chelating cations in the soil, which disrupts its mineral complex and releases the organic matter into the soil (White, Mattina, Lee, Eitzer & Iannucci-Berger, 2003). Although legumes are thought to possess higher concentrations of root exudates and thus an increased ability to degrade soil contaminants, this study found no significant difference in soil dieldrin concentrations when plant species were grouped by plant type (ANOVA, $F_{(2,46)} = 1.353$, $p = 0.269$). Therefore, factors other than root exudate profiles are driving the degradation of dieldrin in the rhizosphere of white lupins.

4.2 Detritusphere assisted degradation?

Interestingly, the decline in plant health of the white lupin treatment coincided with a decrease of dieldrin concentration in the soil, therefore potentially assisting the degradation process. The detritusphere, soil influenced by decaying matter, provides a further niche for microbes capable of degrading complex organic material (Zhou et al., 2020). Where root detritus increased functional diversity in the rhizosphere of *Avena fatua* (wild oat) (Nuccio et al., 2020). The alteration of functional diversity due to detritus is supported by Zhou et al. (2020), who found that in the presence of decaying roots, the rhizosphere was enriched with genes encoding membrane transporters and the metabolism of carbohydrates. Investigation is required into the effect of detritus, and its potential use in the bioremediation of soil contaminants.

4.3 No correlations between microbial abundance or diversity to dieldrin degradation

Increasing the abundance and diversity of microbes in the soil can result in degradation of organic contaminants (Jain et al., 2005). The addition of a rhizosphere has shown to increase Shannon diversity and abundance of microbes in the soil, which has the potential to facilitate this increased degradation (Tam, Derry, Kevan & Trevors, 2001 and Kirk, Klironomos, Lee & Trevors, 2005). A correlation between the presence of a rhizosphere and dieldrin degradation has been reported for sorghum grass. The presence of microorganisms in sorghum sudanese rhizosphere decreased dieldrin by 79% its initial concentration, compared to 18% in the control (Pan, Lei, Wu & He, 2017). Contrary to previous findings, this study found no relationship between the degradation of dieldrin and the diversity of microorganisms in the rhizosphere.

4.4 Significance of differentially abundant microbes

Because little evidence linking dieldrin degradation to community-level parameters such as abundance or diversity was observed in this study, we hypothesized that the degradation of dieldrin in the white lupin treatment was likely due to specific microbial species, rather than whole communal interactions. However, whether these species are intrinsically selected for by the white lupin root exudate profile, or whether their abundance was linked to the decline in white lupin health, remains to be investigated. Two such microbes, *Penicillium* and *Trichoderma*, have previously documented to aid dieldrin degradation. Their enrichment in the white lupin rhizosphere supports the hypothesis that distinct taxa were responsible for the degradation of dieldrin. *Penicillium* is an active decomposer of dieldrin capable of degrading up to 90% of the available concentration (Birolli et al., 2015). Although not as potent as *Penicillium*, fungi of the genera *Trichoderma* are more widely capable of its degradation and currently there are three different strains known to metabolize dieldrin: *viride*, *koningii* & *strain 91355* (Matsumura & Boush, 1967; Bixby, Boush & Matsumura, 1971 and Kataoka, Takagi, Kamei, Kiyota & Sato, 2010). The potency of *Penicillium* and the variety of *Trichoderma* may contribute to degradation of dieldrin within the white lupin treatment observed in this study. Although there has been prior research into microbes capable of dieldrin degradation (Purnomo, 2017), those identified to date do not constitute a comprehensive list and there are likely more microbes yet to be characterized. It is therefore likely that fungal genera which were significantly and specifically increased within the white lupin treatment are potential degraders of dieldrin. *Saitozyma* from the

Trimorphomycetaceae family was significantly increased in only the white lupin rhizosphere. *Saitozyma* was only recently classified in 2015 (Liu et al., 2015), however it has already been recognized as a highly tolerant microbe in the presence of heavy metal soil contamination (Torres-Cruz, Hesse, Kuske & Porras-Alfaro, 2018). The ability for *Saitozyma* to persist in this environment highlights its potential ability to degrade soil contaminants and allow it to survive in adverse environments (Torres-Cruz, Hesse, Kuske & Porras-Alfaro, 2018). *Umbelopsis* was another microbe to be significantly increased in only the white lupin rhizosphere. The fungi from the Umbelopsidaceae family have demonstrated a high potency for remediation, due to its ability to remove selected heavy metals from contaminated sites (Janicki, Długoński & Krupiński, 2018). Although the literature supports the potential ability of *Saitozyma* and *Umbelopsis* to bioremediate soil contaminants, it subsequently highlights the lack of information regarding microorganisms capable of dieldrin degradation.

4.5 Key indicator species

Through the indicator species analysis, this study hypothesises that *Cortinarius* spp. are also capable of dieldrin degradation. *Cortinarius* is a genus of fungi within the Agaricles class and is unique to the white lupin treatment, therefore it is suggested to be capable of dieldrin metabolism. Prior research supports the ability of *Cortinarius* to degrade soil pollutants, as it was demonstrated to exhibit petroleum hydrocarbons and polyphenolic compounds degradation (Petkovšek & Pokorny, 2013 and Bai, Wang, Zong, Lei & Gao, 2013). Previous literature supports the potential ability for Cortinarius to degrade dieldrin, but this again highlights a lack of knowledge regarding dieldrin degrading microorganisms.

5 Conclusion

In summary, the presence of a rhizosphere did not ubiquitously influence the degradation of dieldrin in the soil. Only one of twelve treatments resulted in a significant decrease in the concentration of dieldrin. Contrary to similar studies, microbial abundance and diversity were statistically void of any correlation to the concentrations of dieldrin. Analysis at the genus level indicated dieldrin degradation was occurring via individual microorganisms rather than whole communities. *Trichoderma* and *Penicillium* were found to be significantly increased in abundance in the white lupin treatment. This increased abundance, coupled with a known ability to degrade dieldrin suggests that these genera of microbes may be responsible for the decreased concentration of dieldrin in the soil. Additionally, *Saitozyma* and *Umbelopsis* were found to be significantly enriched in the white lupin rhizosphere. Although there is no literature correlating these microbes to dieldrin degradation, there are studies that infer their capability to degrade pollutants in the soil and suggest that these organisms may also contribute to dieldrin degradation. The fungal genus *Cortinarius* was identified as an indicator of dieldrin degradation in the white lupin rhizosphere. Future investigation is recommended into the ability of the aforementioned microorganisms to degrade dieldrin, an investigation that was beyond the scope of this study. This study found that dieldrin degradation was not a general community response to the presence of a rhizosphere, but rather a product of specific microorganisms found within that community. Analysing microorganisms individually rather than as a community highlights the potential to discover novel dieldrin degraders.

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Authors Contributions

M.B., C.K., A.E.F. and C.T. planned and designed the study; M.B. and C.K. managed the rhizosphere experiment; C.K. and P.Z. conducted the dieldrin analysis; M.B. and J.L.W. analyzed the data. M.B. created the figures, completed the qPCR analysis, performed the 16S/ITS Illumina sequencing and wrote the manuscript. All the authors contributed to the revisions and gave approval for the publication.

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Notes

The authors declare no competing financial interest.

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Chapter 3: Additional Materials & Methods

This section contains additional information regarding materials and methods of collecting data and qualitative analyses not discussed in Chapter 2 that were used to generate the results in Chapter 4. Also not included in Chapter 2 is a visual representation of the sampling site that can be found in Appendix 1.

3.1 Sampling Dry Weights and pH

After the rhizosphere soil was extracted, each plant was cut at the shoot–root interface. The shoots were washed sequentially in three separate buckets of reverse osmosis (RO) water, replacing the water after each treatment. The roots were washed in a 2 mm sieve to remove any remaining debris. Once the shoots and roots were thoroughly washed, each replicate was placed in a paper bag with their corresponding labels. To obtain dry-weight measurements of both the shoots and the roots, the paper bags were placed in a 65°C oven for one week with weights being recorded daily. The final weight measurement was obtained after one week when the weights stopped decreasing due to loss of moisture.

pH measurements for each treatment were obtained by diluting 4 g of soil in 20 ml of 0.01 M $CaCl_2$ and shaking end-on-end for 1 hr. The soil was settled to the bottom of the falcon tube via centrifugation at 2000 rpm for 2 min. After calibration, pH measurements were read using a ThermoFisher Scientific^{TM} OrionStarA215 with a Hannaelectrode.

Chapter 4: Microbial Ecology of Grasses and Legumes

The results presented in this chapter explore the microbial ecology of grasses and legumes. For clarification, the term 'environmental plant factors' used in the following two sections encompasses: shoot weight, root weight, transpiration and pH.

4.1 Changes in Microbial Abundance in the Rhizosphere

4.1.1 Legumes Contain a Higher Bacterial Abundance than Grasses

When comparing the bacterial abundance of different plant species (Figure 4.1 (a)), statistical analysis reveals that there were significant differences present in the data, (Kruskal-Wallis, X^2 = 39.238, df = 12, p = 0.00009). Pairwise comparisons show that the wheat (- citrate) treatment had a significantly lower bacterial abundance (7.05 ng/µl) compared to annual ryegrass (7.61 ng/µl), cocks foot (7.58 ng/ µl), lucerne (7.75 ng/µl), sub clover (7.72 ng/µl) and white lupin (7.5 ng/µl). Lucerne and sub clover also showed significant increase in bacterial abundance compared to phalaris (7.39 ng/µl) and the soil only control (7.17 ng/µl).

Plant species were grouped by plant type to examine their effect on bacterial abundance (Figure 4.1 (b)), in which there are statistically significant differences present (Kruskal-Wallis, X^2 = 15.9, df = 2, p = 0.0003). Both grasses (7.51 ng/µl) and legumes (7.67 ng/µl) had significantly larger bacterial abundances compared to the control (7.17 ng/µl), and although legumes had the highest bacterial abundance, this difference was not significant. There are 4 outliers in the grass boxplot which correspond to the wheat (- citrate) treatment, this sample was removed from the statistical testing as it skewed the data causing a significant result.

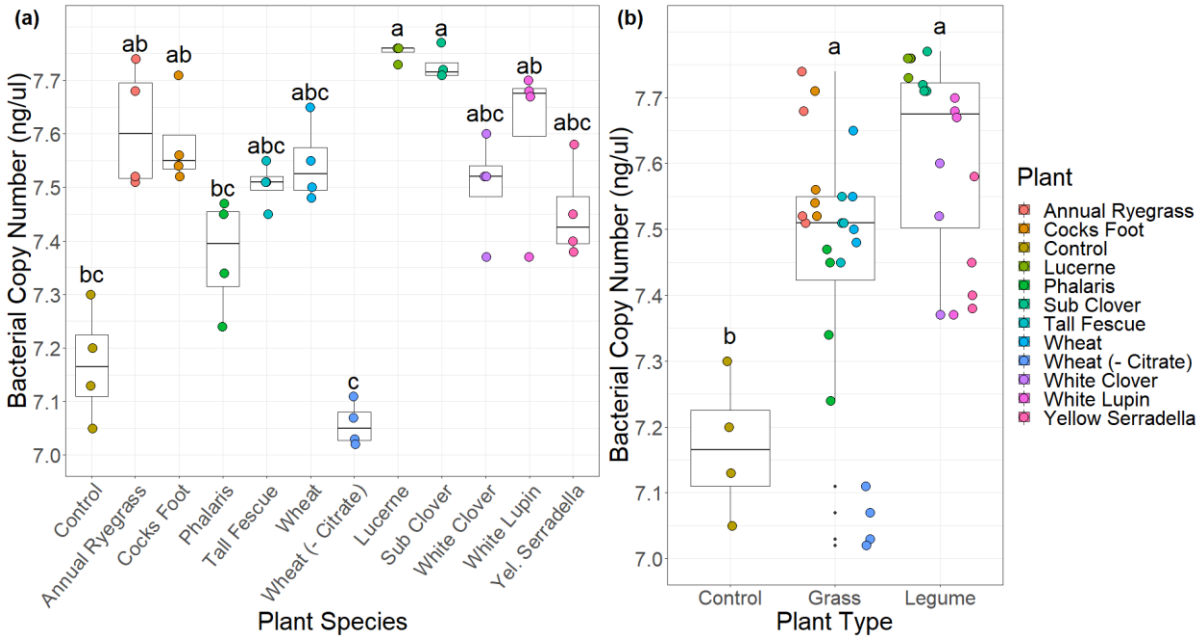

Figure 4.1. Bacterial copy number in different species (a) and types (b) of plant (mean ± standard deviation). Different letters above plots indicate statistical significance ($p < 0.05$). Letters are in order of highest to lowest mean value.

4.1.2 Fungal abundance is Greater in Legumes than Grasses

There were statistically significant differences present between plant species and fungal abundance (Figure 4.2 (a), Kruskal-Wallis, $X^2 = 30.34$, df = 12, $p = 0.0014$). Only one legume showed significant results in the post-hoc Dunn test, with lucerne having a significantly larger fungal abundance (9.05 ng/µl) than phalaris (8.66 ng/µl). None of the treatments showed any statistical significance from the control group.

When plant species were grouped by plant type (Figure 4.2 (b)) there was statistically significant differences present (Kruskal-Wallis, $X^2 = 9.37$, df = 2, $p = 0.009$). Pairwise analysis revealed that legumes had a significantly higher fungal abundance (8.94 ng/µl) compared to grasses (8.66 ng/µl), with no significance present in between the control (8.72 ng/µl). Three annual ryegrass outliers in the grass boxplot remained in the statistical analysis as they did not skew the p-value.

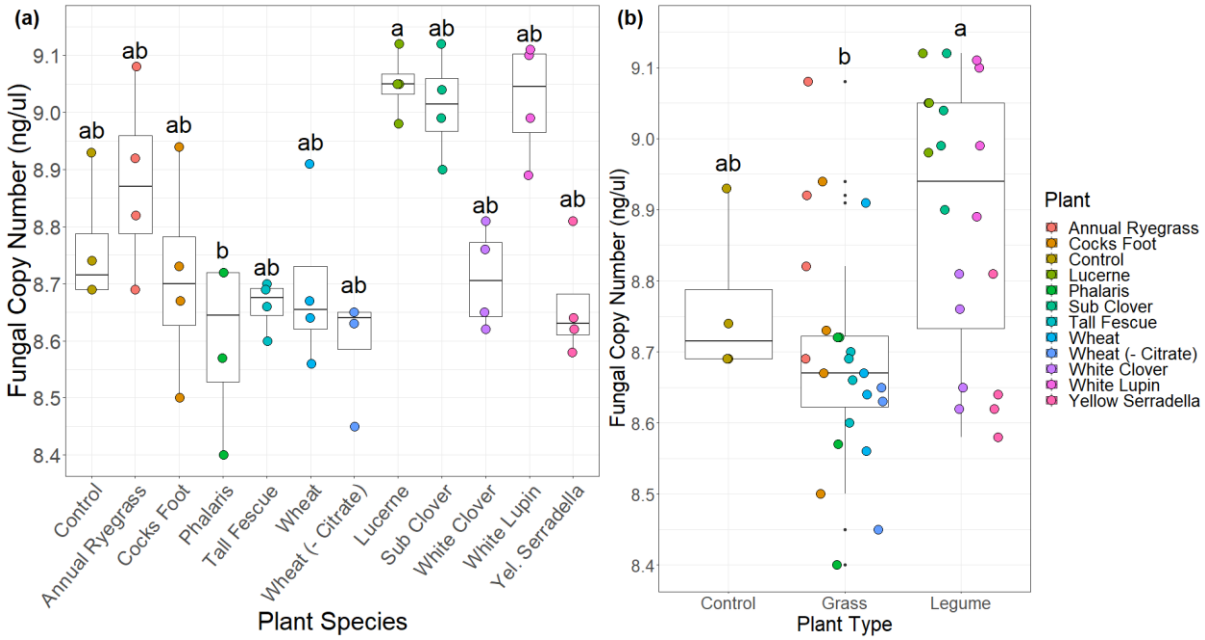

Figure 4.2. Fungal copy number in different species (a) and types (b) of plant (mean ± standard deviation). Different letters above plot indicate statistical significance ($p < 0.05$). Letters are in order of highest to lowest mean value.

4.2 Changes in Microbial Diversity in the Rhizosphere

4.2.1 Variation of Alpha and Beta Diversity of Bacteria in the Rhizosphere

4.2.1.1 Legumes have a Higher Bacterial Alpha Diversity Compared to Grasses

A Shannon index was applied to the data to examine the bacterial diversity between plant species and plant type. There were statistically significant differences between plant species and their bacterial diversity (Figure 4.3 (a), ANOVA, $F_{(12,34)} = 2.899$, $p = 0.007$). A Tukey post-hoc test shows that the microbial diversity seen in phalaris (4.45) was significantly lower than lucerne (4.75), sub clover (4.77) and white clover (4.74). There were no significant changes between the treatment groups and the control.

When grouping species by plant type (Figure 4.3 (b)) there are statistically significant differences in the bacterial diversity (ANOVA, $F_{(2,44)} = 4.945$, $p = 0.0116$). The legumes had a significantly higher Shannon diversity (4.72) compared to the grasses (4.58), with no significant difference from the control (4.56).

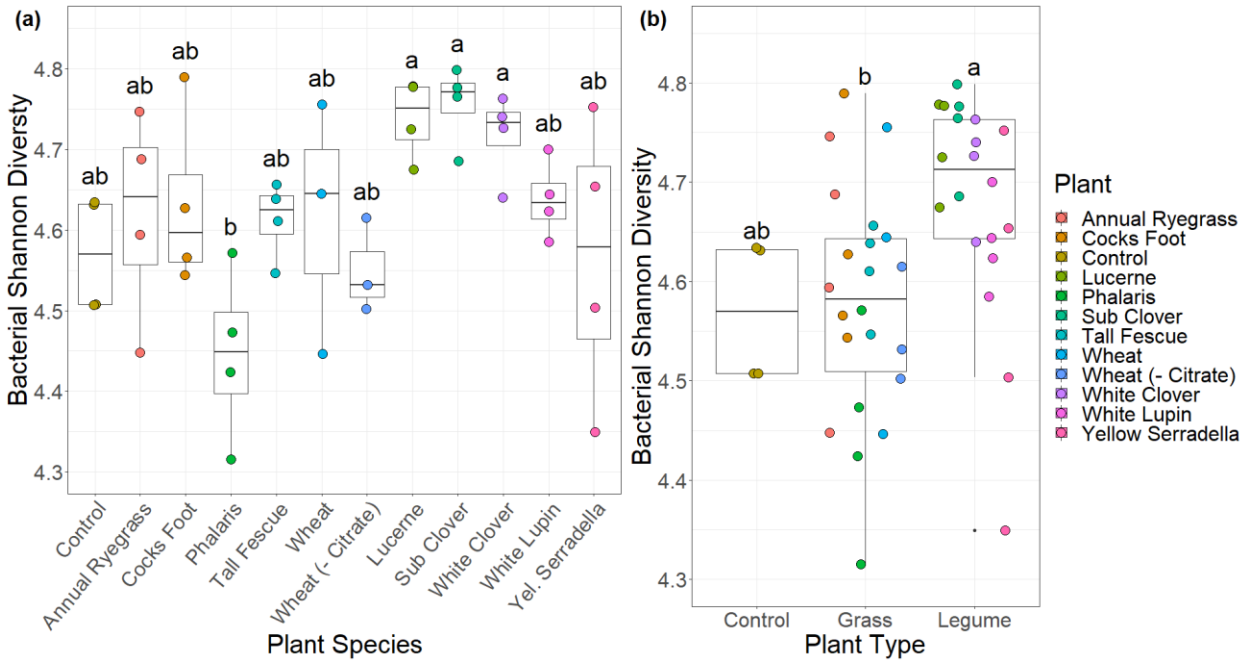

Figure 4.3. Bacterial Shannon diversity in different species (a) and types (b) of plant (mean ± standard deviation). Different letters above plots indicate statistical significance ($p < 0.05$). Letters are in order of highest to lowest mean value.

A Chao1 indices was applied to the data to compare the bacterial species richness of the treatments. There were statistically significant differences between plant species and the bacterial species richness (Figure 4.4 (a), ANOVA, $F_{(12,34)} = 2.902$, $p = 0.007$). The species richness in the sub clover (280) is significantly higher than annual ryegrass (225), phalaris (229), wheat (223) and wheat (- citrate) (212). There were no significant differences between the treatment groups and the control.

When plant species were grouped by plant type (Figure 4.4 (b)) there is statistically significant differences present in bacterial richness (ANOVA, $F_{(2,44)} = 8.571$, $p = 0.0007$). The legume treatment (253) had a significantly higher species richness compared to the grass group (224), with no significance present with the control (226).

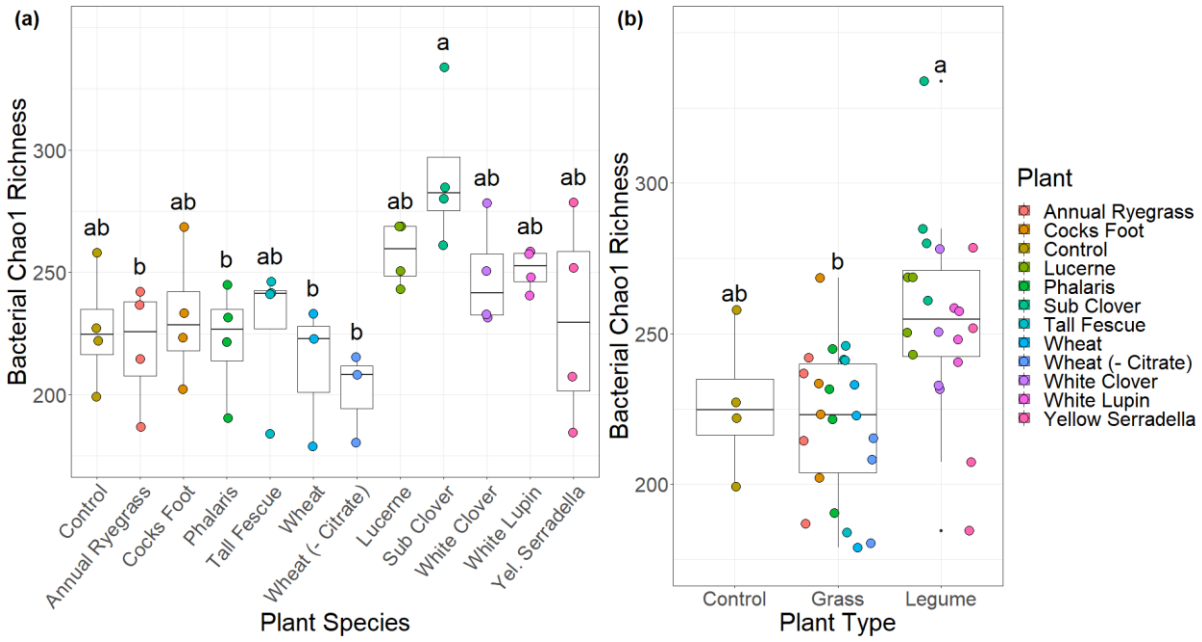

Figure 4.4. Bacterial Chao1 richness in different species (a) and types (b) of plant (mean ± standard deviation). Different letters above plots indicate statistical significance ($p < 0.05$). Letters are in order of highest to lowest mean value.

A Simpson index was applied to the data to compare bacterial species evenness across the treatments (Figure 4.5). There were no significant differences present in the relationship between plant species (Kruskal-Wallis, $X^2 = 17.847$, df = 2, $p = 0.12$) or plant type (Kruskal-Wallis, $X^2 = 5.45$, df = 2, $p = 0.06$) with bacterial species evenness.

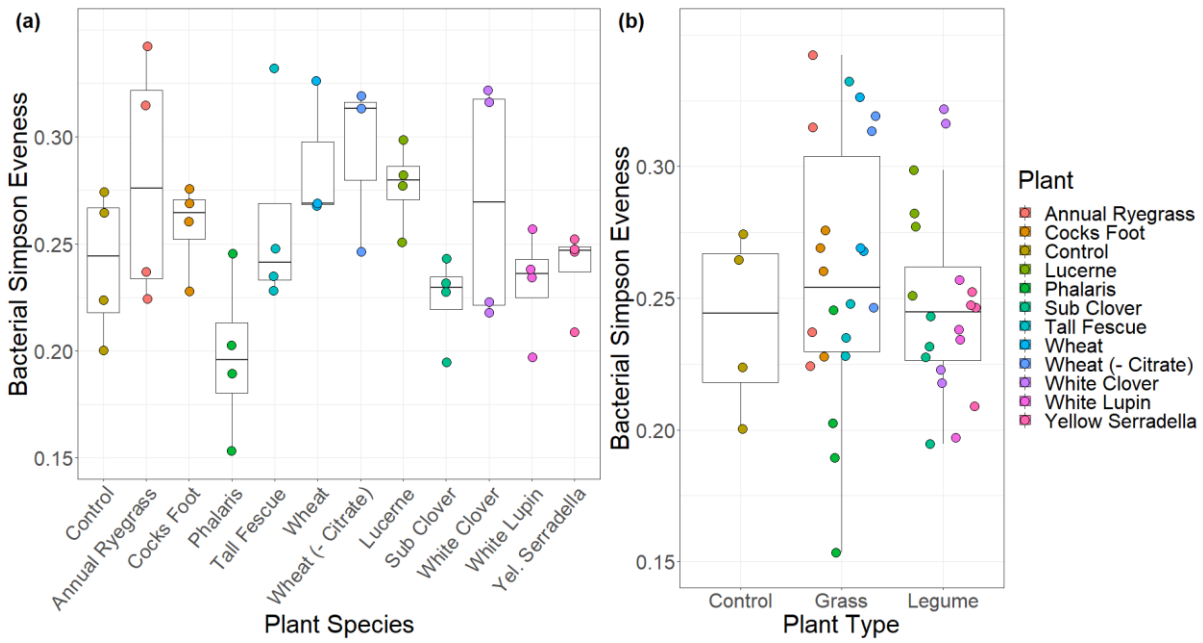

Figure 4.5. Bacterial Simpson evenness in different species (a) and types (b) of plant (mean ± standard deviation). No letters indicate no statistical significance.

4.2.1.2 Presence of a Rhizosphere Increases Bacterial Beta Diversity Between Treatments

A NMDS was created using a Bray-Curtis dissimilarity matrix to examine the similarities in bacterial species between treatments. The space between points in the ordination denotes how dissimilar the bacterial species are in one treatment compared to another. The differences within the NMDS (Figure 4.6) were statistically significant (p = 0.001) with a weak to moderate correlation (R^2 = 0.33) according to an ANOSIM analysis.

The NMDS shows that the presence of a rhizosphere alters the bacterial species diversity, as seen by the separation of replicates in the control treatment compared to the legumes and grasses. The replicates in the legumes were closely grouped together, indicating that the plants in the legume treatment contain similar bacterial species. The legumes were also grouped within the grasses, showing that the bacterial species that are present within the legumes were also present within the grasses. Contrary to what is seen in the legumes, there is more space between the replicates in the grasses. This shows that although similar, there is a larger variation in bacterial species within the grass treatments.

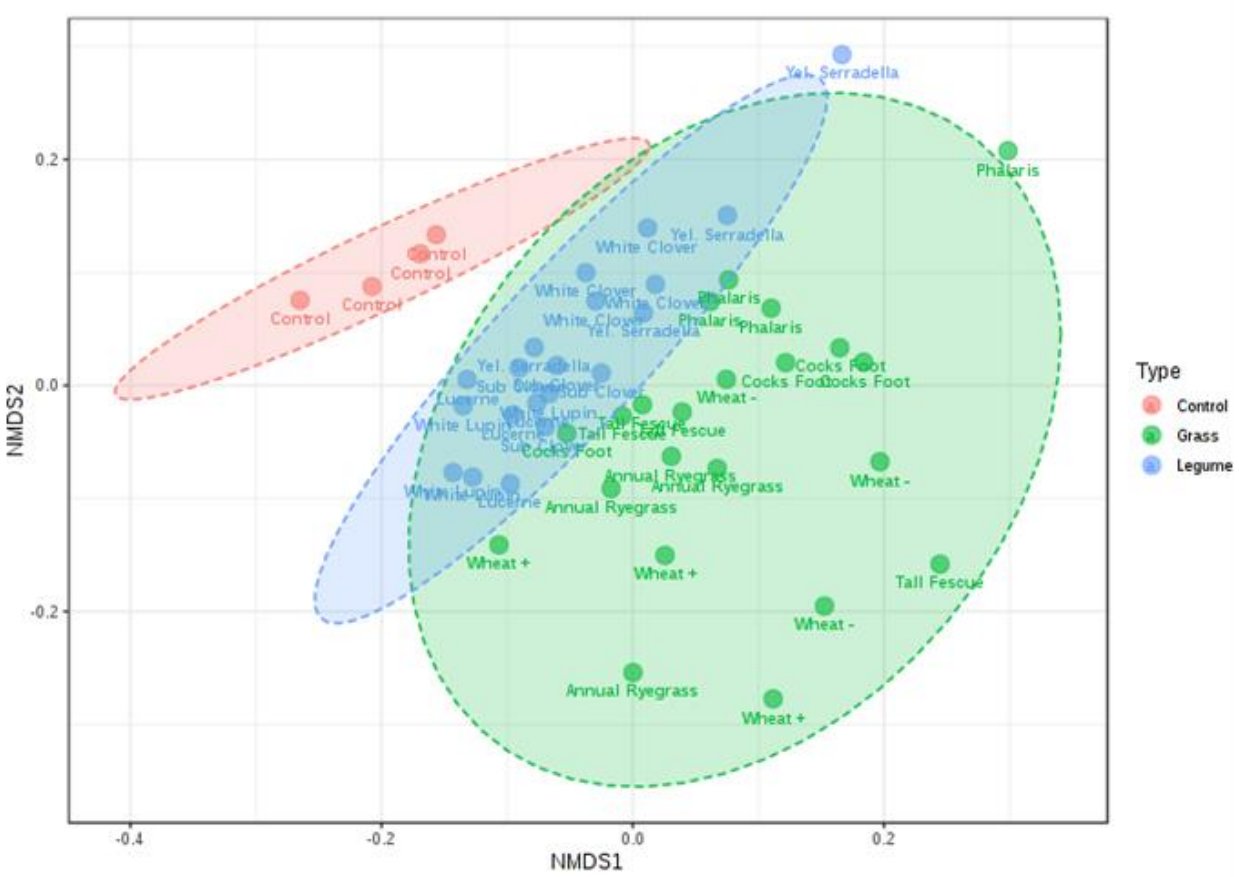

Figure 4.6. NMDS of the dissimilarity in bacterial species between treatments coloured by plant type and labelled by plant species.

The bar plot below (Figure 4.7) represents relative abundance of the bacterial species taxonomy (sorted by phylum) arranged by plant species. All the treatments share a similar core relative abundance, with the Actinobacteria, Proteobacteria, Chloroflexi and Acidobacteria dominating these soils. Large variations begin to occur within Patescibacteria, which was increased in the phalaris rhizosphere compared to the soil only control. At lower abundances there is little variation between treatments, where the smaller variations were mainly present within Verrucomicrobia, Firmicutes and Bacteroidetes.

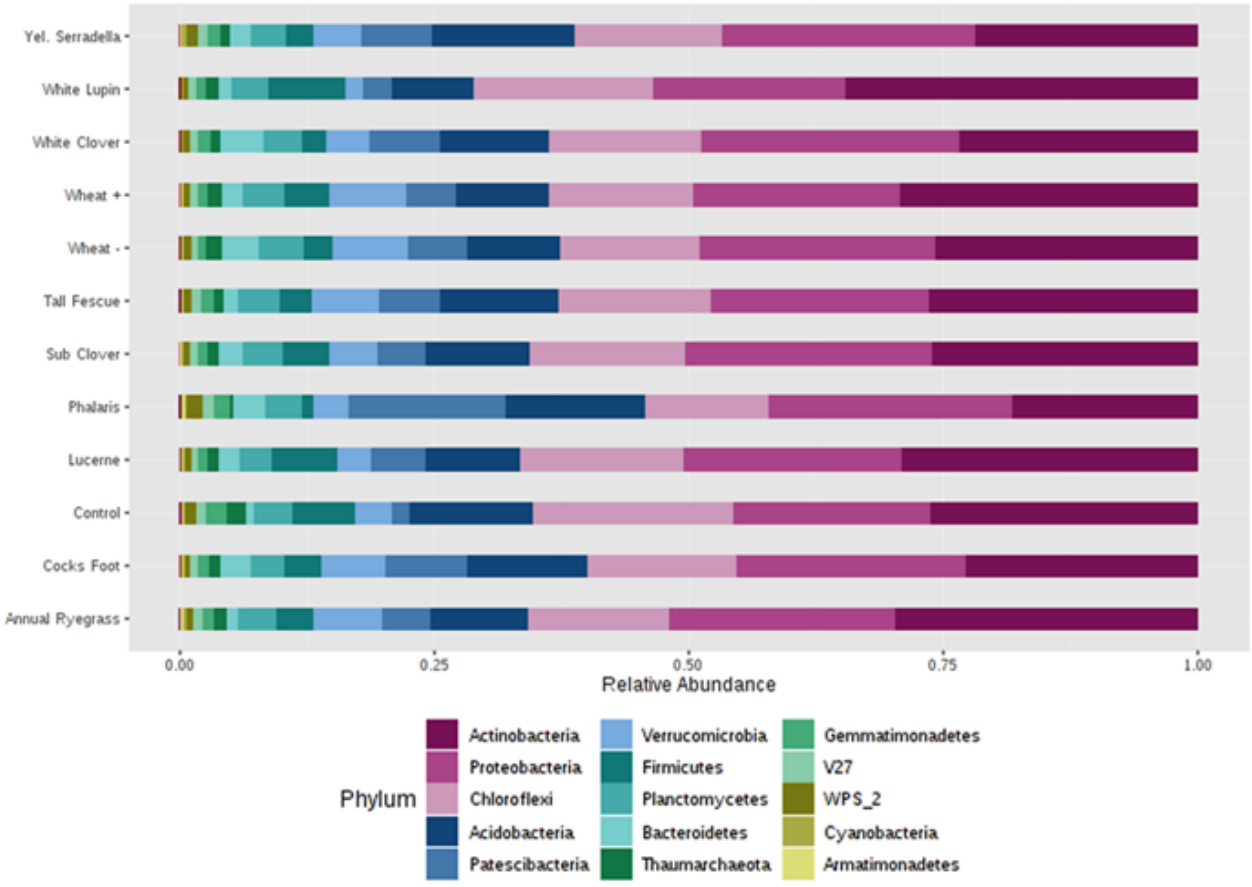

Figure 4.7. Bar plot of bacterial taxonomic composition in different plant species, sorted by phylum.

The correlations of relative abundance evident in plant species was also present when grouping by plant type (Figure 4.8). The composition of Phylum between the groups were largely similar, with the largest division seen in the Patescibacteria phyla. The abundance of this phyla approximately triples in legumes compared to the control, and almost doubles in abundance from legumes to grasses. Smaller changes in abundance can be seen in the Bacteriodies and Gemmatimonadetes phylum, where the abundance of Bacteriodies are doubled in grasses and legumes compared to the control and Gemmatimonadetes are twice as large in the control compared to grasses and legumes.

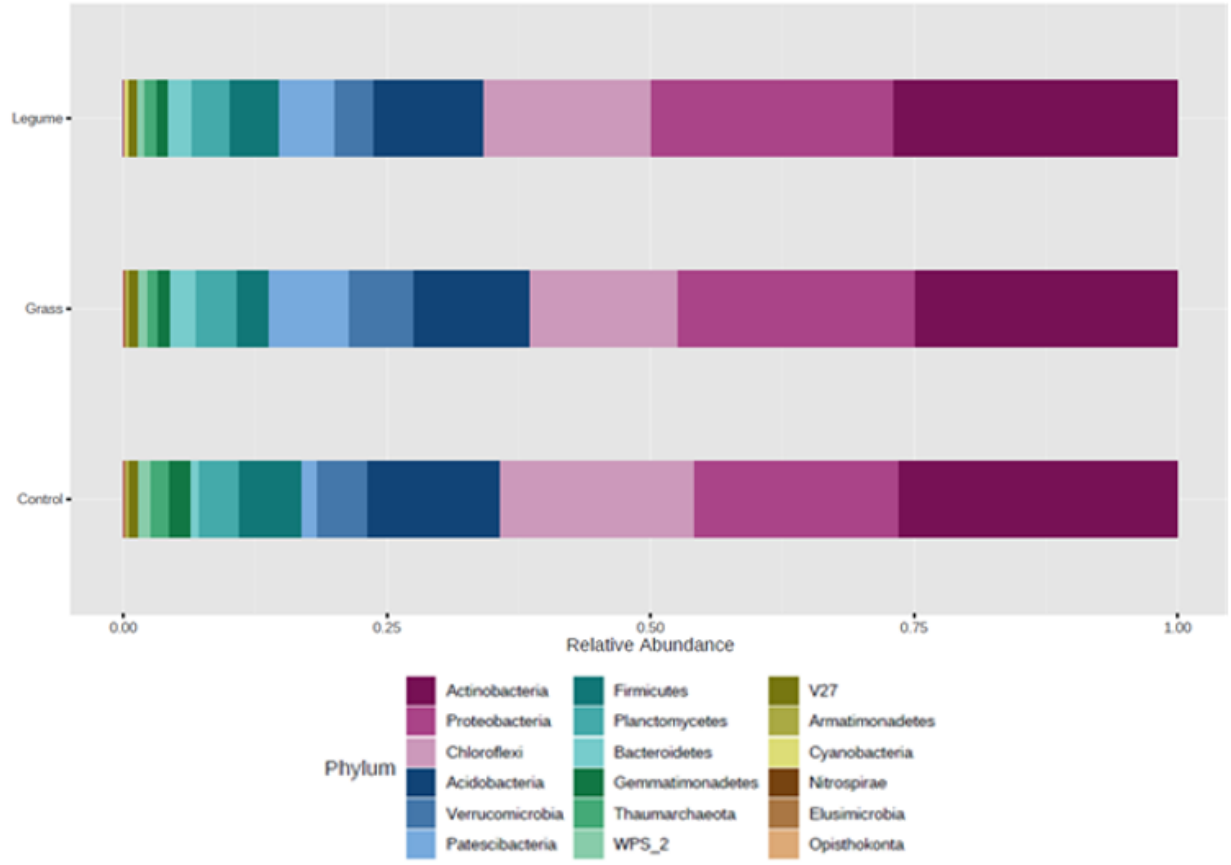

Figure 4.8. Bar plot of bacterial taxonomic composition in different plant types, sorted by phylum.

4.2.2 Variation of Alpha and Beta Diversity of Fungi in the Rhizosphere

4.2.2.1 Homogenous Fungal Alpha Diversity Between Plant Groups

A Shannon index was applied to the data to examine the fungal species diversity of the treatments. There were statistically significant differences between plant species and their fungal species diversity (Figure 4.9 (a), ANOVA, $F_{(12,36)} = 2.655$, $p = 0.01$). A Tukey post-hoc test shows that the fungal species diversity seen in phalaris (3.24) was significantly lower than the sub clover (3.58). There were no significant differences between treatments and the control group.

When grouping species by plant type (Figure 4.9 (b)) there was no statistically significant differences present in the fungal diversity (ANOVA, $F_{(2,46)} = 0.015$, $p = 0.98$).

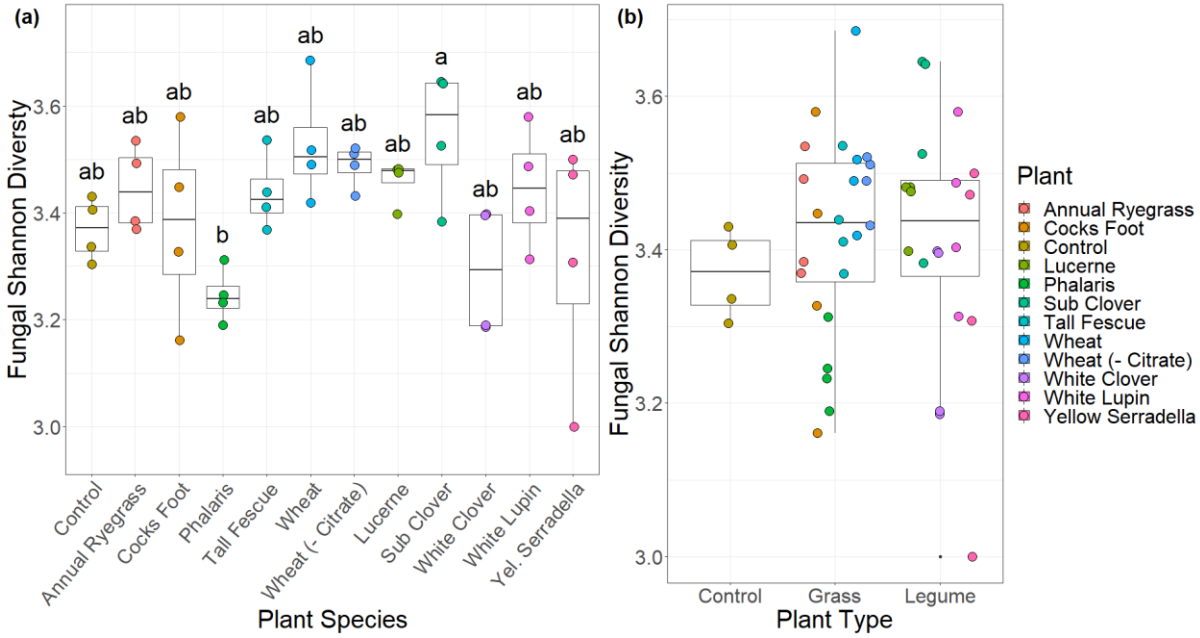

Figure 4.9. Fungal Shannon diversity in different species (a) and types (b) of plant (mean ± standard deviation). Different letters above plots indicate statistical significance ($p < 0.05$). Letters are in order of highest to lowest mean value. No letters indicate no significance.

A Chao1 indices was applied to the data to compare the fungal species richness between plant species and plant type. There were statistically significant differences present between plant species and the fungal species richness (Figure 4.10 (a), ANOVA, $F_{(12,34)} = 2.902$, $p = 0.007$). Pairwise comparisons show that fungal species richness in the phalaris (91) was significantly lower than the sub clover (184). There were no significant differences between the treatment groups and the control samples.

When grouping plant species by plant type (Figure 4.10 (b)), there were statistically significant differences in fungal species richness (Kruskal-Wallis, $X^2 = 9.06$, df = 2, $p = 0.01$). Where both the control (173) and legume (174) had a significantly higher fungal species richness compared to grass (150). Two yellow serradella outliers in the legume boxplot were included in the statistical analysis as they had no effect on the *p*-value.

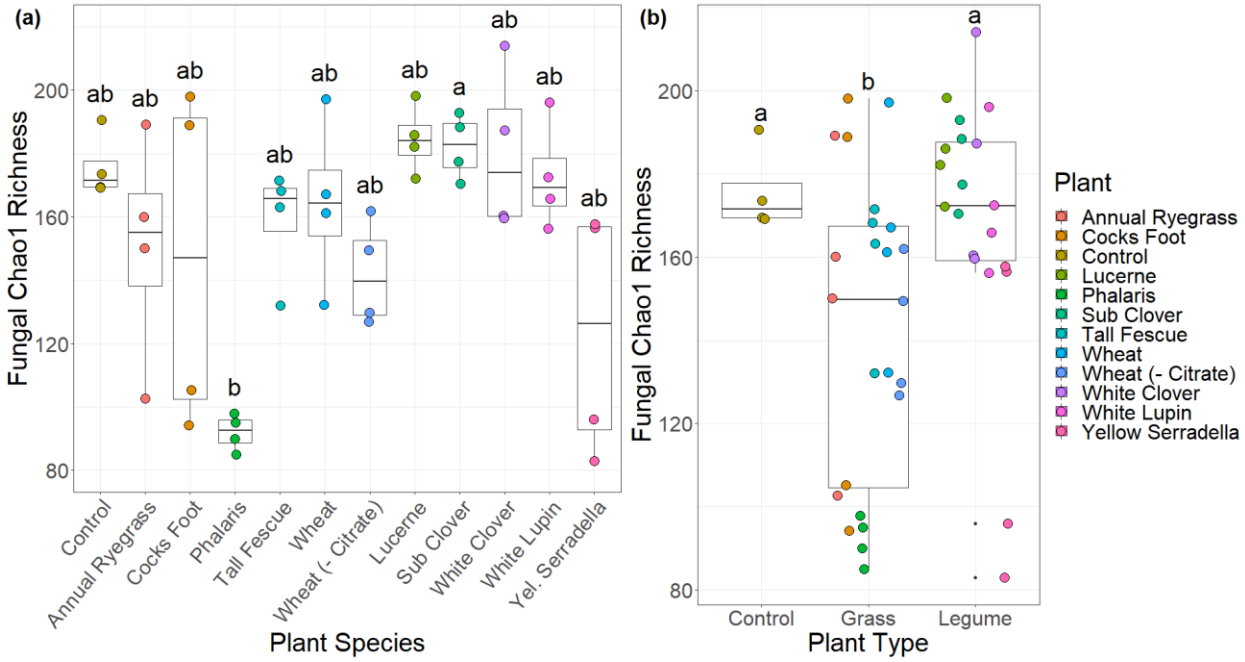

Figure 4.10. Fungal Chao1 richness in different species (a) and types (b) of plant (mean ± standard deviation). Different letters above error bars indicate statistical significance ($p < 0.05$). Letters are in order of highest to lowest mean value.

A Simpson index was applied to the data to compare fungal species evenness across samples. There were significant differences between species (Figure 4.11 (a), Kruskal-Wallis, X^2 (12) = 23.192, df = 12, p = 0.02), however the Dunn post-hoc test did not reveal any significant changes.

There was no statistical significance between plant type and Simpson evenness (Figure 4.11 (b), Kruskal-Wallis, X^2 = 4.47, df = 2, p = 0.11).

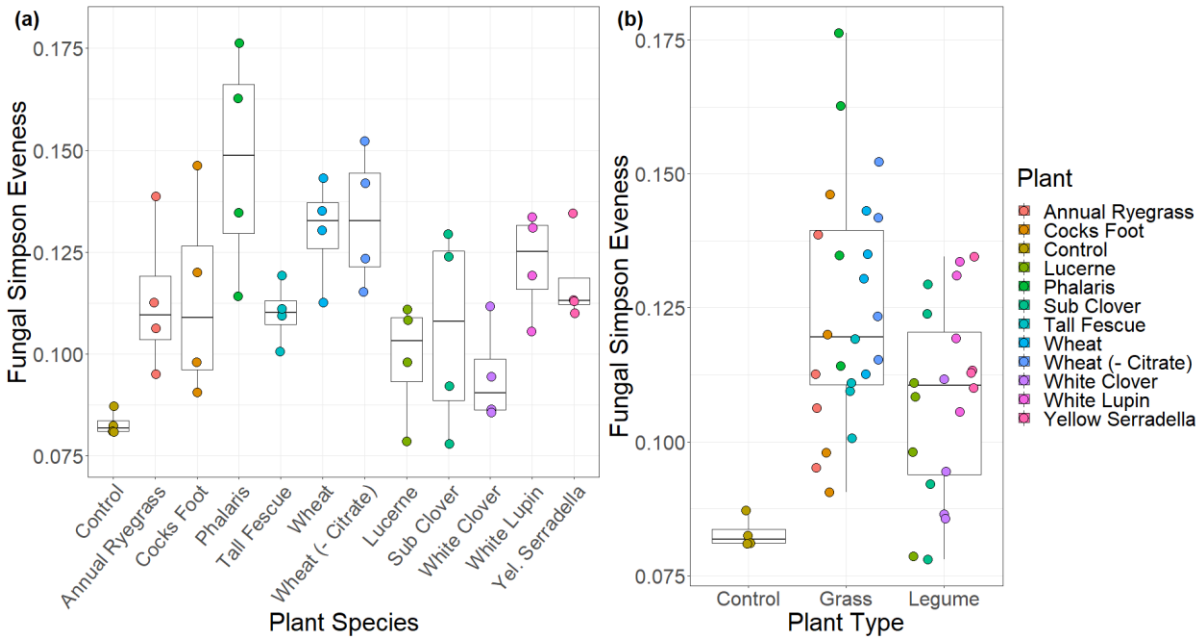

Figure 4.11. Fungal Simpson evenness in different species (a) and types (b) of plant (mean ± standard deviation). No letters indicate no statistical significance.

4.2.2.2 Similarities Between Treatments in Fungal Beta Diversity

A NMDS was created using a Bray-Curtis dissimilarity matrix to examine the similarity of fungal species between treatments. The space between points in the ordination denotes how dissimilar the fungal species are. The differences seen in Figure 4.12 below were statistically significant (p = 0.001) with a weak to moderate correlation (R^2 = 0.27) between the treatments according to an ANOSIM analysis.

The NMDS shows that the control group is dispersed within both the legumes and grasses, indicating that the presence of a rhizosphere did not alter the fungal communities. Except for

yellow serradella and a few outliers, most replicates in legumes were closely grouped together, revealing that the legumes share similar fungal species between treatments. This trend is also present in the grasses, where the replicates are mostly grouped together, except for phalaris and a few outliers. Overall, except for some outliers, the fungal communities across the control, legumes and grass treatments are relatively similar, as indicated by their close grouping in the ordination.

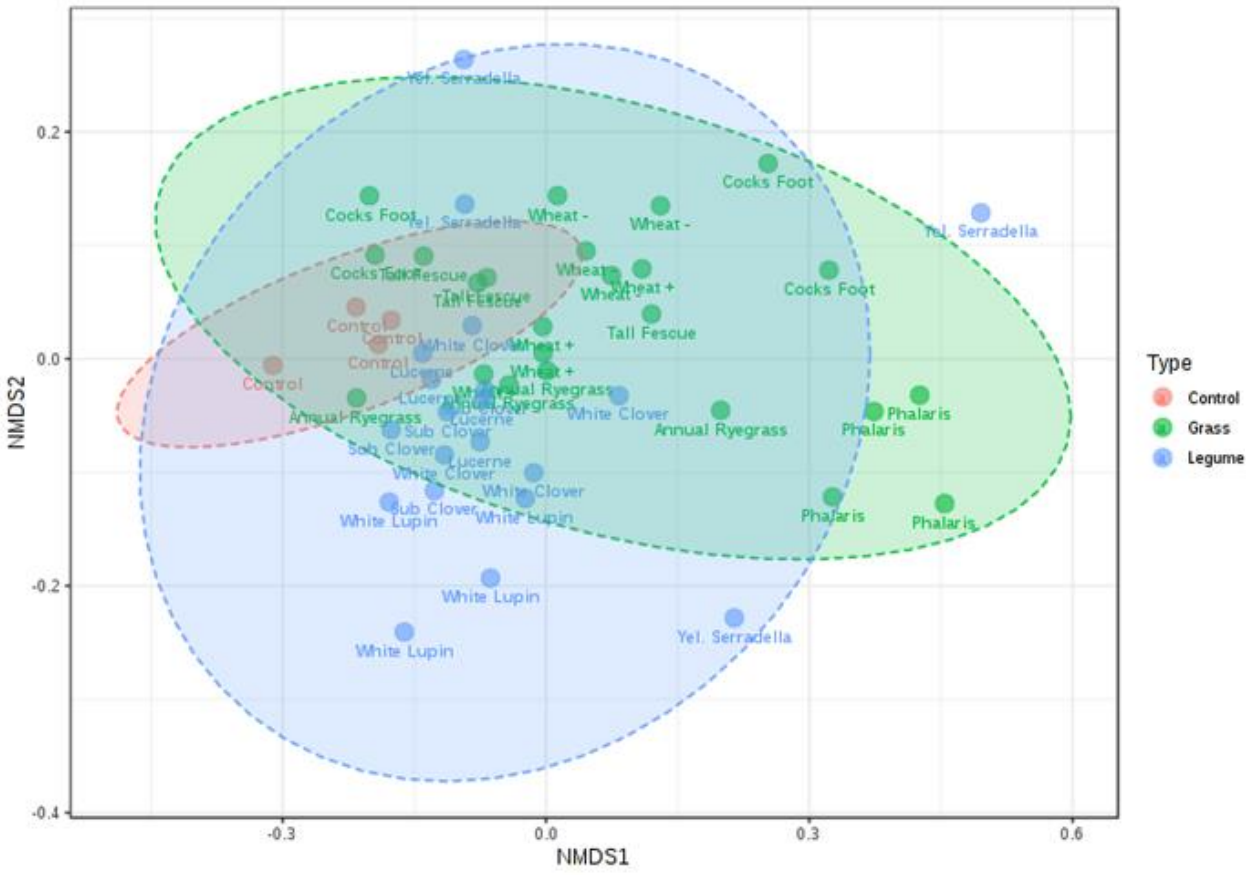

Figure 4.12. NMDS of the dissimilarity in fungal species between treatments coloured by plant type and labelled by plant species.

The bar plot below (Figure 4.13) represents relative abundance of fungal species taxonomy (sorted by class) compared by plant species. There were large similarities between treatments, as they were all dominated by the presence of Mortierellomycetes, Sordariomycetes and V1. The largest variations of relative abundance can be seen in Agaricomycetes with the largest abundances seen in phalaris, white clover and white lupin and the lowest evident in the control and yellow serradella.

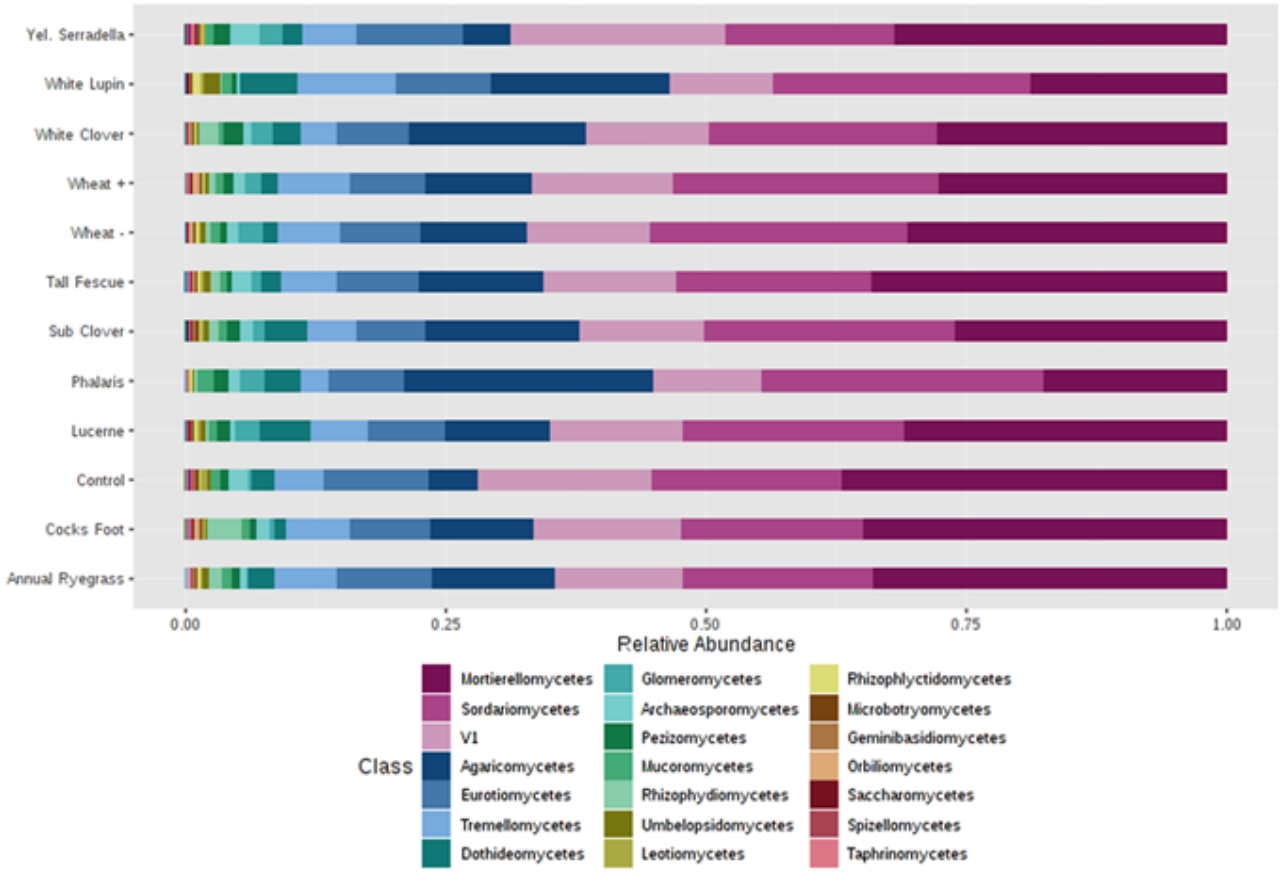

Figure 4.13. Bar plot of fungal taxonomic composition in different plant species, sorted by class.

The correlations of relative abundance seen in plant species was also present when grouping by plant type (Figure 4.14). The composition of Phylum between the groups were largely similar with the largest differences seen in Agaricomycetes, where the abundance of this phyla approximately triples in both grasses and legumes compared to the control. Smaller changes in abundance were evident in Glomeromycetes and Microbotryomycetes, where the abundance of Glomeromycetes was doubled in grasses and legumes compared to the control and Microbotryomycetes was twice as large in the control compared to grasses and legumes.

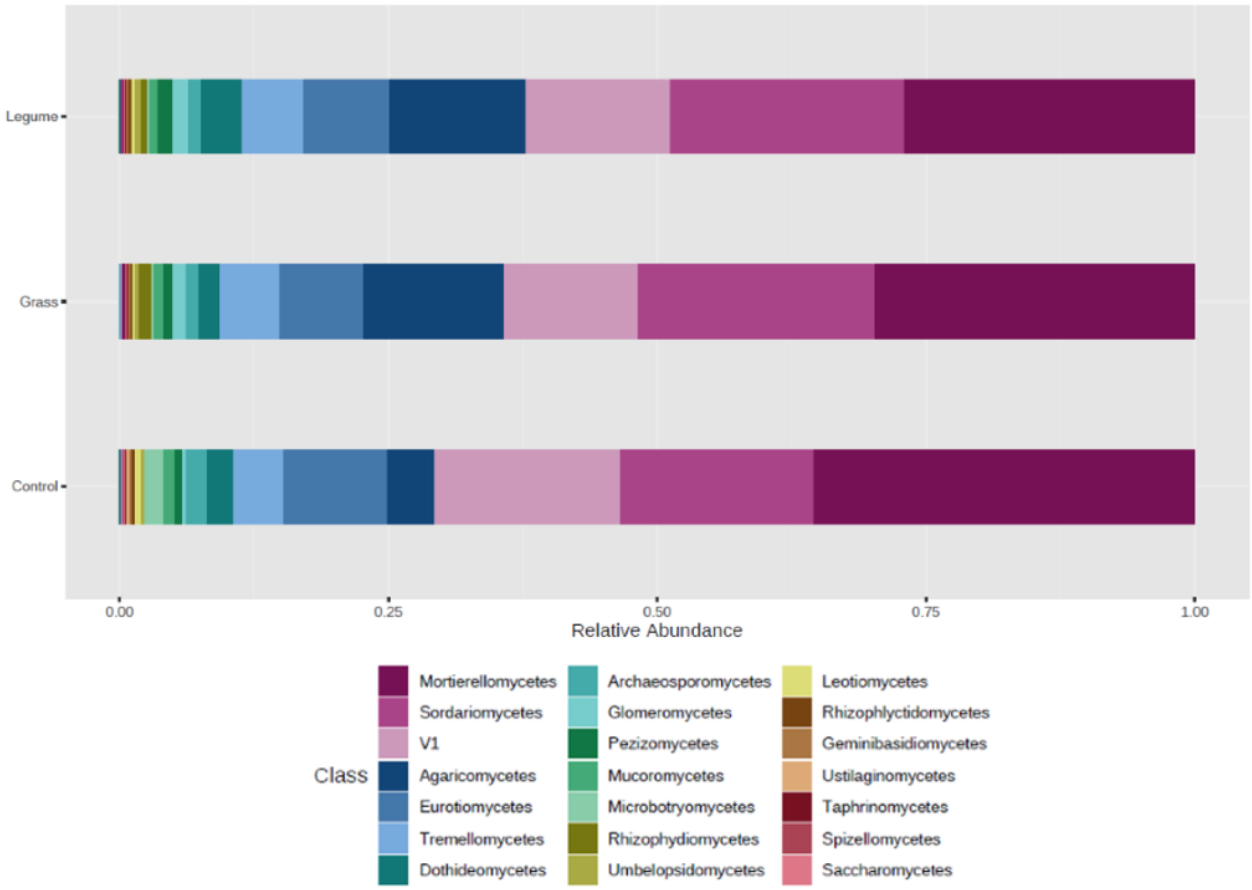

Figure 4.14. Bar plot of fungal taxonomic composition in different plant type, sorted by class.

4.3 Environmental Plant Factors of Grasses and Legumes

The effects of biomass for plant species and type were examined in this study, the root weight results were examined in Chapter 2, whereas the shoot weight results are displayed in Figure 4.15 below. Statistical testing indicates that there were significant differences between plant species (Figure 4.15 (a), Kruskal-Wallis, $X^2 = 31.291$, df = 10, $p = 0.0005$). Dunn post-hoc analysis reveals that annual ryegrass (7.47 g) and tall fescue (6.71 g) pertain the largest shoot biomass, being significantly larger than wheat (3.67 g), lucerne (3.46 g), white clover (3.41 g) and white lupin (1.96 g). The four of which have the lowest shoot weights in the study.

Grass having a large shoot mass was not isolated to those two species, as grasses had a significantly greater mean shoot weight (5.47 g) compared to the legumes (3.5 g) (Figure 4.15 (b), t-test, $F_{(1,342)} = 18.63$, $p = 0.00009$).

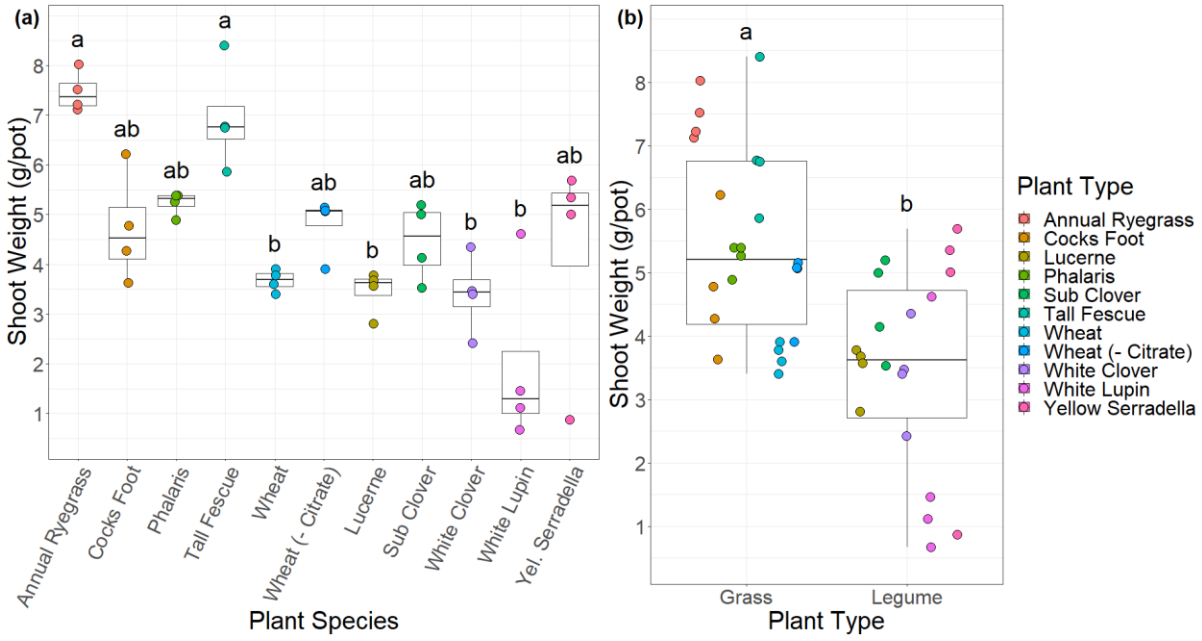

Figure 4.15. Plant shoot weight (g/pot) in different species (a) and types (b) of plant (mean ± standard deviation). Different letters above error bars indicate statistical significance ($p < 0.05$). Letters are in order of highest to lowest mean value.

When comparing the pH in the rhizosphere in each treatment, Kruskal Wallis testing found significant differences were present between the pH and plant species (Figure 4.16 (a), X^2 = 36.14, df = 11, p = 0.0001). The pH values for most the species were similar, ranging between 4.5 to 4.6. However, the control and yellow serradella treatments had pH values of less than 4.4, the lowest of any species, both of which are significantly lower than six other species.

The same trend for the control treatment was replicated when analysing by plant type, with the control group pertaining significantly decreased pH compared to both grass and legume (Figure 4.16 (b), Kruskal-Wallis, X^2 = 11.11, df = 2, p = 0.003).

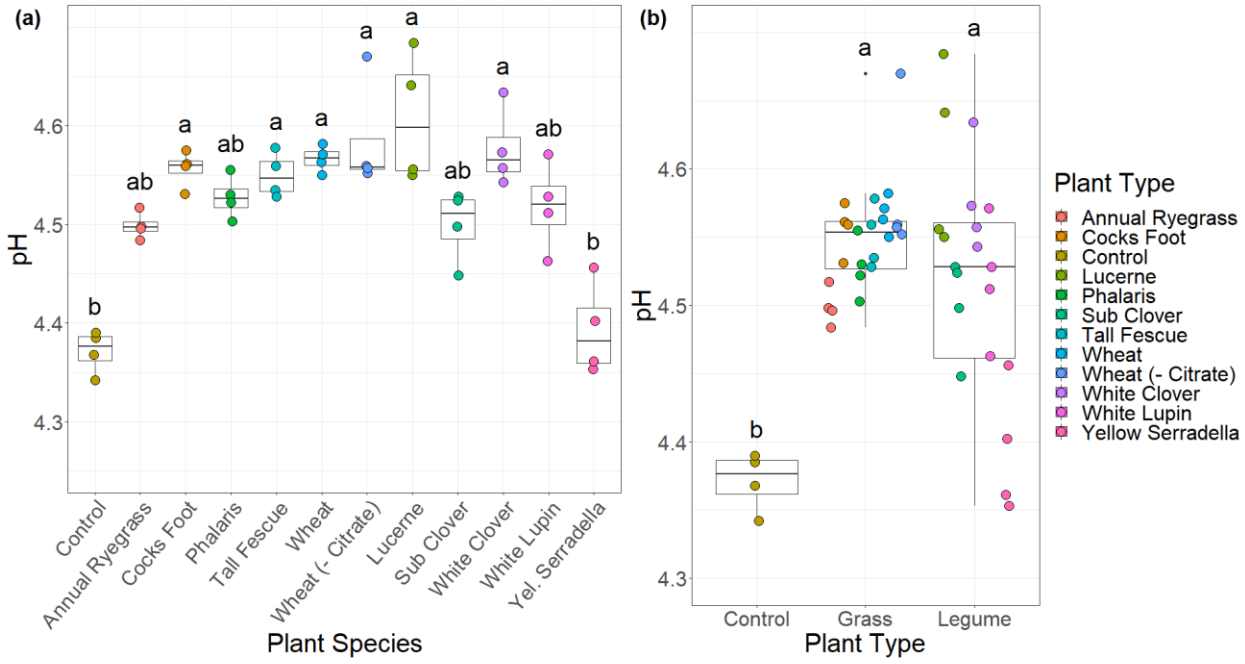

Figure 4.16. pH in different species (a) and types (b) of plant (mean ± standard deviation). Different letters above error bars indicate statistical significance (p < 0.05). Letters are in order of highest to lowest mean value.

The transpiration of each replicate was monitored throughout the study and its results displayed below in Figure 4.17. Statistical analysis indicates that there were significant differences between transpiration and plant species (Figure 4.17 (a), Kruskal-Wallis, X^2 = 37.46, df = 11, p = 0.00009). Annual ryegrass, cocks foot and tall fescue contained the highest water usage, with

68.77, 60.36 and 61.99 mL/day respectively. All three of these highest values were significantly larger than the lowest value of the control (18.22 ml/day), and excluding tall fescue, were also significantly higher than white lupin (31.82 ml/day).

Kruskal Wallis testing states that there are also differences between transpiration and plant type (Figure 4.17 (b), $X^2 = 19.94$, df = 2, $p = 0.00004$). Where the grass had a significantly higher water usage (56.49 ml/day) over legumes (45.35 ml/day), and both of which were significantly greater than the control (18.22 ml/day).

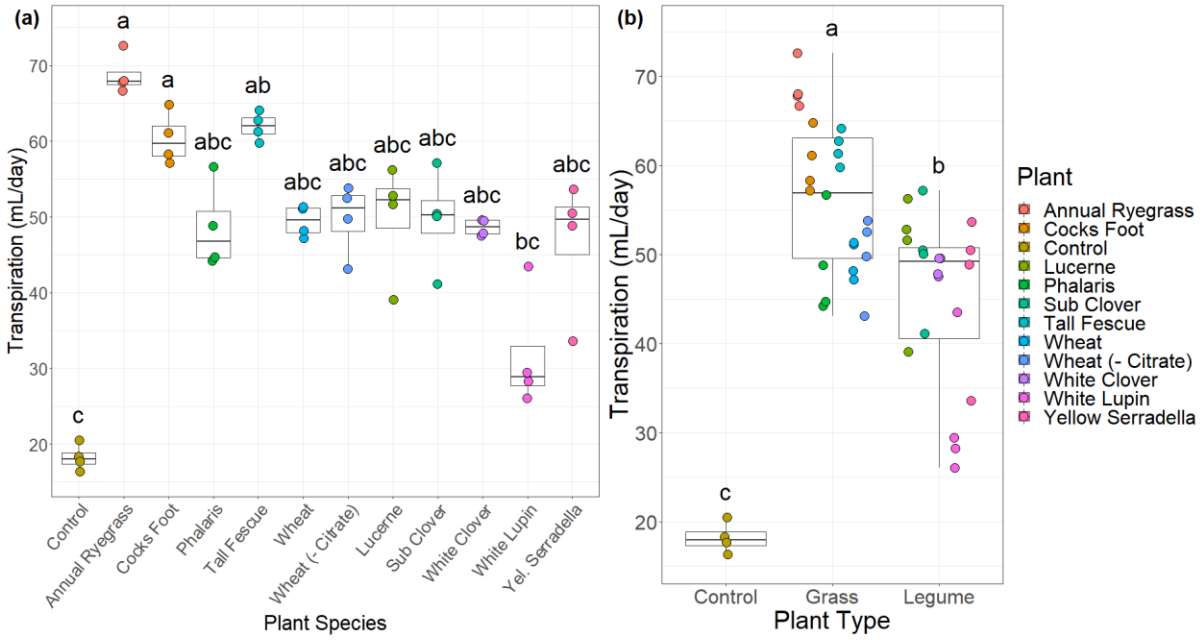

Figure 4.17. Transpiration (ml/day) in different species (a) and types (b) of plant (mean ± standard deviation). Different letters above error bars indicate statistical significance. Letters are in order of highest to lowest mean value.

4.4 Correlation between Shoot Weight and the Concentration of Dieldrin

To investigate any effects between environmental plant factors and dieldrin concentrations, regression analyses were conducted. A linear regression analysis between dieldrin concentrations

and pH indicates that there was no correlation or significance between these factors (Figure 4.18 (a), $F_{(1,46)} = 0.02$, $R^2 = 0.0006$, $p = 0.86$). Similar results were found when comparing the effect of transpiration on dieldrin concentrations (Figure 4.18 (b)). Where a regression analysis indicates no correlation or significant differences ($F_{(1,46)} = 0.001$, $R^2 = 0.00003$, $p = 0.96$).

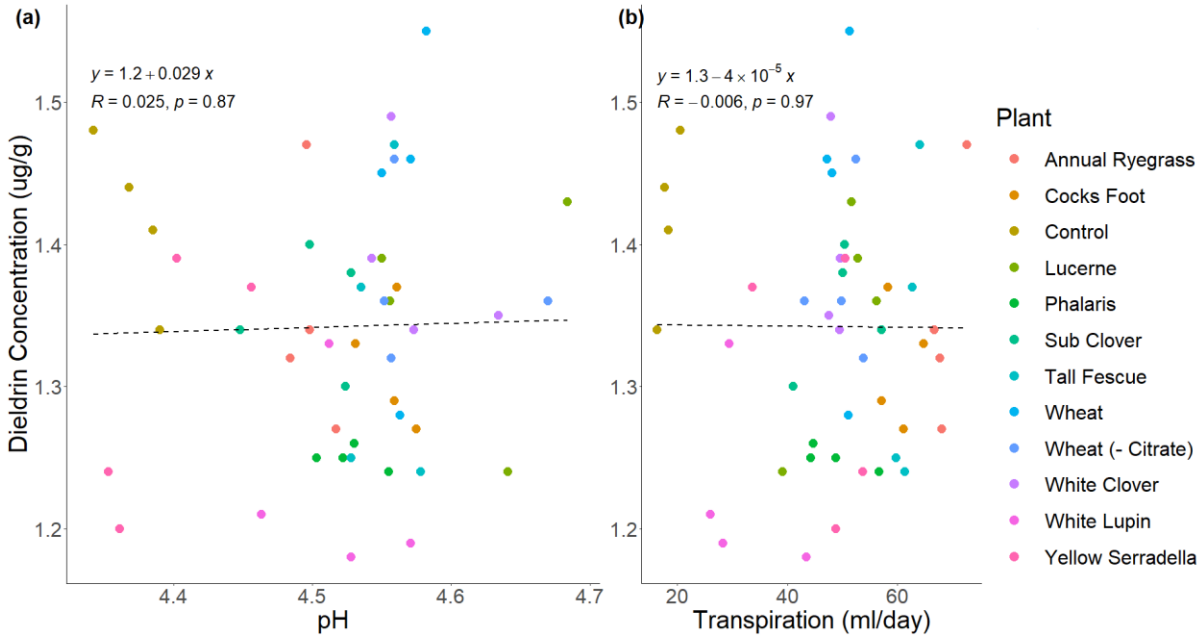

Figure 4.18. Dieldrin concentration as a function of pH (a) and Transpiration (b).

A multiple regression analysis was utilized to compare the effects of plant biomass to dieldrin concentrations. The results indicate that there was a significant, albeit low correlation between plant biomass and dieldrin concentrations ($F_{(2,46)} = 3.72$, $R^2 = 0.15$, $p = 0.03$). Outlined by the equation below, these results indicate that there was a small negative correlation between root weight and dieldrin concentration, whereas there was a slight positive correlation between shoot weight and dieldrin concentrations.

$$Dieldrin = 1.32 + (0.03 \times Root\ Weight) - (0.01 \times Shoot\ Weight)$$

4.5 Relationship Between Environmental Plant Factors and Microbial Abundance

4.5.1 Bacterial Abundance Driven by Transpiration

A linear regression analysis was used to examine the effects of pH on bacterial abundance and found no significance or correlation between these two variables (Figure 4.19 (a), $F_{(1,46)} = 3.14$, $R^2 = 0.06$, $p = 0.08$). However, a linear regression assessing the effect on transpiration on bacterial abundance found a significance, although low correlation between these factors (Figure 4.19 (b), $F_{(1,46)} = 5.48$, $R^2 = 0.1$, $p = 0.02$). The regression equation in Figure 4.19 (b) highlights a small positive correlation between the two variables.

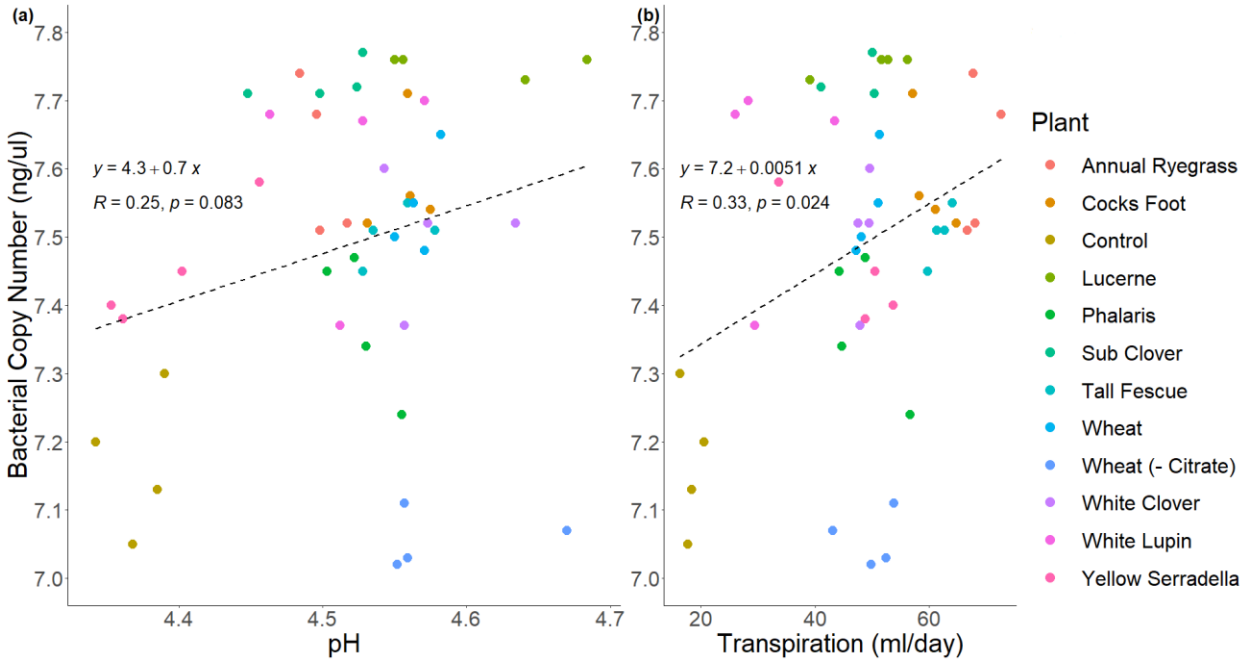

Figure 4.19. Bacterial abundance as a function of pH (a) and Transpiration (b).

The multiple regression analysis shows that there were no significant correlations between environmental plant factors and bacterial abundance ($F_{(2,46)} = 0.7$, $R^2 = 0.03$, $p = 0.49$).

$$Bacterial\ Abundance = 7.5 - (0.02 \times \text{Shoot Weight}) + (0.02 \times \text{Root Weight})$$

4.5.2 Environmental Plant Factors do not Influence Fungal Abundance

Linear regression analysis between environmental plant factors and fungal abundance are shown in Figure 4.20. This relationship states that there were no significant correlations between fungal abundance and pH (Figure 4.20 (a), $F_{(1,46)} = 0.31$, $R^2 = 0.006$, $p = 0.58$). Similar results were found with transpiration, as there were no significant correlations between water usage and fungal abundance (Figure 4.20 (b), $F_{(1,46)} = 1$, $R^2 = 0.02$, $p = 0.32$).

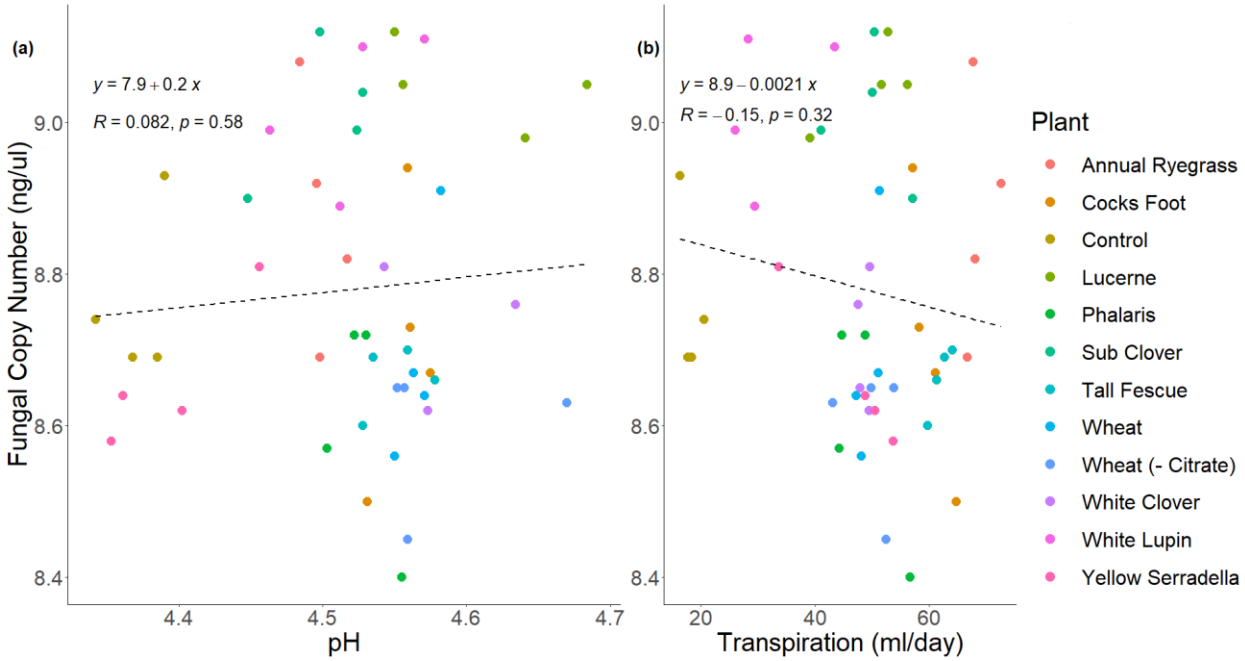

Figure 4.20. Fungal abundance as a function of pH (a) and Transpiration (b).

A multiple regression analysis, shown in the equation below shows that there were no significant correlations between environmental plant factors and fungal abundance ($F_{(2,46)} = 1.2$, $R^2 = 0.05$, $p = 0.28$).

$$Fungal\ Abundance = 8.9 - (0.02 \times \text{Shoot Weight}) + (0.01 \times \text{Root Weight})$$

4.6 Relationship Between Environmental Plant Factors and Microbial Diversity

4.6.1 Environmental Plant Factors do not Influence Bacterial Diversity

A linear regression analysis indicated that there were no significant correlations between bacterial diversity and pH (Figure 4.21 (a), $F_{(1,46)} = 0.53$, $R^2 = 0.14$, $p = 0.46$). Similar results were found with transpiration, as there were no significant correlations between water usage and bacterial diversity (Figure 4.21 (b), $F_{(1,46)} = 0.01$, $R^2 = 0.14$, $p = 0.89$).

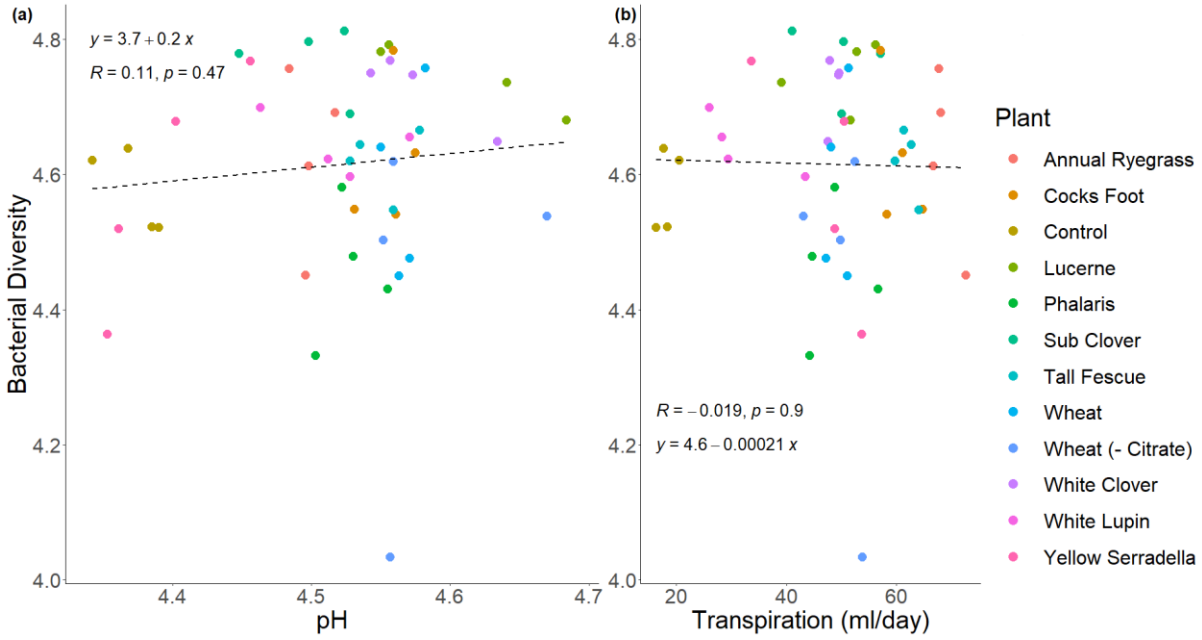

Figure 4.21. Bacterial diversity as a function of pH (a) and Transpiration (b).

A multiple regression analysis, shown in the equation below shows that there were no significant correlations between environmental plant factors and bacterial diversity ($F_{(2,46)} = 0.9$, $R^2 = 0.04$, $p = 0.39$).

$$Bacterial\ Diversity = 4.6 - (0.01 \times \text{Shoot Weight}) + (0.01 \times \text{Root Weight})$$

4.6.2 Fungal Diversity not Impacted by Environmental Plant Factors

Linear regression analysis between environmental plant factors and fungal diversity are shown in Figure 4.22. This relationship states that there were no significant correlations between fungal diversity and pH (Figure 4.22 (a), $F_{(1,46)} = 1.39$, $R^2 = 0.02$, $p = 0.24$). Similar results were found with transpiration, as there were no significant correlations between water usage and fungal diversity (Figure 4.22 (b), $F_{(1,46)} = 0.41$, $R^2 = 0.008$, $p = 0.52$).

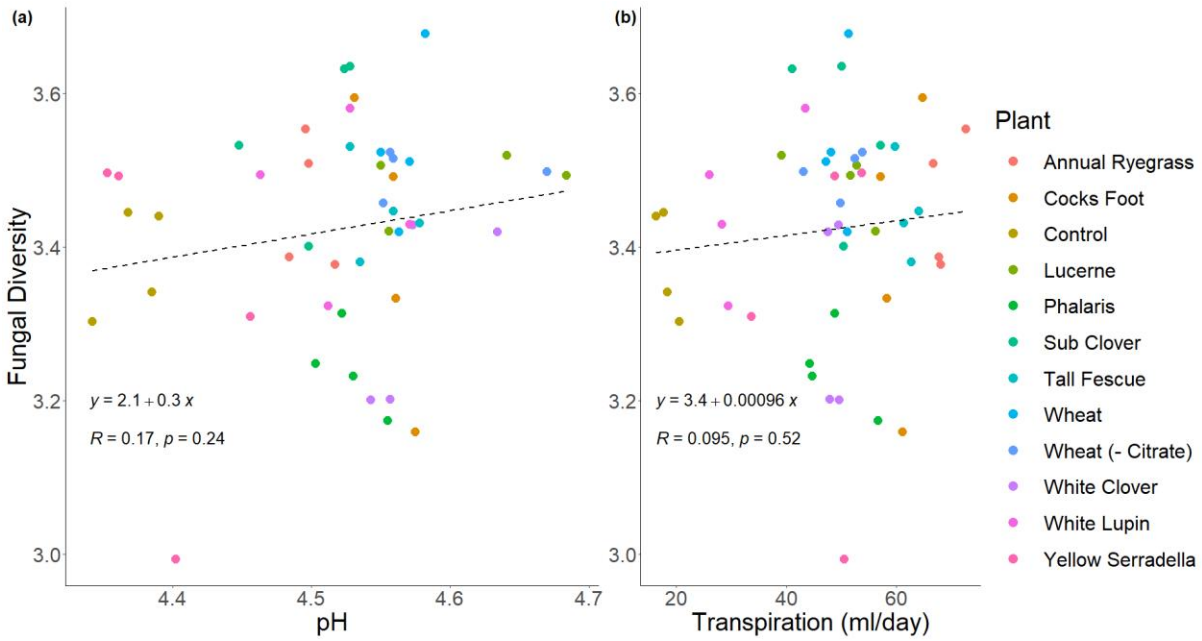

Figure 4.22. Fungal diversity as a function of pH (a) and Transpiration (b).

A multiple regression analysis, shown in the equation below shows that there are no significant correlations between environmental plant factors and fungal diversity ($F_{(2,46)} = 0.05$, $R^2 = 0.14$, $p = 0.95$).

$$Fungal\ Diversity = 3.41 - (0.001 \times \text{Shoot Weight}) + (0.004 \times \text{Root Weight})$$

Chapter 5: What's Driving Ecological Changes in the Rhizosphere?

Dieldrin is a persistent organic pollutant that was utilised internationally as a pesticide from the 1950s to the 1970s. Although dieldrin has not been used commercially in the last half century, the concentration of dieldrin in contaminated soils can be high enough to cause concern for human health (Western Australia Government, 2016). Despite the known health concerns of dieldrin contamination, there is a fundamental lack of research investigating the remediation of dieldrin from the soil. This study investigated the ability of microorganisms within the rhizosphere of grasses and legumes to degrade dieldrin.

5.1 Bacterial Abundance Driven by pH and Transpiration in the Rhizosphere

This study found that the presence of a rhizosphere significantly increased bacterial abundance compared to the soil only control, in which the legume rhizosphere had a greater effect on bacterial abundance in comparison to grasses (Figure 4.1 (b)). The increased bacterial abundance evident in the rhizosphere can be linked to environmental traits measured from this study, where there is a trend between bacterial abundance and pH (Figure 4.19 (a)) and a significant correlation between bacterial abundance and transpiration (Figure 4.19 (b)). The potential for increased bacterial abundance in the rhizosphere due to an increase in pH is supported by He et al. (2017) whom found a positive correlation between the abundance of bacteria and the pH in the rhizosphere when compared to soil only treatments. Unlike pH, transpiration does not directly affect bacterial abundance, rather, it is a cascading effect where increasing water usage by plants is positively correlated to relative yield (Hanks, 1974). Increased plant productivity, as shown by the increased transpiration, will increase root exudation (Aulakh *et al.*, 2001), thus increasing microbial activity in the rhizosphere.

The ability for root exudates to affect bacterial abundance is further supported in this study by the inclusion of the two isogenic wheat lines. Although not significant, the wheat (- citrate) mutant contained a reduced abundance of bacteria compared the wheat treatment (Figure 4.1

(a)). Citrate is a common root exudate amongst grass species and by removing this organic acid it reduces the abundance of bacteria within the rhizosphere (Lipton, Blanchar & Blevins, 1987). Although the removal of Citrate correlates to bacterial abundance, there was no such relation to the concentration of dieldrin in the soil.

5.2 Diverse Root Exudate Profiles may promote higher Fungal Abundance in Legumes

Variations of microbial abundance in soils can be due to several factors and unlike the bacterial abundance, there are no correlations between fungal abundance and environmental plant factors in this study. However, it is suggested one of the largest contributors for change in microbial abundance are root exudates (Donnarumma *et al.*, 2008). Three legume species: lucerne, sub clover and white lupin had a higher fungal abundance when compared to other treatment groups (Figure 4.2 (a)). Both lucerne and sub clover have been found to contain highly diverse root exudate profiles, rich in sugars and flavonoids (Sugiyama & Yazaki, 2012). Similarly for white lupin, where they have root clusters exuding high concentrations of cis-aconitic, citric and malic acids (Marschner *et al.*, 2002). Isobe et al. (2001) found that legumes secreted more amino acids, sugars and flavonoids compared to grasses and found flavonoids are responsible for attracting rhizobia to the rhizosphere. These low molecular weight organic acid molecules can stimulate chemotactic microbes, signalling them to move to the nutrient rich rhizosphere (Parke, Rivelli & Ornston, 1985). These literature studies support findings in this study where legumes have a significantly higher fungal abundance compared to grasses (Figure 4.2 (b)). The increased fungal abundance evident within is study is likely driven by the diverse root exudate profiles in the rhizosphere of legumes.

5.3 Presence of a Rhizosphere Increases Bacterial Diversity

A diversity index was applied to the data as it allows a comparison of the phylogenetic relationships between treatments. There are a number of diversity indices that can be applied for different analyses, however it is common that the calculations used in Shannon, Chao1 and Simpson indices are used for comparing diversity, richness and evenness respectively (Lemos *et al.*, 2011). Results from the Shannon index show that legumes have a significantly higher bacterial diversity compared to grasses (Figure 4.3 (b)). This variation can be explained by the

Chao1 species richness which is significantly larger in legumes compared to grasses (Figure 4.4 (b)). This increase in species richness likely indicates that the legumes have more unique species of bacteria present compared to grasses, which is likely driving an increase in the bacterial diversity. This increase in species richness is likely due to the increased concentrations of root exudates in the legume rhizosphere. Where it has been found that the concentrations of acetic and succinic acids were positively correlated to bacterial richness (R^2 = 0.892 and 0.897 respectively) (Wu *et al.*, 2010). This is particularly evident in legumes where the presence of root nodule associated bacteria increased the diversity of microorganisms in the rhizosphere in chickpea, particularly in the order of *Rhizobiales* (Shcherbakova *et al.*, 2017).

Although legumes have a greater bacterial diversity compared to grasses, this study found the overall presence of a rhizosphere significantly increased the bacterial diversity within the soil. This is evident in the Bray-Curtis NMDS ordination (Figure 4.6), which shows that the control samples were separated from the grasses and legumes, indicating that the presence of a rhizosphere in the soil alters the diversity of bacterial species. This is supported by literature findings which suggest that an increase of bacteria in the rhizosphere is due to the symbiotic relationships present between bacteria and the roots of a plant (McNear Jr, 2013).

This dominance of soil bacteria is visualised in Figure 4.8, when assessing the differences in taxonomy between plant type. The control, grasses and legumes are each dominated by a core community of three bacterial phyla: Actinobacteria, Proteobacteria and Chloroflexi. Actinobacteria play a key role in soil ecosystems as it assists decomposition of organic matter and dead organisms (Janssen, 2006). Proteobacteria encompass a large quantity of metabolic diversity and are vital to carbon and nitrogen cycling (Janssen, 2006). Chloroflexi are predominately comprised of photoheterotrophs which have been recently found to be capable of nitrate and iron reduction (Ward *et al.*, 2018).

The largest variation in the taxonomy is in the Patescibacteria phylum, where it is twice as abundant in both grasses and legumes compared to the control soil. Recent advances in next generation sequencing have precluded Patescibacteria to its own phylum consisting of 14 classes (Herrmann *et al.*, 2019), and although relatively new, there are some studies explaining differences of composition in soils. Patescibacteria has been found in soils and groundwater sources where its abundance is dependent on hydrochemical conditions, predominately the

availability of organic resources (Herrmann *et al.*, 2019). The presence of the rhizosphere in grass and legume treatments provide Patescibacteria the organic resources it requires and is likely driving its composition in these treatments compared to controls.

5.4 Increased Richness Insufficient in Driving Fungal Diversity

Contrary to the findings in bacterial diversity, the presence of the rhizosphere had a minimal impact on fungal diversity. Although, the legumes had a significantly higher Chao1 richness compared to grasses (Figure 4.10 (b)), this change was not enough to alter the significance in the Shannon diversity results (Figure 4.9 (b)). This indicates that although there were significantly more fungal species present in the legumes compared to the grasses, the increased richness was not enough to significantly increase fungal species diversity. The lack of species diversity is reflected in the fungal NMDS (Figure 4.12) where all three treatments were overlapping, indicating that the control, grass, and legume treatments share a core community of fungi.

Lack of diversity within the fungi is reflected in the taxonomic composition with each treatment sharing three core classes: Mortierellomycetes, Sordariomycetes and V1 (Figure 17). Mortierellomycetes are saprophytic fungi that consume decaying leaves and other organic material (Carris, Little & Stiles, 2012). Sordariomycetes are a class of fungi that decompose wood and survive as parasites (Carris, Little & Stiles, 2012). It is suggested that the V1 classification is comprised of fungi that are unculturable. This suggestion is made as there are a series of similar bacterial classes (WS4, TM6 and OP1) that are attributed to uncultivated species (Federhen, 2011).

The largest variation is seen in Agaricomycetes class of fungi where the abundance is twice as large in grasses and legumes than it is the control groups. Agaricomycetes are a class of the Basidiomycota division, who are largely studied as wood decomposers and ectomycorrhizal symbionts (Carris, Little & Stiles, 2012). It is presumably the ability to form a mutualistic relationship with roots that causes its selection in the rhizosphere, and therefore driving the increased abundance in the grass and legume treatments.

5.5 Significant Increase of Shoot Weight, Transpiration and pH in Grasses

Grasses appear to have a significantly greater shoot weight and transpiration compared to legumes (Figure 4.15 (b) & Figure 4.16 (b) respectively). However, it is likely that this is just an artefact as increased transpiration is likely a result of the increased shoot weight. This is likely a result of experimental design, where a greater amount of seeds was sowed in the grass treatment, resulting in increased biomass.

However, statistical testing in this study found that the presence of a rhizosphere increases the pH. This result is supported by a study by Schoninger, Gatiboni and Ernani (2012) whom found that the presence of a soybean cultivation increased rhizosphere pH when compared to a soil only control. These results are also replicated in grasses where the presence of a *lolium perenne* rhizosphere increased the pH in the soil (Meharg & Killham, 1990). Therefore, the presence of a rhizosphere is driving the increased pH evident in this study.

5.6 Plant Biomass Significantly Correlated to Dieldrin Degradation

Despite the significant increase in pH due to the presence of a rhizosphere, there was no correlation between dieldrin degradation and pH (Figure 4.18 (a)). Although transpiration did not indicate its capability to degrade dieldrin, there were significant correlations between shoot and root weight with dieldrin degradation. This model indicated that there was a slight positive correlation between the concentration of dieldrin to shoot weight, whereas there was a slight negative correlation with root weight. The trend between root weight and decreased pollutant concentration supports findings by Hong et al. (2010) who found that a 2.5-fold increase of root weight in Zea mays resulted in a 100-fold decrease in total petroleum hydrocarbons. Although there is evidence to support the negative correlation found between dieldrin concentrations and root weight in this study, this regression analysis generated a low to moderate correlation factor (0.15) revealing a weak relationship between these variables. Therefore, there are likely more significant factors influencing dieldrin degradation in this study.

5.7 Summary of Key Ecological Findings

Investigation of the abundance and diversity of bacteria and fungi, and environmental plant factors in the rhizosphere allowed an insight into ecological changes occurring in the rhizosphere of grasses and legumes in dieldrin-contaminated soils. It was found that the abundance of microorganisms is driven by different processes, with bacterial abundance being positively correlated to pH and transpiration, whereas fungi was impacted by the diversity of root exudates. It was shown that the presence of a rhizosphere increased bacterial diversity, however, the profile was dependent on the type and species of plant. There were significant correlations present between shoot weight and transpiration within the grasses and legumes, however this was sought to be an artefact, a result due to experimental design. There was a significant correlation between plant biomass and the concentration of dieldrin, however this correlation was weak and likely not enough to impact the degradation of dieldrin. Therefore, there must be additional variables driving the degradation of dieldrin.

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Chapter 6: Conclusion

This thesis summarises the investigation into the degradation of dieldrin in the rhizosphere. Key findings that are set for publication are presented in the form of a research article in Chapter 2. Additionally, these findings are supported by supplementary results that investigate the ecological changes in the rhizosphere and are presented in Chapter 4. Chapter 5 examines these findings and discusses what forces are driving those ecological changes within the rhizosphere. This final chapter aims to combine these sections and outline the major findings within a cohesive syntax, that which reflects the entire thesis. In Chapter 2, it is discussed that although degradation of dieldrin is minimal, one species of legume, the white lupin, showed the ability to degrade dieldrin in the rhizosphere. When investigating factors responsible for this degradation, it is likely that individual microorganisms in the rhizosphere, rather than the whole community, are driving this process.

This study found that the differences in root exudates was driving the changes in microbial abundance and diversity in the rhizosphere. However, the presence of a rhizosphere had a statistically significant impact on only the bacterial abundance. This increased abundance was linked to an increase in pH and transpiration in the rhizosphere, where the concentration of root exudates was indirectly increased due to the increased transpiration. Although there was not a distinct rhizosphere effect, it was suggested that the increased fungal abundance in the legume rhizosphere compared to the grass rhizosphere was likely due to the concentration of root exudates. Furthermore, the fungal abundance results were replicated in bacterial diversity, with legumes containing a greater diversity compared to grasses. It was therefore suggested that this result was also due to the concentration of root exudates, which was supported by literature findings that found a correlation between an increase in root exudation to bacterial richness, which subsequently drove bacterial diversity. Despite the significant changes present within microbial abundance and diversity within this study, a multiple regression analysis within Chapter 2 denotes that there was no significant changes between these microbial abundances and diversities to a decrease in the concentration of dieldrin in the soil.

If the microbial communities were not driving this decrease in dieldrin concentrations within white lupin, then there must be other factors present. Environmental plant factors were measured to investigate their impact on dieldrin degradation. This study found a significant increase in

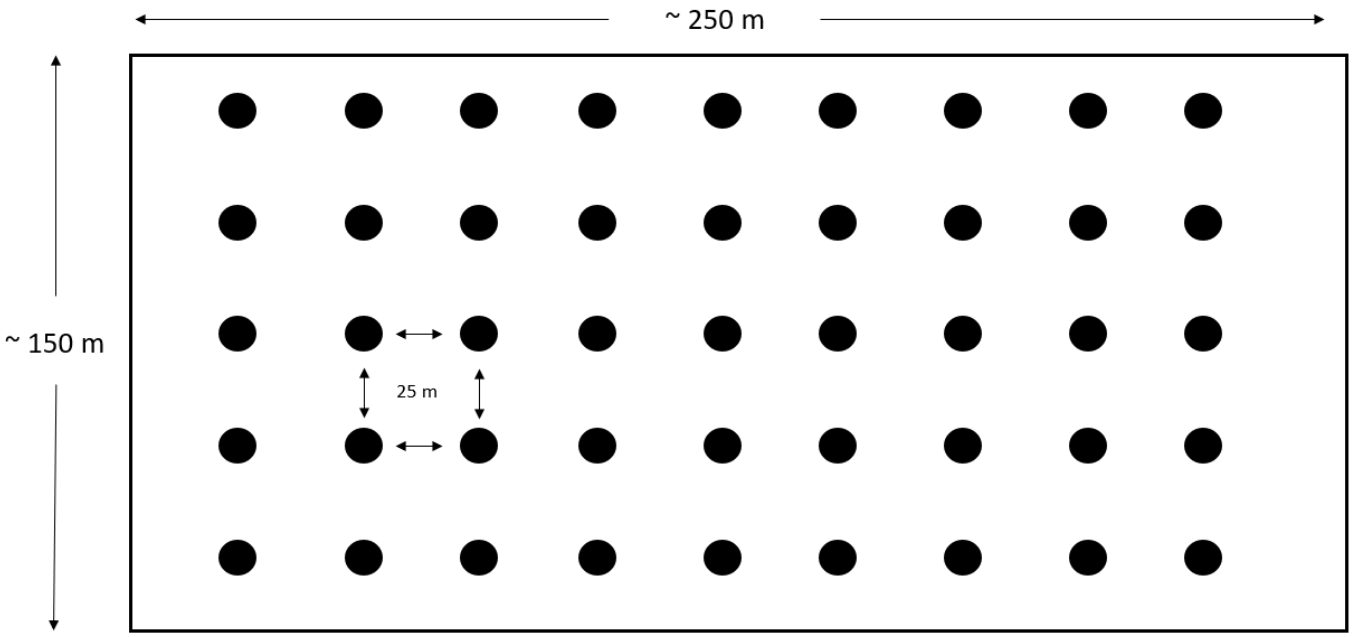
both the shoot weight and transpiration of grasses compared to legumes. However, it was discussed that this is likely just an artefact, a result due to the experimental design, where the error was overestimating the biomass of grass species, and as a result of increased biomass there was an increase in transpiration. It was also found that the presence of a rhizosphere increased the pH of the soil, which was supported by literature findings. Despite the increases in transpiration and pH, there was no correlation between these factors and dieldrin degradation. However, there was a significant correlation between shoot and root weight with dieldrin degradation, where there was a slight negative relationship between root weight and dieldrin concentrations. However, this relationship was only low (0.15), indicating that although a factor, it was likely not sufficient enough to drive dieldrin degradation.

The research article in Chapter 2 was supplemented with additional results in Chapter 4 to conclude in that neither the microbial communities nor the environmental factors were key drivers in the degradation of dieldrin in the rhizosphere. Differential abundance testing and an indicator species analysis indicated that it is the presence of individual microorganisms that is driving this degradation of dieldrin. Differential abundance testing revealed that white lupin has the ability to recruit *Trichoderma* and *Penicillium* to the rhizosphere, as they were found to be significantly increased in abundance compared to the other treatments. This increased abundance, coupled with a known ability to degrade dieldrin suggests that these genera of microbes are likely responsible for the decreased concentration of dieldrin in the soil. Additionally, *Saitozyma* and *Umbelopsis* were found to be significantly enriched in the white lupin rhizosphere. Although there is no literature correlating these microbes to dieldrin degradation, there are studies that infer their capability to degrade pollutants in the soil. This author suggests that these organisms contribute to the dieldrin degradation evident in this study. The fungal genus *Cortinarius* was identified as a species indicator in the white lupin rhizosphere, as white lupin was the only treatment to degrade dieldrin, the author suggests that this species may possess the capacity to metabolise dieldrin. This study found that dieldrin degradation was not a general community response to the presence of a rhizosphere, but rather a product of specific microorganisms found within that community. Analysing microorganisms individually rather than as a community highlights the potential to discover novel dieldrin degraders.

The author recommends that further investigation be conducted into the ability for the highlighted microorganisms (*Saitozyma*, *Umbelopsis* and *Cortinarius*) to degrade dieldrin. Such a study would be similar to Matsumoto (1964) where the selected microorganisms are subject to strict nutritional conditions, where their ability to metabolise dieldrin is examined. The author further recommends investigation into the inoculation of dieldrin degrading microorganisms into the rhizosphere to further promote their abundance. This would be similar to a study from Zhang et al. (2018) where the cucumber rhizosphere was inoculated with a known herbicide degrader in which they found increased degradation of chlorimuron-ethyl. In addition to the above focus on microbial studies, environmental factors should also be considered when designing research. As discussed in Chapter 1, chlorinated hydrocarbons, like dieldrin, degrade faster in higher temperature and moisture conditions (Komprda *et al.*, 2013) and persist longer in acidic soils compared to alkaline soils (Rhodes, 2014). Although the links between soil temperature and pH conditions to chlorinated hydrocarbons are present, no such study exists for dieldrin, and should therefore be considered for future manipulative experiments.

Appendix

Appendix 1: Soil sampling sites from a farmland in Emerald, Victoria. Each dot represents a sampling location. Three replicates were extracted at each sampling location.



Appendix 2.1: Quality Checking Primers and Standard Curves

Confirmation of Primers for Next-Generation Sequencing

To ensure the primers were amplifying the correct sequences in the Illumina MiSeq, confirmation of the primer lengths was confirmed via agarose gel electrophoresis. The bacterial primers (Chapter 2) are estimated to result in an amplicon size between 300 bp to 400 bp (Caporaso *et al.*, 2011). Results from the gel electrophoresis confirm amplification of DNA resulting in a band of just over 400 bp (Figure 2.1), slightly over but approximately equal to the estimated size of the band. The quality of the gel electrophoresis could have been improved, as indicated by the smearing of the sample and ladder wells. Possible reasons for the poor gel include poorly prepared gel, too much DNA in each well and/or poor-quality sample including contamination. However, contamination can be ruled out as there was no amplification present within the negative control well. Despite the poor quality of the gel, the lack of contamination and approximate size of the band was sufficient to continue the sequencing protocol.

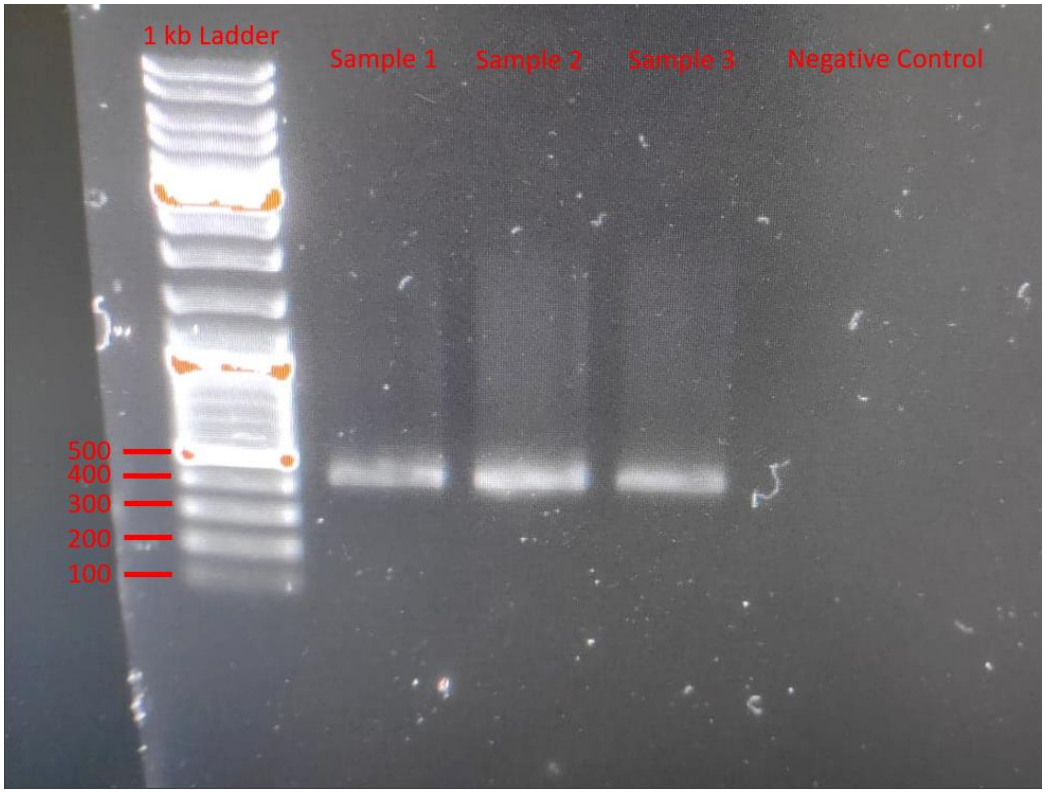


Figure 2.1. Picture of gel electrophoresis for bacterial primers used in next-generation sequencing. Units for the 1 kb ladder are in bp.

The specificity of the fungal primers (Chapter 2) were also confirmed in this study via gel electrophoresis, with an approximate amplicon size of 125 bp – 200 bp (Lear *et al.*, 2018). These are confirmed in Figure 2.2 below. However, this gel electrophoresis shows signs of either smearing or double bands. Reasons for the presence of smearing have been previously outlined, but the presence of double bands can be due to incorrect binding of fluorescent dyes. Due to the correct expected amplicon size and lack of contamination in the negative control, results were

deemed sufficient to continue with the sequencing protocol.

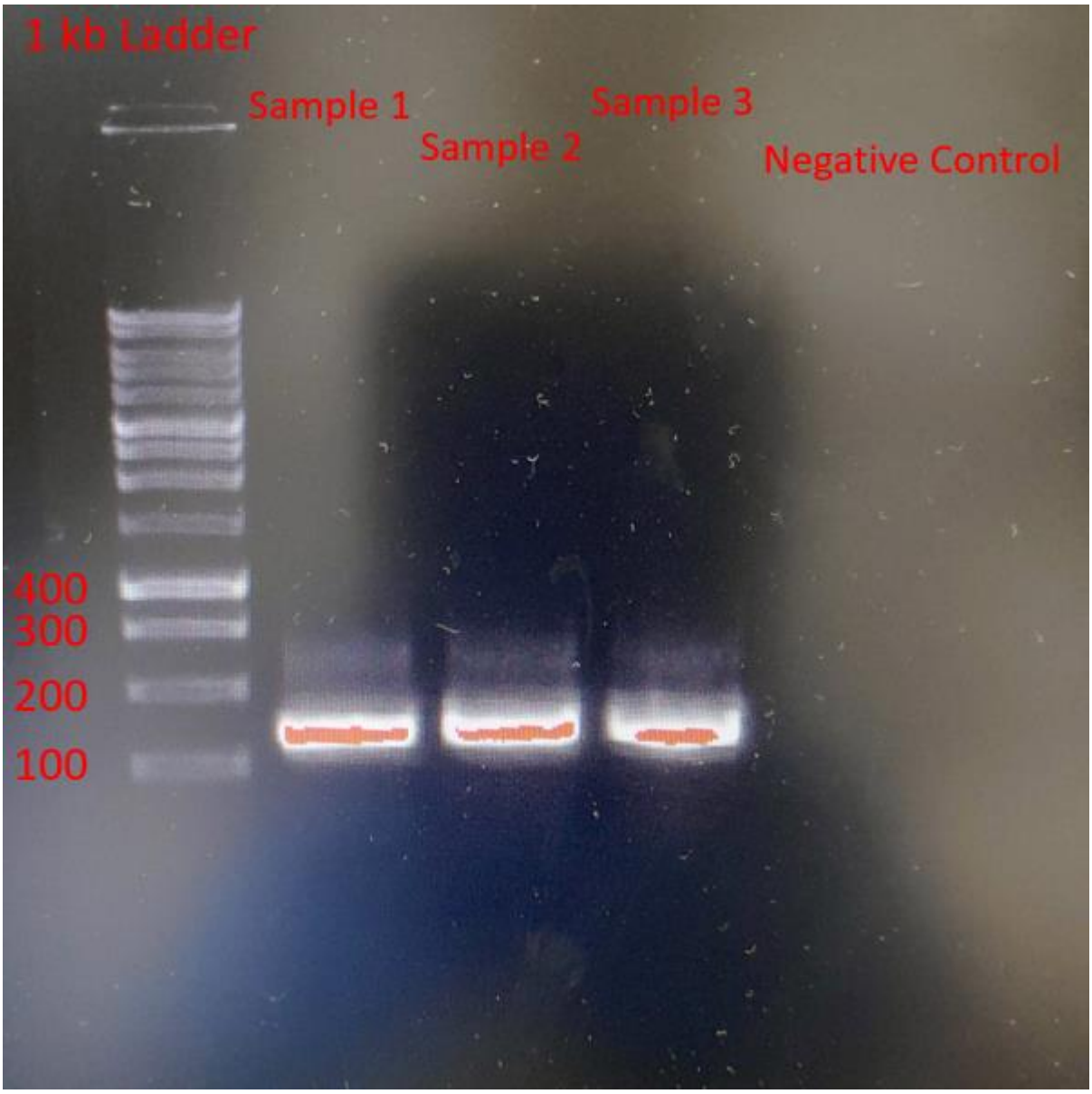


Figure 2.2. Picture of gel electrophoresis for fungal primers used in next-generation sequencing. Units for the 1 kb ladder are in bp.

Appendix 2.2: Standards Curves for qPCR

Qualitative analysis results are included in the output of the qPCR to ensure that there is an adequate fit between the standard curve and the samples. The standard curves for both bacteria and fungi are included in Appendix 3 and 4 respectively, with an example standard curve in

Figure 2.3 below. All the standard curves have R^2 values over greater than .95. Where there is always at least a correlation of 95% between samples to the standards, or per Figure 3.3 below, a correlation of 97.3%.

Note, that the qPCR efficiency (E) of the standard curve ranges from 69% to 120%. Ideally E is 100%, which occurs when molecules in the target sequence double during each replication cycle. E can drop below 100% for a number of reasons, such as reaction conditions, non-optimal reagent concentrations and/or bad primer design. Additionally, secondary structures present in the same can affect the melting temperature, which will reduce E. It is also possible for E to exceed 100%, this can result from certain contaminants or excessive amounts of DNA present in the sample which results in polymerase inhibition. This causes the efficiency plot to flatten out driving and amplification of over 100%.

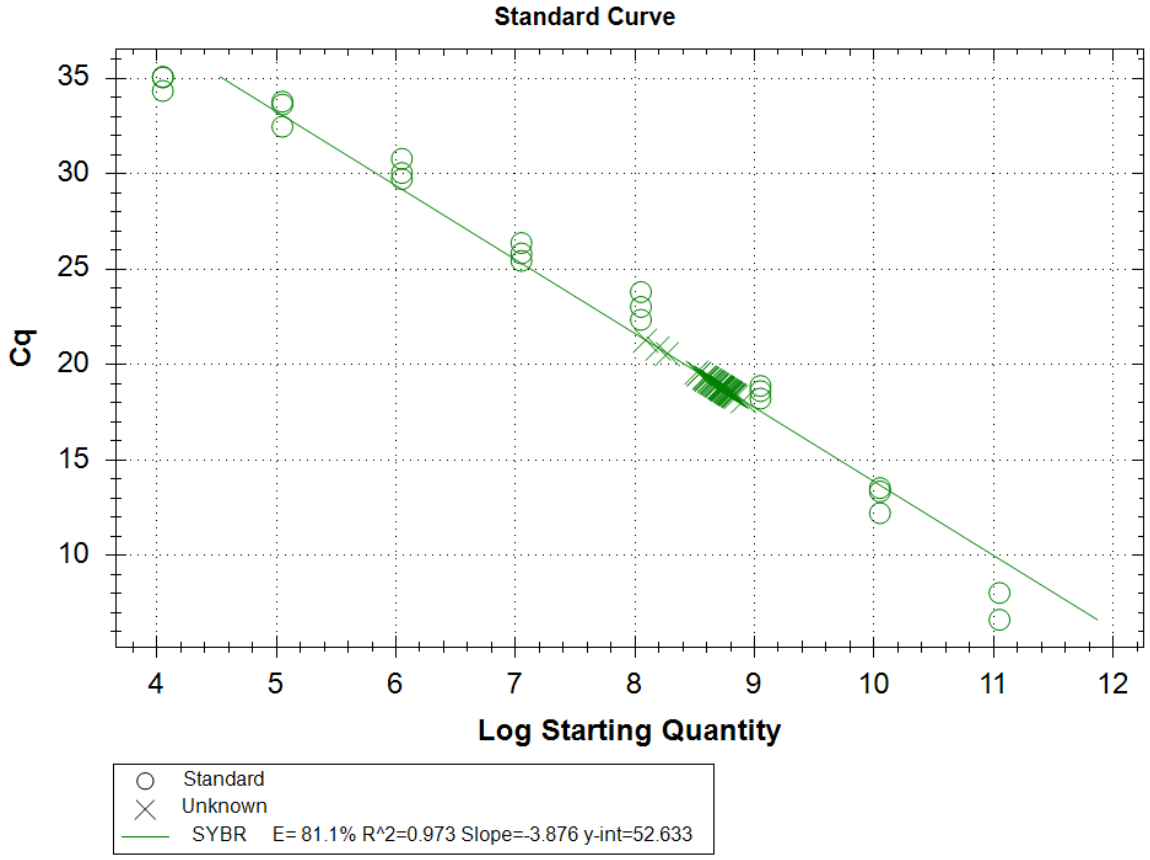


Figure 2.3. Standard curve for bacterial samples 1 – 20.

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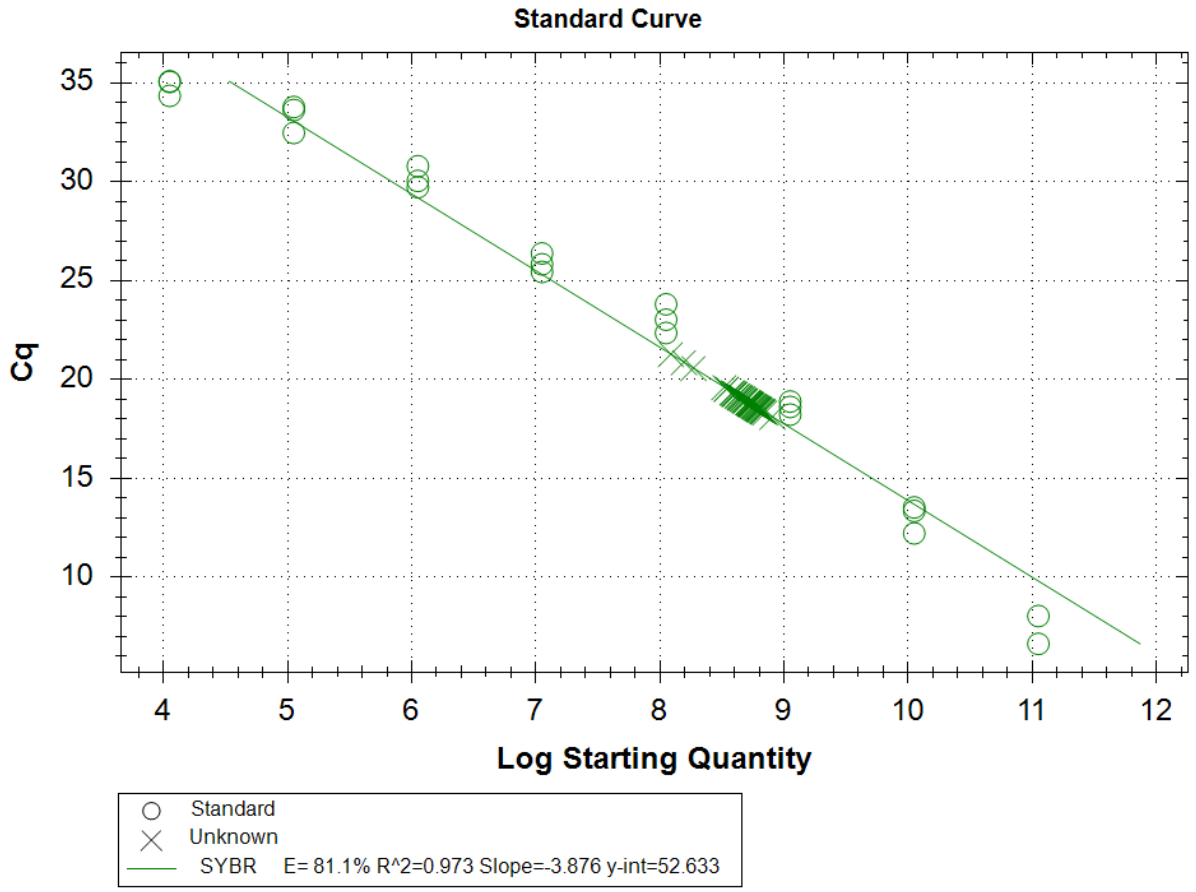
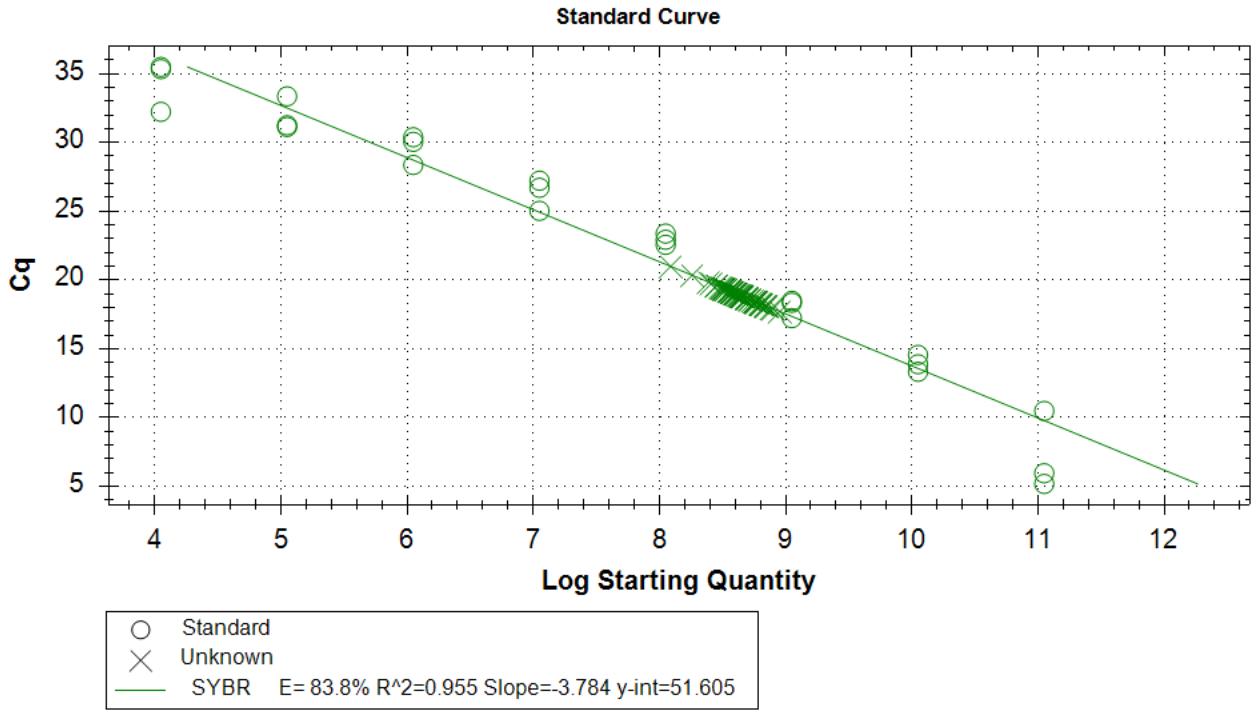
Appendix 3: Standard curves from the qPCR to predict bacterial copy numbers.

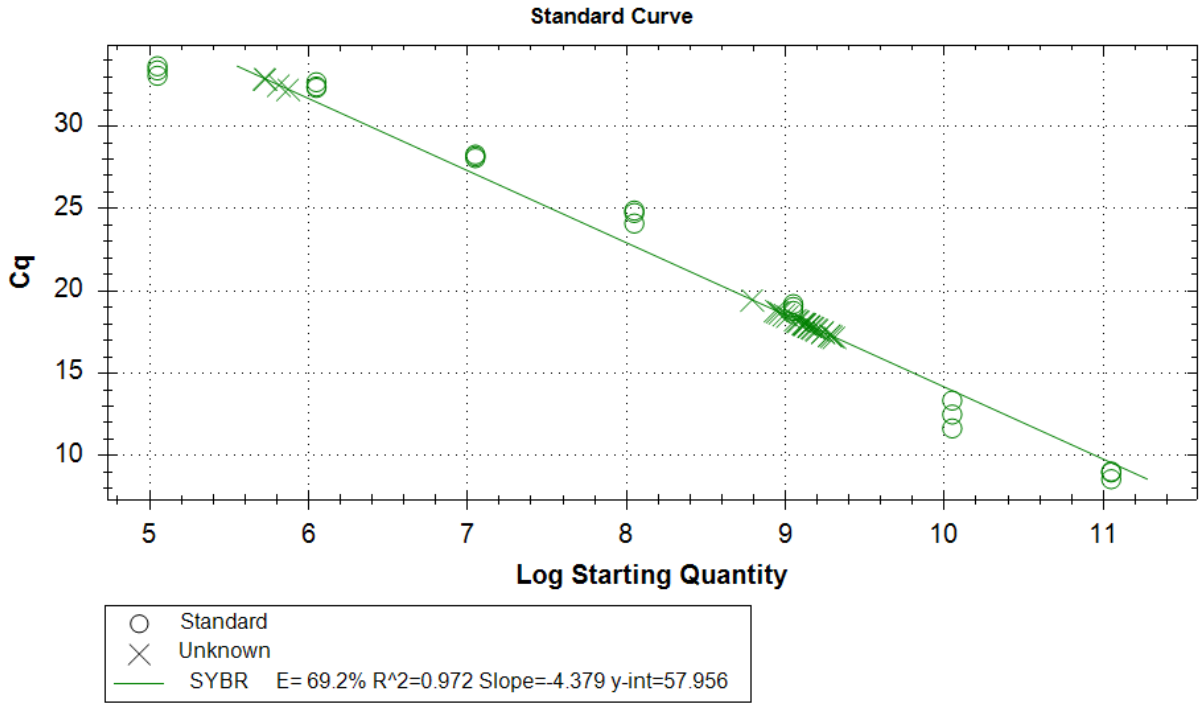
Appendix 3.1: Bacterial copy number standard curves for Lucerne, White Clover, Sub Clover and Annual Ryegrass.



Appendix 3.2: Bacterial copy number standard curves for Phalaris, Yellow Serradella, Cocks foot and Tall Fescue.

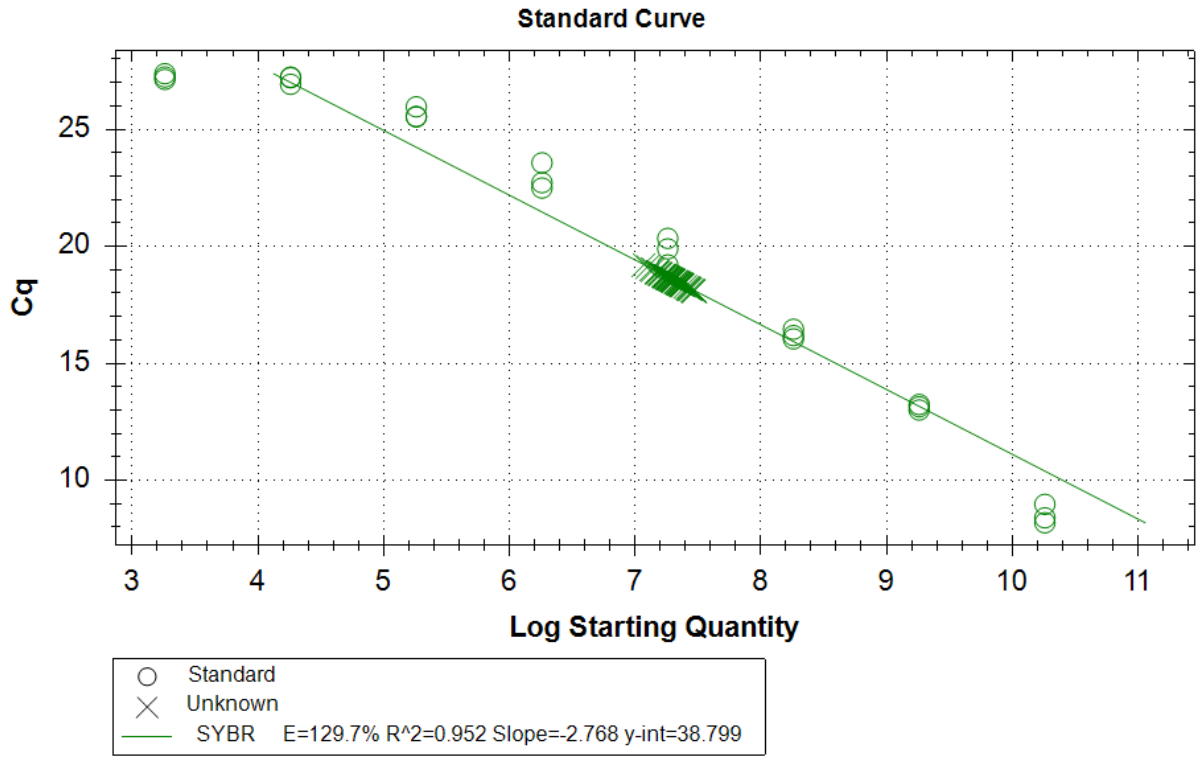


Appendix 3.3: Bacterial copy number standard curves for Wheat, Wheat (- citrate), soil only Control, and qPCR negative control.

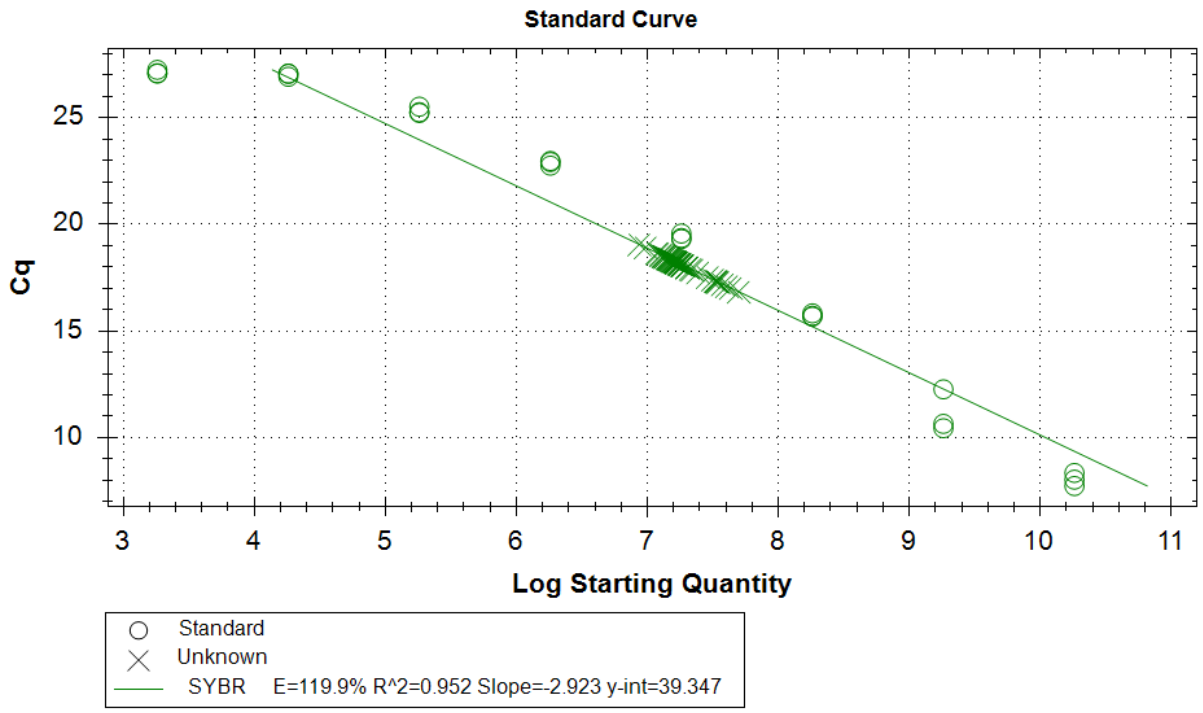


Appendix 4: Standard curves from the qPCR to predict fungal copy numbers.

Appendix 4.1: Fungal copy number standard curves for Lucerne, White Clover, Sub Clover and Annual Ryegrass.



Appendix 4.2: Fungal copy number standard curves for Phalaris, Yellow Serradella, Cocks foot and Tall Fescue.



Appendix 4.3: Fungal copy number standard curves for Wheat, Wheat (- citrate), soil only Control, and qPCR negative control.