The influence of aboveground diversity on plant-microbe interactions: insights from the field to agriculture.

Kirsty Matthews Nicholass

A thesis submitted for the degree of Doctor of Philosophy in Environmental Sciences.

School of Life Sciences

University of Essex

October 2020

“I will argue that every scrap of biological diversity is priceless, to be learned and cherished, and never to be surrendered without a struggle.”

- A quote from E.O.Wilson, author of “The Diversity of Life” (1992).

Abstract

Understanding species coexistence in diverse plant communities is a central aim of theoretical ecology. Similar to Janzen-Connell effects reported in tropical forests, negative Plant-Soil-Feedback and Negative-Density-Dependent type effects have recently been implied to promote diversity in temperate grasslands by preventing conspecific species from repeatedly occupying the same site. I, therefore, use a range of methodological approaches (observational, experimental, long-term biodiversity trials) to explore plant-microbe interactions from natural ecosystems and apply this knowledge, 'learned from nature,' within an agricultural and sustainability framework. In natural grasslands, I show root-associated mycobiomes are host-specific, driven by phylogenetic host distance operating at the level of plant functional group (i.e. grasses and forbs), and a positive correlation between local plant density and pathogenic fungal diversity, suggesting host-specific pathogens select for rare species by elevating mortality when plant density is high. Next, host-specificity of the mycobiome is weaker in low diversity grasslands, implying the strength of host-specificity could be important for overall plant diversity. I build on these findings and test for within species host-specificity and NDD across a biodiversity gradient in agricultural production systems. I demonstrate vast potential to exploit beneficial plant-microbial interactions both within and between crop cycles with evidence to imply mixing of within species cultivars could dilute the accumulation of soil-borne pathogens. Finally, I explore the effect of management strategies, creating an intrinsic biodiversity gradient, on soil-microbiomes in the world's largest agricultural system, palm oil. I show that increasing the diversity of the understory in oil palm plantations causes shifts in microbial diversity and composition. I conclude knowledge of plant-microbe interactions will enable improved predictions of vegetation dynamics, including nature restoration, and incorporation of this

framework into agri-ecosystems could see a shift of conventional agriculture to microbe driven self-sufficient systems and ultimately increase overall sustainability.

Acknowledgments

I must begin by thanking my supervisor, Alex Dumbrell, for his unwavering support, invaluable opportunities and continual interest in both my research and personal development throughout my journey as an undergraduate student to the completion of my PhD. Thank you to postdocs Dr Kate Randall, Dr Alessia Bani and Dr Robert Ferguson who have offered me invaluable advice from the field to analysis continually throughout my PhD. I also thank Dr Dave Clark for his instrumental training and guidance on the statistical analysis approaches utilised throughout all my data chapters of my thesis. Many thanks also to the technical staff, Tania Cresswell-Maynard, John Green and Mellissa Anderson. A huge thank you to my collaborators at the University of Ljubljana and the University of Cambridge for providing a supportive environment for me to undertake fieldwork in otherwise inaccessible regions of the world.

On a personal level, I must thank my friends who have kept me sane over the past four years, in particular, Fionnuala McKenna, Shellie Wall, Jacob Pullin, Hannah Bingham, Tayler Fordham, Will Arundel, Isabell Lord, Ella Shepherd, Alice Curtis, and Megan Lawler.

I would like to express my deepest gratitude to my family who have continually been supportive, and more importantly patient, with me throughout my PhD. Without their support this would not have otherwise been possible.

Finally, a thank you to you, the reader. I hope you find pleasure in reading my research as a reflection of the journey I have undertaken in the past four years.

Declaration

I declare that the work contained within this thesis is the result of my own work and was entirely written by myself under the supervision of Professor Alex Dumbrell.

Chapter 3 samples were collected in 2016 and 2017 from Slovenia with the help of Assistant Professor Irena Maček and Assistant Professor Klemen Eler (University of Ljubljana).

Chapter 4 samples from Norway and the UK were collected with the help of Dr Kate Randall.

Chapter 6 samples were collected by the field and research team, led by Jassica Pranja, at SMARTRI, Indonesia.

Bioinformatic processing of raw NGS reads followed the standard pipeline used in Profs Alex Dumbrell's laboratory (following section 3.8 of Dumbrell *et al.*, 2016).

Kirsty Matthews Nicholass

University of Essex

2020

Contents

Abstract 3

Acknowledgments 5

Declaration 6

Chapter 1 22

Introduction 22

1.1 | Summary 22

1.2 | Biodiversity and grasslands 23

1.2.1 | Drivers of plant community assembly 24

1.3 | Evidence for plant-Soil-feedback in temperate ecosystems 28

1.3.1 | The Janzen-Connell hypothesis 29

1.3.2 | Fungal mediated regulation of vegetation dynamics 31

1.3.3 | The 'pathogen-hypothesis' 34

1.4 | Community ecology of microbes 36

1.5 | Fungi as pathogens 38

1.6 | Implications for management, conservation and a more sustainable future. 40

1.7 | Molecular methods and ecoinformatic tools 43

1.8 | Conclusion 45

1.9 | Thesis rationale 45

1.10 | Thesis structure 46

Chapter 2 48

General methods 48

2.1 | Molecular methods 48

2.2 | Targeted amplicon sequencing 50

2.3 | Bioinformatics 54

2.4 | Soil chemistry 55

2.4.1 | Preparation 55

2.4.2 | pH 55

2.4.3 | Nutrient analysis 55

2.4.4 | Total carbon 56

Chapter 3 57

Evidence for fungal host-specificity and NDD from a natural hyperdiverse grassland ecosystem. 57

3.1 | Abstract 57

3.2 | Introduction 59

3.3 | Methods 64

3.3.1 | Study site 64

3.3.2 | Sampling 64

3.3.3 | Root DNA Extraction 66

3.3.4 | Molecular analysis 66

3.2.5 | Soil chemistry 66

3.2.6 | Statistical analysis 66

3.4 | Results 69

3.4.1 | Soil variables 69

3.4.2 | Root-associated fungi 72

3.4.2.1 | Summary 72

3.4.3 | Alpha-diversity 73

3.4.4 | Beta diversity 80

3.4.5 | MV-GLM. Multivariate species analysis between grasses and forbs. 85

3.5 | Discussion 88

3.5.1 | Host-specificity of fungal mycobiomes 88

3.5.2 | Pathogenic fungi 89

3.5.3 | Frequency and VMR - NDD effects 91

3.6 | Spatial and temporal dynamics within grassland ecosystems 93

3.7 | Positive PSF and vegetation dynamics? 95

3.8 | Conclusion 96

3.9 | Limitations 95

Chapter 4 99

Characterisation of belowground plant species-specific fungal communities across an aboveground grassland diversity gradient. 99

4.1 | Abstract 99

4.2 | Introduction 101

4.3 | Methods 105

4.3.1 | Study site 105

4.3.2 | Sample collection 106

4.3.3 | Root DNA Extraction 106

4.3.4 | Molecular analysis 106

4.3.5 | Soil chemistry 106

4.4.6 | Statistical analysis 107

4.4 | Results 109

4.4.1 | Soil variables 109

4.4.2 | Root-associated fungi 115

4.4.3 | Alpha-diversity between Norway and the UK. 117

4.4.4 | Alpha-diversity 121

4.4.5 | Beta-diversity: composition 131

4.4.6 | Fungal composition by guild. 134

4.4.7 | MV-GLM results 138

4.4.8 | Chapter 3 and 4 results summary 139

4.5 | Discussion 141

4.5.1 | Fungal diversity across a plant diversity gradient and large geographical scale 141

4.5.2 | Alpha-diversity 142

4.5.2.1 | Alpha-diversity: Plant species effects 142

4.5.2.1 | Alpha-diversity: plant functional group effects 144

4.5.3 | Beta diversity 146

4.6 | Conclusion 148

4.7 | Limitations 149

Chapter 5 152

A shift towards sustainable agri-ecosytems: unveiling plant-microbe associations and the potential for their exploitation within and between cropping cycles. ... 152

5.1 | Abstract 152

5.2 | Introduction 154

5.3 | Methods 159

5.3.1 | Experimental setup 159

5.3.2 | Wheat cultivars – biodiversity experiment 159

5.3.3 | Cover-crop trial 161

5.3.4 | DNA Extraction 163

5.3.5 | Molecular analysis 163

5.3.6 | Soil chemistry 163

5.3.7 | Statistical analysis 163

5.4 | Results 167

5.4.1 | Wheat cultivars - Biodiversity experiment 167

5.4.2 | Cover crop trial 181

5.5 | Discussion 187

5.5.1 | Wheat cultivars - Biodiversity experiment 187

5.5.1.1 | Effects of soil fraction: Root-associated vs soil-borne microbial communities 188

5.5.1.2 | Host specificity of microbial communities from wheat cultivars in monoculture 190

5.5.1.3 | Aboveground diversity gradient effects on soil-borne and root-associated microbial communities 191

5.5.1.4 | Conclusion 192

5.5.2 | Cover crop trial 193

5.5.2.1 | Host specificity of root-associated bacterial and fungal communities 194

5.5.2.2 | Functional response – fungi 195

5.5.2.4 | Conclusion 200

5.6 | Limitations 201

Chapter 6 203

Managing oil palm plantations more sustainably – Effects of understory vegetation management on soil-borne microbial communities 210

6.1 | Abstract..... 203

6.2 | Introduction 205

6.3 | Methods 211

6.3.1 | Study site..... 211

6.3.2 | Soil Microbial Sampling..... 215

6.3.3 | Soil Abiotic Sampling 216

6.3.4 | DNA extraction 216

6.3.5 | Molecular analysis 216

6.3.6 | Quantitative PCR 217

6.3.6 | Statistical analysis 218

6.4 | Results..... 221

6.4.1 | Effects of understory management treatment on soil abiotic variables 221

6.4.2 | qPCR analysis of 16S rRNA genes (bacteria and archaea), 18S rRNA and ITS..... 226

6.4.3 | Impacts of treatment on measures of microbial alpha-diversity 228

6.4.4 | Effects of understory management on soil microbial composition ... 236

6.4.5 | MV-GLM results..... 238

6.5 | Discussion..... 250

6.5.1 | The effect of oil palm management on microbial diversity and composition 250

6.6 | Conclusion..... 257

6.7 | Limitations 258

Chapter 7 260

General discussion 260

7.1 | Summary of thesis findings 260

7.1.1 | Chapter 3 summary 260

7.1.2 | Chapter 4 summary 261

7.1.3 | Chapter 5 summary 261

7.1.4 | Chapter 6 summary 263

7.2 | Key insights 264

7.2.1 | NGS as a means to explore ecosystem functioning as driven by fungal microbes 264

7.2.2 | Fungal-mediated regulation of aboveground vegetation dynamics. 265

7.2.3 | A new understanding of plant density - density as a function of neighbouring identity. 266

7.2.4 | Agriculture and sustainability – the future. 268

List of Figures

Figure 1.1. Schematic diagram referring to the theory of negative density dependence driven by belowground fungal pathogens. 36

Figure 1.2. Schematic for community assembly of the rhizosphere microbiota based on a review by Philippot et al (2013). 38

Figure 2.1. Schematic visualising the environmental source material and phylogenetic marker genes of interest explored throughout data chapters 3-6. 49

Figure 2.2. First stage amplicon PCR conditions utilised throughout each data chapter. 53

Figure 3.1. Location of sampling sites.. 65

Figure 3.2. Total fungal; A) OTU richness; B) OTU Shannon's Diversity Index; C) OTU Simpson's Diversity Index across plant species across each 3 natural grassland sites in Slovenia. 74

Figure 3.3. Plant-species specific pathogenic fungal communities assemblies. 78

Figure 3.4. Linear-modelled results of pathogenic alpha diversity with average VMR.. 79

Figure 3.5. Plant-species specific root-associated total fungal communities across three natural grassland sites in Slovenia.. 81

Figure 3.6. Plant-species specific root-associated pathogenic fungal communities across 3 natural grassland sites in Slovenia. 84

Figure 3.7. Volcano plot of MV-GLM modelled shifts in total fungal phyla (left) and pathogenic fungal phyla (right) abundances in grasses when compared to forbs. .. 86

Figure 4.1. Root associated fungal community assemblies; A) OTU richness, B) OTU Shannon's Diversity Index, C) OTU Simpson's Diversity Index between Norway and the UK. 117

Figure 4.2. Root associated fungal taxonomic richness. 118

Figure 4.3. Root associated fungal taxonomic diversity.. 119

Figure 4.4. Root associated fungal taxonomic evenness.. 120

Figure 4.5. Plant-species specific total fungal communities assemblies; A) OTU richness, B) OTU Shannon's Diversity Index, C) OTU Simpson's Diversity Index across plant species across each 3 natural grassland sites in Norway.. 123

Figure 4.6. Plant-species specific total fungal communities assemblies; A) OTU richness, B) OTU Shannon's Diversity Index, C) OTU Simpson's Diversity Index across plant species across each 3 natural grassland sites in the UK……..124

Figure 4.7. Plant family effects for plant-species specific root-associated total fungal communities across 3 natural grassland sites in A) Norway (stress = 0.1790) and B) the UK..... 132

Figure 4.8. Plant functional group effects for plant-species specific root-associated total fungal communities across 3 natural grassland……...129

Figure 4.9. Plant family effects for plant-species specific root-associated pathogenic fungal communities across three natural grassland sites in A) Norway (stress: 0.1972) and B) the UK 137

Figure 4.10. Plant functional group effects for plant-species specific root-associated pathogenic fungal communities across three natural grassland sites……...133

Figure 4.11. Volcano plot of MV-GLM modelled shifts in pathogenic fungal phyla relative abundances in the UK when compared to Norway..... 138

Figure 4.12. Schematic to illustrate the role of plant species and plant functional group effects on measures of alpha diversity for fungal guilds in the regulation of aboveground diversity.. 145

Figure 5.1. Schematic detailing the random block design of nine (1-9) wheat cultivars grown in single (monoculture), triple or nine variety yield plots (see coloured key), n = 27..... 160

Figure 5.2. Venn diagram showing the overlap in operational taxonomic units (OTUs) between root-associated and soil-borne bacterial and fungal communities.. 168

Figure 5.3. Volcano plot of MV-GLM modelled shifts in total fungal phyla abundances in root-associated microbes when compared to soil-borne microbes..... 170

Figure 5.4. Host specific effects of plant wheat variety on alpha-diversity measure of root-associated (A-C; grey) and soil-borne (D-F; white) bacterial communities of plants in monoculture.. 172

Figure 5.5. Host specific effects of plant wheat variety on alpha-diversity measure of root-associated (A-C; grey) and soil-borne (D-F; white) fungal communities. 173

Figure 5.6. Total root-associated (circles) and soil-borne (triangles) bacterial communities from nine wheat varieties grown in monoculture.. 174

Figure 5.7. Total root-associated (circles) and soil-borne (triangles) fungal communities from nine wheat varieties grown in monoculture. 175

Figure 5.8. Total soil-borne bacterial communities from nine wheat varieties grown in monoculture.. 176

Figure 5.9. Total bacterial (A-C) and fungal (D-F) alpha-diversity across a wheat variety diversity gradient (monoculture, triplicate and nine species mix). 179

Figure 5.10. Venn diagram showing the overlap in operational taxonomic units (OTUs) among wheat variety levels for bacterial and fungal root associated and soil-borne microbial communities. Numbers represent total OTU counts……173

Figure 5.11. Comparison of bacterial taxonomic distributions between cover-crop treatment plots.. 182

Figure 5.12. Root associated bacterial (A) richness, (B) diversity and (C) evenness effects from cover crop varieties..... 183

Figure 5.13. Root associated fungal (A) richness, (B) diversity and (C) evenness effects from cover crop varieties. 184

Figure 5.14. Effects of cover crop variety on OTU richness of (A) saprotrophs and (B) endophytes of root-associated fungal communities. 185

Figure 5.15. Specific root-associated bacterial communities (left) and fungal communities (right) from four cover varieties. 186

Figure 6.1. Tripartite interaction between plant host, beneficial and pathogenic microbiota within the soil profile and rhizosphere. 208

Figure 6.2. Schematic to show the design of the BEFTA Understory Vegetation Project. 214

Figure 6.3. Schematic to illustrate sampling protocol for phylogenetic soil microbial profiling. 215

Figure 6.4. Plots of soil variables across treatments and sampling depth. 225

Figure 6.5. Copy number per 1 g of fresh weight soil based on qPCR quantification for 16S rRNA genes; bacteria (a) and archaea (b), 18S rRNA eukaryotes (c) and ITS fungi (d)..... 227

Figure 6.6. Differences in bacterial (A) OTU richness, (B) diversity – Shannnon's Diversity Index and (C) evenness - Simpson's Diversity Index) between understory treatments at BEFTA..... 229

Figure 6.7. Differences in archaeal (A) OTU richness, (B) diversity – Shannnon's Diversity Index and (C) evenness - Simpson's Diversity Index) between understory treatments at BEFTA..... 230

Figure 6.8. Differences in total microbial-eukaryote (A) OTU richness, (B) diversity – Shannon's Diversity Index and (C) evenness - Simpson's Diversity Index, and heterotrophic microbial eukaryotes (D-F), autotrophic eukaryotes (G-I) and fungi (J-L) between understory treatments at BEFTA……225

Figure 6.9. Differences in total fungal (A) OTU richness, (B) diversity – Shannnon's Diversity Index and (C) evenness - Simpson's Diversity Index, and pathogenic fungi (D-F), and saprotrophic fungi (G-I) between understory treatments at BEFTA. 234

Figure 6.10. Differences in total fungal (A) bacterial, (B) archaeal, (C) micro-eukaryote and (D) fungal composition between treatment plots..... 237

Figure 6.11. Volcano plot of MV-GLM modelled shifts in bacterial phyla abundances under A) reduced management, and B) enhanced management. 239

Figure 6.12. Volcano plot of MV-GLM modelled shifts in archael phyla abundances under A) reduced management, and B) enhanced management. 241
Figure 6.13. Volcano plot of MV-GLM modelled shifts in microbial eukaryote phyla abundances under A) reduced management, and B) enhanced management. 243
Figure 6.14. Volcano plot of MV-GLM modelled shifts in autotrophic phyla abundances under A) reduced management, and B) enhanced management.. 245
Figure 6.15. Volcano plot of MV-GLM modelled shifts in heterotrophic phyla abundances under A) reduced management, and B) enhanced management. 247
Figure 6.16. Volcano plot of MV-GLM modelled shifts in ITS fungal phyla abundances under A) reduced management, and B) enhanced management.. 249

List of Tables

Table 2.1. Sequences of PCR primers for phylogenetic marker genes. 51

Table 3.1. Mean and standard error of pH, moisture and total carbon across three sampling sites in Slovenia.. 69

Table 3.2. Mean and standard error of cations across three sampling sites in Slovenia. 70

Table 3.3. Mean and standard error of anions across three sampling sites in Slovenia.. 71

Table 3.4. Linear-modelled analysis of fungal richness, diversity and evenness as an effect of plant species and plant functional group (i.e. forbs and grasses). 75

Table 3.5. Linear-modelled analysis of fungal richness, diversity and evenness as an effect of plant species and plant functional group (i.e. forbs and grasses). 77

Table 3.6. PERMANOVA analysis of assemblies of fungal OTUS (including; Ascomycota, Basidiomycota, Chytridiomycota, Zygomycota and Glomeromycota) across differing plant taxonomic levels and between plant functional group. 82

Table 3.7. PERMANOVA analysis of assemblies of pathogenic, symbiotic and saprotroph fungal OTUS across differing plant taxonomic levels and between plant functional group.. 83

Table 3.8. MV-GLM modelled shifts of pathogenic taxa occurring in significantly higher abundance in grasses when compared to forbs. 87

Table 4.1. Summary of diversity patterns and locations in three geographical field sites. 105

Table 4.2. Mean and standard error of pH, moisture and total carbon across three sampling sites in Norway.. 109

Table 4.3. Mean and standard error of cations across three sampling sites in Norway.. 110

Table 4.4. Mean and standard error of anions across three sampling sites in Norway. 111

Table 4.5. Mean and standard error of pH, moisture and total carbon across three sampling sites in the UK..... 112

Table 4.6. Mean and standard error of cations across three sampling sites in the UK. 113

Table 4.7. Mean and standard error of anions across three sampling sites in the UK. 114

Table 4.8. Linear-modelled results for plant species effects on fungal communities split by phyla and fungal guild for Norway... 122

Table 4.9. Linear-modelled results for plant functional group effects of species-specific fungal communities split by phyla and fungal guild for Norway. 125

Table 4.10. Linear-modelled results for plant species effects on fungal communities split by phyla and fungal guild for the UK.……..…..123

Table 4.11. Linear-modelled results for plant functional group effects of species-specific fungal communities split by phyla and fungal guild for the UK. 130

Table 4.12. PERMANOVA analysis of Norway for assemblies of pathogenic, symbiotic and saprotrophic fungal OTUS across differing plant taxonomic levels and between plant functional group..... 134

Table 4.13. PERMANOVA analysis of the UK for assemblies of pathogenic, symbiotic and saprotrophic fungal OTUS across differing plant taxonomic levels and between plant functional group..... 136

Table 4.14. Summary of significant plant species specific and plant functional group specific fungal communities across Norway (N), the United Kingdom (UK) and Slovenia (S).. 139

Table 4.15. Summary of distinct fungal communities for Norway (N), the United Kingdom (UK) and Slovenia (S) across all plant taxonomic levels..... 140

Table 5.1. Details of cover crops used in trial at RAGT Seeds, UK, including sample ID, plant species and brand name.. 162

Table 5.2. Linear modelled results of soil variables across treatment plots. 167

Table 5.3. Linear-modelled analysis of bacterial and fungal alpha-diversity across a wheat variety richness gradient for both root-associated and soil-borne microbial communities.. 178

Table 6.1. Mean values (+ SEs) for 13 soil variables.. 222

Table 6.2. Linear-modelled test statistics for 13 soil variables.. 223

Table 6.3. Summary of alpha-diversity measures indicating a significant increase(↑) /significant decrease(↓) or no significance (NS) in comparison to normal treatment plots.. 235

Chapter 1

Introduction

1.1 | Summary

Global biodiversity is declining rapidly and there is an urgent need for sustainable management approaches to stem this decline. The paradox of biodiversity and how plant species stably coexist is one of the most enduring questions in ecology. As such, a solid understanding of the ecological mechanisms that maintain and promote biodiversity is required to inform these management options. Historically, interspecific competition has formed the basis of community assembly, but difficulty in confirming resource partitioning has fuelled interest in other assembly mechanisms (Petermann *et al.*, 2008; Mommer *et al.*, 2010, 2018). In the diverse grassland communities common across much of Europe, recent research has highlighted the potential for belowground interactions to be important in maintaining biodiversity. Similar to Janzen-Connell effects reported in tropical forests, negative Plant-Soil-Feedback (PSF) maintains diversity by resisting the forces of competitive exclusion. Plant species progressively make their local soil environment less suitable for members of the same species, avoiding any one species dominating the community. Underlying this mechanism are two assumptions; 1) host-specificity between plants and the drivers of PSF, and 2) Negative Density Dependence (NDD). This means abundant plant populations are kept in check by an accumulating pathogen load. There is strong evidence to suggest root associated pathogenic fungi are the main pathogenic drivers of plant community assembly (Maron *et al.*, 2011; Bagchi *et al.*, 2014; Mommer *et al.*, 2018), however, studies to date have taken a 'black-box' approach in which the taxonomic identity of the microbial players involved remains unknown. Understanding the drivers of aboveground vegetation dynamics not only has significance for nature

restoration (Koziol *et al.*, 2020), but insights learned from natural ecosystems could inform more sustainable agri-ecosystems.

1.2 | Biodiversity and grasslands

Biodiversity is described as the variety of life on Earth; it encompasses the variability in genes, singular organisms, species and populations. Through the conditions and processes of natural ecosystems, and the species and diversity they contain, biodiversity provides humankind with ecosystem services that are a requirement to sustain and fulfil human life (Daily, 1997). However, worldwide biodiversity is declining at a rapid rate, largely due to land conversion and agricultural expansion coupled with limited habitat protection (Hoekstra *et al.*, 2005). Species loss results in modifications in key processes important to the productivity and sustainability of Earth's ecosystems; degradation of biodiversity denies future generations of genetic resources, ecosystem services and as such, compromises sustainability. A solid understanding of the drivers underpinning biodiversity is essential to mitigate any potential species losses, maintain or even restore biodiversity for a more sustainable future.

How a large number of competing plant species coexist remains one of the most enduring questions in ecology (Gause, 1932; Aarssen, 1989; Zobel, 1992; Chase *et al.*, 2002; Petermann *et al.*, 2008). Temperate grasslands, encompassing grasses, clover, leguminous species, dicots, herbs and shrubs, contribute to a vast number of ecosystem services including water purification from fertilisers and pesticides, protection from erosion, contributions to recreation and aesthetics and food for livestock (Carlie*r et al*., 2009). Unfortunately, temperate grasslands are the most converted but least protected biomes on the Earth (Hoekstra *et al.*, 2005). Therefore, understanding the mechanisms of coexistence in diverse plant ecosystems is attracting renewed interest in conservation and sustainability research. To understand

coexistence we must understand why competitive exclusion does not occur as commonly as we might predict. Competitive exclusion describes the inevitable elimination of a species from a habitat due to competition between species with identical needs for resources. This proposes competitive exclusion would be common in grassland communities due to similarities in requirements, such as water, CO_2, light, N, P and differences in overall competitive ability (Silvertown *et al.*, 2004). However, this is rarely the case as demonstrated by the frequently diverse grassland ecosystems we see today (Wilson *et al.*, 2012). As such, the study of plant ecology increasingly looks for mechanisms that stabilize fitness differences between competing species (Bever *et al*., 2010).

1.2.1| Drivers of plant community assembly

Understanding community composition, diversity and species coexistence in plant communities is still an unsolved problem. We must understand how multiple species co-exist despite similar needs for resources and differences in fitness abilities. Drivers of community assembly and vegetation dynamics refer to large-scale biogeographical processes, including those that occur over evolutionary time, and abiotic, biotic, dispersal limitations and interspecific interactions that help to explain local biodiversity patterns.

Conditions allowing coexistence fall into two separate categories, those in which habitats change through space, and those in which habitat changes through time. In stable environments equilibrium communities establish by uninterrupted succession over several years therefore species are able to coexist in competitive equilibrium if they differ in niche (Pickett, 1980). Niche differentiation refers to two separate entities (Shmida & Ellner, 1984). Firstly, habitat differentiation occurs when cohabitating species utilise different portions of the habitat. However, root trait differences with regards to differences in rooting depth and composition between species are often not

large enough to account for separate species-specific niches (Mommer *et al*., 2011). Secondly, resource differentiation occurs when species partition available resources in a way that each species is not in direct competition for a single resource. Niche differentiation and resource differentiation are widely accepted by zoologists, but those researching lower trophic levels, such as plants, find it difficult to accept that each plant species in a highly diverse environment could occupy a separate and distinct niche, i.e. there are more plants than there are niche spaces. As such, ecologists such as Silvertown & Law, (1987) challenge the generality of equilibrium theory and propose a number of alternative mechanisms, such as transient density-independent mortality and various non-equilibrium theories, that could explain plant species coexistence.

Alternatively, non-equilibrium theory assumes habitats differ over time. It has been widely shown non-equilibrium communities are more species rich in comparison to equilibrium communities (Grime, 1981; Tilman, 1982). Heterogeneity through time is produced by disturbances that can be biotic in the form of predation and disease, or abiotic, such as fire and drought (Pickett, 1980). These disturbances prevent a competitive equilibrium from being reached. Inferior competitors are therefore favoured in periods of stress due to the decreased competition from superior competitors. This allows coexistence of a greater number of species with varying competitive abilities that would not otherwise be able to survive the increased levels of interspecific completion in undisturbed environments. This closely mimics the theory behind the Intermediate Disturbance Hypothesis (Connell, 1978; Roxburgh *et al.*, 2004). However, non-equilibrium theory and the IDH have since been strongly criticised. For example, Chesson & Huntly (1997) argues that coexistence is only favoured when disturbances create spatial or temporal niche opportunities and a test of 116 published diversity-disturbance relationships in which a non-significant relationship was the most common finding (Mackey & Currie, 2001). For this reason,

non-equilibrium theory no longer receives much consideration concerning plant species coexistence.

Separate to the two above entities, the balanced competition concept is based on converging competitive ability under natural selection. This results in a reciprocal balance of competitive ability for the resource that is being competed for (Aarssen, 1984, 1989). As such, competitive exclusion is avoided when individuals of one population respond to the individuals of another population in an arms race. This theory was developed to explain how different species can hold the same degree of niche differentiation, but yet their probability to coexist based on relative differences in competitive ability is different. Aarssen (1983) suggests this is a result of 'competitive combining' ability. This is explained by plants holding a variety of biological attributes differing in competitive ability, which when summed, confer the same competitive ability. It is thought this is a fine tuning process in which coevolution adjusts the arrangement of biological attributes and therefore regulates the way in which plants respond to one another.

Grubb (1977) expands on the theory of niche differentiation and competitive combining abilities by highlighting the role of regeneration in species coexistence. Gaps created by plant death and disturbance create colonisation opportunities (regeneration niches) for species that are unable to colonise in later successional stages (Kimmerer & Young, 1996), with the size of the clearing determining what species will re-occupy the site. Most research concerning the regeneration concept is centred around tropical trees, when a large tree disappears, species with a high demand for light might be the likely new co-habitants of that area. In contrast, if the gap is small, shade tolerant species would likely invade the opening resulting in the coexistence of shade tolerant species with larger species requiring a high demand for light. These differential establishments across a range of regeneration opportunities promotes coexistence (Denslow, 1980). However, evidence for patterns of

regeneration in grassland communities is limited, though it is accepted abiotic conditions and life history traits of plant species play a major role in the success of regeneration.

Finally, focussing on a larger scale, the theory of island biogeography (MacArthur, 1960; MacArthur & Wilson, 1963) and relative species abundance was unified by Hubbell (1997). Collectively, this suggests community diversity is not only regulated by present environmental and ecological factors, but is controlled and limited by immigration of species from meta-community source areas and the extinction of species within the local community (Volkov *et al.*, 2003). This proposes the inhabitants of a local community are in diversity, but not taxonomic equilibrium, controlled by continual species turnover. As such, the diversity of the local community is a subsample of the meta-community. Moreover, Taylor *et al.*, (1990) proposes the number of species in any habitat is determined by the commonness of that habitat type. The larger the regional or global habitat size and the older the geographical age, the greater the time allowed for natural selection and speciation and therefore the greater the number of potential species that could occupy that site (Zobel, 1992; Pärtel *et al*., 1996). This suggests species richness is dependent on the regional species pool and therefore this may influence diversity and coexistence.

As evidenced, it is likely there are multiple mechanisms underpinning plant species coexistence. However, an increasingly rich body of evidence highlights negative plant-soil-feedback (PSF) interactions involving soil microbes, especially fungal pathogens (Bagchi *et al*., 2014a), as a significant driver of grassland diversity, a process that has previously been overlooked. Interspecific competition has commonly been invoked as the central force to explain the organisation of plant communities (Tilman, 1982). However, on a local scale, current co-existence theory is increasingly centred on stabilising of fitness differences and on how plant heterogeneity is maintained despite few differences in both niche space and requirements.

1.3 | Evidence for plant-Soil-feedback in temperate ecosystems

Whilst the above theories have historically carried weight regarding coexistence theory amongst ecologists, the developing evidence for co-existence theories such as niche differentiation and non-equilibrium theory is frequently challenged (Silvertown & Law, 1987; Chesson & Huntly, 1997; Mackey & Currie, 2001; Mommer *et al.*, 2010). More recently, the incorporation of a microbial perspective is having greater success in describing plant coexistence. More specifically, plant-soil-feedback (PSF) has received growing attention as a mechanism that underlies a number of ecological phenomena including, soil sickness, species dominance, species invasion (Huang *et al.*, 2013), and more recently, species coexistence (Mills & Bever, 1998; Bonanomi *et al*., 2005; Bagchi *et al*., 2014). PSF is a process by which plants modify soil conditions (physical, chemical, or biological), which in turn affects the growth, survival and overall fitness of subsequent members of the same species in that area. This interaction can have positive, negative or neutral effects on conspecific plant populations, but, commonly occurs producing negative conditions for the establishment, growth and reproduction of conspecific juvenile species (Bonanomi *et al.*, 2005). This theory was investigated by (Bever, 1994) in which four perennial plant species demonstrated lower growth and root-shoot ratios when grown on soil inoculate previously conditioned by their own species.

Developing ecological theory and the use of advanced molecular tools is beginning to shed light on the role of negative PSF in species coexistence. Nonetheless, PSF is not a 'new' phenomenon. Negative PSF has been described since ancient times, formally recognized by agriculturalists as early as the 1960's, referred to the phenomena as 'soil sickness' (Börner, 1960; Wensley, 1956). Crop rotation schemes, as part of the agricultural revolution, have been implemented

frequently since the nineteenth century to overcome some of the difficulties caused by such negative feedbacks. For instance, European crop rotations commonly involved wheat, barley, and turnips with clover or peas (Overton, 1996). It was understood crop rotation schemes aided crop production by preventing build-up of specific pests and soil borne pathogens thus preventing the suppression of subsequent yields of similar crops. Petermann *et al.* (2008) explored this interaction utilising a controlled greenhouse experiment and proposed that such negative PSF effects could be a type of Janzen-Connell effect that promotes species coexistence by preventing heterospecific species repeatedly occupying the same site due to the build-up of soil pathogens. Interestingly, microbial regulated negative PSF has rarely been considered as a driver of community dynamics in natural ecosystems despite early demonstrations of its presence.

1.3.1 | The Janzen-Connell hypothesis

Over fifty years ago, Janzen (1970) and Connell (1971) described how a density dependent mechanism as a result of negative PSF contributes to the high species diversity seen in tropical forest communities. Janzen (1970) investigated community structure in tropical forests, recognising lowland tropical trees do not produce new adults within the immediate vicinity of the parent tree. Adults appeared to be more uniformly distributed than would be expected if the probability of a new adult species being present correlated with the number of seeds arriving at that point. This observation was consistent in Central and South American forests. Janzen attributed high species richness, low abundance and a more uniform distribution than expected to two observations common to most forests; (1) the number of seeds arriving at a given point from the parent tree declines with distance, (2) the adult tree, its seeds and seedlings are a food source for several host specific parasites and herbivores.

Connell (1971) simultaneously investigated the role of natural enemies in preventing competitive exclusion in both marine and rain forest ecosystems. Connell's theory focuses on the idea that competitive exclusion is rarely seen in nature (Miller, 1967). Initially it was thought species coexistence occurred due to divergent strategies that allowed sharing of a common resource. However, Connell stresses the presence of coexistence despite lack of obvious divergences in character. Connell proposes prey populations are maintained at low densities by efficient predators so that overall competition is reduced. Thereby, no population reaches a high enough density to exclude another through competition for resources. In the example of tropical trees, Connell suggests each tree species supports a large population of host-specific enemies, without detriment to itself, but which attack seedlings of the same species. Therefore, for a tree species to persist seedlings must be dispersed to a point in which attack by natural enemies does not impede survival. Both Janzen and Connell agree on the fundamental point that density dependent mortality, which is capable of increasing diversity, decreases with distance from the parent tree. In addition to this distance/survival relationship, Connell highlights environmental conditions directly affect the efficiency of the predator population in regulating prey density. Environments with few irregular events such as extreme weather, and a low amplitude of change in abiotic variables favour the ability of natural enemies to efficiently regulate prey populations and thus preventing exclusion between competing species.

To summarise, the Janzen-Connell hypothesis jointly proposes local build-up of insect and fungal pathogens allows for high plant species diversity by promoting mortality when density is high (negative density dependence; NDD), this makes limited resources more available to heterospecific and potentially rarer species. Individuals are expected to experience lower rates of recruitment and survival near conspecific neighbours as a result of (1) pest and pathogen accumulation from adults to their

nearby offspring, and (2) the greater abundance of host-specific herbivores and pathogens among hosts with higher densities (Peters, 2003).

The phenomena that areas with higher densities of few common species are more prone to the negative effects of natural enemies has been recognised for decades (Charles, 1958). Thereby, low diversity ecosystems and those that have close phylogenetically related species are also likely to share pathogen suites (Liu *et al.*, 2012), hence proliferation and transmission of pathogens will be greater and detrimental effects on growth and production will be enhanced. This acts as a stabilizing mechanism when plant fitness is unequal and can promote species coexistence and maintain diversity by slowing down competitive exclusion through altering the ability of plants to compete with neighbouring species (Rigg *et al.*, 2016). Studies show even very low levels of negative feedback enables the survival of inferior competitors (Bonanomi *et al.*, 2005), although intermediate levels of feedback are required to allow for stable coexistence in communities that are frequently disturbed. Increasing intensities of negative feedback have been shown to prompt a greater species turnover both in time and space, also owing to increased species diversity.

1.3.2 | Fungal mediated regulation of vegetation dynamics

Current research for understanding plant ecology has an aboveground bias that until recently has underestimated the potential of microorganisms as drivers of community assembly and coexistence. However, treatment of soil with fungicide has revealed complete removal of NDD effects, hence a link can be established between fungal pathogens and NDD (Bagchi et al., 2010, 2014). This study highlights fungal pathogens as the main driver behind negative PSF in tropical plant ecosystems by demonstrating higher growth and survival associated with fungicide treated soils. Moreover, fungal suppression translated into ecosystems of lower diversity. This study showed variations in tropical plant diversity and species composition are caused by

fungal pathogens and insect herbivores. Treatment with insecticides demonstrated that insects significantly regulate the species composition of a community, however, insects attack irrespective of the host plant density therefore minimally effect the overall diversity. As such, a NDD component is essential to create significant shifts in overall species diversity. However, these 'black box' approaches by where the taxonomic identity remains unknown are only the starting point of this developing field in ecology. Treatment of soils with fungicides causes elimination of pathogenic fungi, but also mutualistic fungi such as arbuscular mycorrhizal fungi (AMF). It therefore cannot be said with full certainty that there is a causal link between fungal pathogens and NDD. Liang *et al.*, (2015) highlights the importance in considering other belowground fungal relationships in which it is shown positive feedback, as driven by AMF, is enough to counteract Janzen-Connell effects of fungal pathogens.

Based on evidence from tropical forests, it has been suggested Janzen-Connell effects vary along a latitudinal gradient due to abiotic constraints on pathogenic microbiota (Givnish, 1999). However, evidence for this theory has been contested in which it is shown temperate tree species demonstrate density-dependent mortality between seed dispersal and seedling establishment at the same proportions as tropical trees (Hille Ris Lambers *et al.*, 2002). Based on the above evidence and experimental work involving temperate grasses, it is probable to suggest Janzen-Connell effects might also play a significant role in maintaining diversity in grasslands. Already, temperate grassland species have been shown to be disadvantaged when trying to recolonize formally occupied sites (Petermann *et al.*, 2008). This observation was attributed to soil microbes in which plant species progressively make the environment unsuitable for members of their own species.

Variations in the strength of negative feedback are also shown to depend on conspecific density or rank abundance of host plants (Packer & Clay, 2000). Liang *et al.*, (2016) provides recent support for the hypothesis that juvenile recruitment is

suppressed by a greater abundance of host-specific herbivores and pathogens associated with hosts of higher densities. Quantitative analysis of tree assemblages in China showed that relative abundance of pathogenic fungi increases with an increase in host tree density. This translated into decreased seedling survival, an apparent effect of NDD. This could mean microbial communities differ both quantitatively and qualitatively between rare and abundant species. Klironomos (2002) showed rare plants suffer enhanced detrimental effects on fitness when grown on 'home' soil (where pathogens have had time to accumulate) in comparison to abundant plants, this reinforces the theory that the strength of NDD is different in plants of different rank abundance. It is predicted the negative effect of these organisms also declines with distance from the adult tree, likely as host density begins to decrease simultaneously with pathogen abundance. This is further supported by a study showing juvenile recruitment is significantly reduced when grown in soil inoculate taken from dense adult tree populations, but recruitment was unaffected when grown in soil of low adult abundance (Liu *et al.*, 2015). Very few studies make a comparison between variations in population densities, particularly in temperate ecosystems, and as such further research would be required to address this.

Despite the obvious influence of negative PSF in stabilising competitive abilities, some research suggests a simultaneous role of both negative and positive PSF results in the greatest measurement of plant diversity (van der Putten, 2017). This study examines the performance of plants with different nutrient acquisition strategies under a number of different soil treatments: home soil, sterilised soil, and soil conditioned from all other plant species. Stepwise addition of plants with different nutrient acquisition strategies revealed a variety of negative and positive PSFs led to the greatest plant species richness. Following this, a field experiment involving two tree species with either AMF or ectomycorrhizal nutrient acquisition strategies demonstrated a difference in local offspring survival, with AMF resulting in enhanced

diversity due to their inability to protect offspring from pathogen accumulation. Work by Albornoz *et al.*, (2017) showed those plants with efficient phosphorus acquisition may have a trade-off with resistance to soil pathogens, hence their superior competitive ability is dampened by that of fungal pathogens. This highlights a new dimension to the Janzen-Connell hypothesis in which the effects vary in strength between species with differing nutrient acquisition strategies.

1.3.3 | The 'pathogen-hypothesis'

Throughout this chapter I have documented evidence Janzen-Connell type effects in tropical forest ecosystems. However, more recent studies spanning temperate trees and grassland ecosystems eludes to the idea that Janzen-Connell type effects may also play a role in regulating community dynamics in grassland ecosystems. In grassland ecosystems, Janzen-Connell type effects have been collectively termed as the 'pathogen hypothesis' (Mommer *et al*., 2018). The 'pathogen hypothesis' is analogous to the Janzen Connell hypothesis as discussed in section 1.5, i.e. the local build-up of insect and fungal pathogens allows for high plant species diversity by promoting mortality when density is high. For the pathogen hypothesis to hold true, two assumptions must be met;

1) Host specificity - plant species accumulate species-specific pathogens. Plant host performance is reduced through an accumulation of species-specific pathogens, but not to the detriment of neighboring plant species. This reduced the competitive ability of superior plant species and opens niche space and resources for heterospecific species.
2) Negative density dependence (NDD) – pathogen accumulation is correlated with a decrease in aboveground diversity gradient, i.e. as a community becomes less diverse, the density of any plant increases, and pathogen load

accumulates. This results in increased negative PSF and decreased survival and fitness.

Once the first requisite has been met i.e. host specificity, effects of NDD has a number of ecological implications. Along a gradient of plant species richness, pathogen accumulation is high at low plant species diversity (such as those in monocultures) and pathogen accumulation is predicted to decrease with increasing plant species diversity. This implies negative PSF selects for rare species by elevating mortality of abundant species in a density dependent manner, known as the 'rare species advantage'. While seldom considered, understanding how PSF contributes to plant rarity may also have further implications for the conservation and management of rare species (Rigg *et al.*, 2016). However, the identities and host specificities of the fungal pathogens involved in this relationship have not yet been determined. Mangan *et al.*, (2010) found tree species that showed stronger negative feedback (due to greater pathogen accumulation) were less common as adults in the forest community, indicating that vulnerability to soil pathogens may determine the relative abundance of species in tropical forests. It may also be the case that less diverse grassland systems hold weaker host specific relationships between fungal pathogens and plant hosts. However, the strength and direction of this relationship in temperate grassland species remains largely untested.

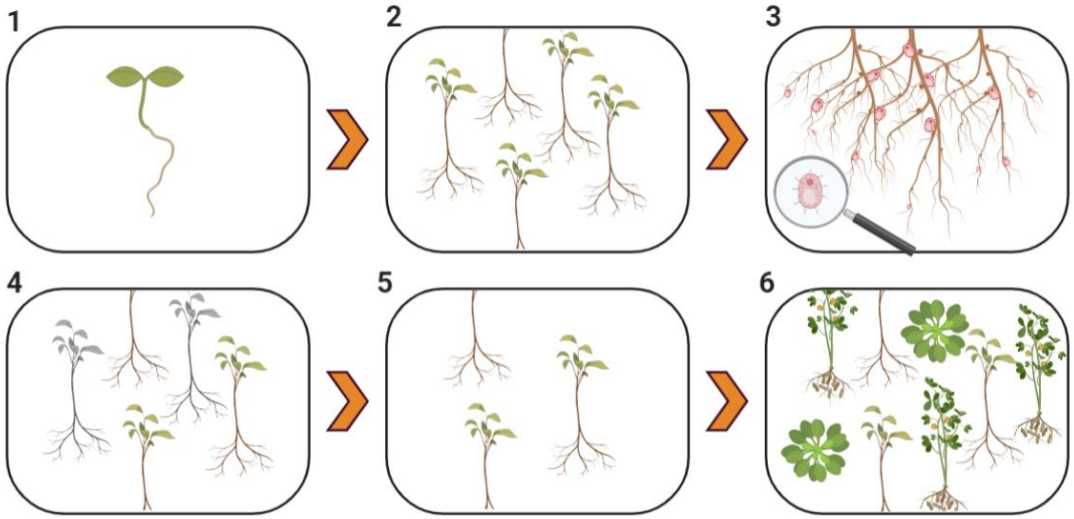


Figure 1.1. Schematic diagram referring to the theory of negative density dependence driven by belowground fungal pathogens. 1) colonization of a juvenile plant species; 2) growth of initial species to high density; 3) accumulation of host specific fungal pathogens; 4) elevated mortality of high density plant populations; 5) death of individuals, space and resources made available to heterospecific juvenile species; 6) growth and establishment of heterospecific plant species- hyperdiverse grassland ecosystem.

1.4 | Community ecology of microbes

Soils sustain an immense diversity of microbes encompassing three significant domains including bacteria, eukaryotes and archaea. Soil microbes are essential to all life on Earth due to their huge diversity in both form and function. It has been estimated one gram of soil contains up to 10^{10}–10^{11} bacteria (Horner-Devine *et al.*, 2003) and 200 million fungal hyphae (Leake *et al.*, 2004). In soils, this complex entanglement of soil microbes plays a major role in regulating key ecological processes, such as nutrient recycling (Kowalchuk & Stephen, 2001), soil formation (Rillig & Mummey, 2006), plant nutrition and more recently, vegetation dynamics (Chapter 1.3). However, due to technical constraints and difficulty in cultivation, soil microbiomes still remain largely unexplored.

The rhizosphere, 'the roots, soil, and everything in between them,' is the most active portion of soil by which microbes influence a host of local, landscape and global scale processes (McNear Jr., 2013). The assembly of rhizosphere microbiota is three fold. I have summarised the role soil plays in the assembly of the rhizosphere based on a review by Philippo*t et al*., (2013), Figure 1.2. The role of soil can be best described as a hierarchy of events. Initially, the soil acts as a reservoir for potential microbes (the microbial seedbank) – the physiochemical properties of the soil such as pore size, nutrient concentration, moisture and temperature will also effect the pool of microbes that occupy each and every site. This stresses microbes are not homogenously distributed in the environment. Secondly, the location of germination and establishment will determine what indigenous microbes the plant roots will be exposed to. During this stage there is potential for a 'maternal effect' in which the biota on or within seeds may also influence the microbes that shape the community occupying the rhizosphere. However, studies regarding the maternal effect remain mixed (Normander & Prosser, 2000) and it is likely only those microbes that grow within seeds influence rhizosphere community assembly by penetrating the root interior to establish on the rhizosphere (Johnston-Monje & Raizada, 2011). Recruitment influenced by microbes native to seeds suggests plant can transfer microbes across generations. Finally, plant species and genotype is the final driving force for rhizosphere community assembly. Plant species differ in their root structural morphology and secretion of quantities and qualities of root exudates, this contributes to the species-specific effect regarding rhizosphere community assembly (Kowalchu*k et al*., 2002) and has key relevance to the underlying assumptions of the 'pathogen hypothesis.' For example, root exudates include polymerised sugar (mucilage), rhizodeposits (utilised as carbon sources by microbes), and secondary metabolites including antimicrobial compounds (Bergsma-Vlami *et al.*, 2005) and flavonoids which are involved in pathogen suppression (Bais *et al.*, 2006). In addition, plant species can influence the rhizosphere across spatial and temporal scales (Dumbrell *et al.*, 2011).

Rhizodeposits can vary at different growth stages, indicative by microbial succession (Van Overbeek & Van Elsas, 2008), and also root development including differences in root zones and movement through the soil profile as the plant grows (Folman *et al.*, 2001). It is this final stage in microbial community assembly that has direct relevance to the 'pathogen hypothesis', i.e. plant rhizospheres accumulate species-specific microbes.

Figure 1.2. Schematic for community assembly of the rhizosphere microbiota based on (Philippot et al, 2013). First, the soil serves as a microbial seedbank, the abiotic properties of the soil, together with largescale biogeographical processes, structure this community. Second, the location where seeds germinate determines which indigenous biota the plant roots are exposed to. Third, plant species and genotype determine which members of the microbial seedbank can grow and proliferate in the rhizosphere.

1.5 | Fungi as pathogens

Whilst there are four main groups of plant pathogens, only fungi (including oomycetes) and nematodes are portrayed as soil-derived pathogens. Whilst bacteria are highly ubiquitous in the soil, non-spore forming bacteria cannot survive long periods in soil and bacterial infection requires penetration through an open wound to cause disease (Raaijmakers et al., 2009). Fungi are the most diverse lineage of

eukaryotes present in total soil biomass and are important biological components in all terrestrial ecosystems. Fungi are key to biological processes such as decomposition, but also constitute an important group of plant pathogens and participate in mutualistic relationships with both heterotrophic and autotrophic organisms (Smith & Read, 2008; Martin *et al*., 2011). Infected plants with soil borne fungi typically exhibit symptoms non-distinct to those associated with abiotic stresses such as drought, stress and nutrient deficiency. Fungi can cause disease through a number of mechanisms operating both pre and post germination including seed decay, dampening of seedling emergence, crown rot and root rot (Raaijmakers *et al.*, 2009). However, soil borne pathogens differ in their dynamics of spread as opposed to their aboveground counterparts. Soil borne pathogens are confined to the soil compartment, with the three dimensional structure of the soil profile, including soil texture, soil particles, the type of organic matter, the living component and finally temperature and moisture determining the spread of fungal through the soil. However, with an estimate of 1.5 million fungal species worldwide, only 6% of fungal species have been studied and described in literature (Hawksworth, 2001), making fungi the least investigated component of biodiversity on Earth. The significance of fungal pathogens is supported by their ubiquitous presence and functional role in plant populations.

The inconspicuous life form of fungi, inaccessibility of their habitats, complex methods of reproduction and our inability to cultivate a large proportion of taxa confounds attempts to study the community ecology of these organisms (Martin *et al.*, 2011). However, modern technological advances have allowed ecologists to overcome these limitations when it comes to studying community assembly of microbes. Early methods of culturing to measure species identity and abundance have been revolutionised in recent years and complex molecular DNA techniques, based on genomic differences, is the common method of practice in such studies. Such

methodological advancements have contributed greatly to our knowledge on the community assembly of fungal species and the functional role these play in terrestrial ecosystems. Fungi as pathogens are earning increasing attention with regards to their role in maintaining plant species abundance and diversity (Mills & Bever, 1998; Maron *et al*., 2011; Bagchi *et al*., 2014). However, knowledge of the identity and specificity of these fungi is limited (Liang *et al.*, 2016).

Despite lack of information on pathogen identity, host specificity of pathogenic fungi has been recognised. Differential responses of plants to pathogenic fungi suggests certain species may be more susceptible to the virulence of certain pathogenic fungi (Mills & Bever, 1998). Packer & Clay (2000) and Schnitzer *et al.*, (2011) also highlight host specificity by demonstrating greater impediment of seedling growth and higher rates of disease when plants were grown in conspecific soil. These host-specific responses form the basis of the Janzen-Connell hypothesis. However, many pathogens have shown their ability to attack multiple hosts when host species demonstrate phylogenetic closeness (Gilbert *et al.*, 2012). Previous studies have however focused on single plant, single pathogen interactions and lack studies that look at overall pathogen diversity. It is possible multi-pathogenic communities may have additive, or interferences, with a functional outcome that differs from a single plant-pathogen relationship (Bradley *et al.*, 2008). This interaction is only something that can fully explored using molecular DNA techniques including bioinformatics pipelines to provide taxonomic assignment.

1.6 | Implications for management, conservation and a more sustainable future.

Addressing the role of soil borne microorganisms, both their beneficial and deleterious roles in PSF, will help to inform management strategies for the conservation of grassland ecosystems. Knowledge of host-specific fungal pathogens

and their phylogenetic host range could help to design optimal seed mixes for the greatest potential species richness (i.e. a rich seed mix consisting of plant species with no known pathogenic overlap). Furthermore, whilst perhaps a distant prospect, there is potential for soils to be 'fertilised' with synthesised soil inoculate containing known fungal-pathogens that can supress superiorly competitive plant species. Careful and planned introductions of select host-specific fungal pathogens could shift low diversity ecosystems to high diversity ecosystems. Moreover, confirmation of host-specificity and developing an understanding of plant-microbial interactions could inform more sustainable agricultural production systems in which beneficial interactions can be exploited. Applying this knowledge to temperate, and even tropical agri-ecosystems, not only has benefits from a biodiversity perspective, but will likely drive improved ecosystem functioning and lessen the reliance on external inputs.

The history regarding agricultural management to suppress, what was assumed to be soil borne pathogens, has benefitted ecologists by opening up the field of ecology regarding plant-pest interactions. However, this has very much come a full circle now, with newly learnt theory developed by years of research into plant-soil-feedbacks, ecologists are now able to drive the management of more sustainable and productive agricultural systems. A lot of this research focusses on the phenomena of increased biomass, known as over-yielding, of plants grown in mixtures compared to those grown in monoculture. Whilst theories regarding resource differentiation have offered limited evidence for over-yielding (Mommer *et al*, 2010), the role of fungal pathogens in driving the diversity-productivity relationship is receiving increased interest. Mommer *et al.*, (2018) provides the first empirical test of the 'pathogen hypothesis' within natural grassland species. Using NGS technology, Mommer demonstrated host-specificity of pathogenic fungal microbiomes associated with roots of grassland plants. However, when plant species were mixed, fungal richness decreased driven by a loss of pathogenic OTUs, this was appropriately described as

a ‘lost in diversity’ effect. This implies the deleterious effects of pathogen accumulation is much stronger in monoculture, where the diversity is low, and the density of the single species is very high. This study has huge scope for the development of more productive and sustainable agri-ecosystems. To address this, a similar study should be repeated in an agricultural field setting.

These findings have huge implications from an agricultural sustainability perspective. Identifying host-specific pathogens of cultivable crops will help to inform more reliable crop rotation schemes and develop potential intercropping strategies both at the species and cultivar level. Evidence would suggest planting crop species that are phylogenetically distant in succession will reduce effects of negative PSF (Gilbert *et al.*, 2012), and as a result crop losses due to fungal pathogens will be reduced. Adopting such strategies will also lessen the reliance on pesticides which not only has economic benefits, but will also reduce deleterious risks associated with the use of heavy herbicide, pesticide and fertilizer inputs. The adoption of intercropping strategies is a relatively new concept in arable agriculture. However, efficient crop production systems could be adopted by selecting plant species with no known shared fungal pathogens. Not only will this reduce overall pathogen accumulation, but it will reduce the transmission of pathogen disease between adjacent plant neighbors. While it is plausible genetically distant crops will not share pathogenic microbes, the profitable and practical implications of intercropping different plant species is questionable. Nonetheless, there is scope to produce genetically similar but distinct cultivars of the same species. Whilst genetic differences in such strategies may be small, these differences might be enough to produce economic and ecologically significant benefits.

1.7 | Molecular methods and ecoinformatic tools

As highlighted throughout, technological advancements in molecular methods have significantly aided the data I was able to produce to address the significant ecological questions within this project. For a comprehensive view of the history of molecular approaches in microbial ecology please see our peer-reviewed paper (Clark *et al.*, 2018).

The journey of microbial community profiling was kick-started by the discovery of DNA polymerases and short strands of nucleotides in *Escherichia coli* by Kleppe *et al*. (1971). Kleppe outlined several features that are hallmarks of current polymerase chain reaction (PCR) technology. However, it took fourteen years for the subsequent development of the Polymerase Chain Reaction (PCR) technique by Mullis *et al* (1986). This technique revolutionised the study of microbial profiling, allowing DNA to be analysed with more sensitivity, accuracy and speed by targeting specific genes within the 'microbial soup'. Simultaneously, molecular advancements were made allowing the characterisation sequence of nucleotides in nucleic acids. The groundwork for sequencing proteins was established by Fred Sanger (Sanger *et al.*, 1965; Brownlee & Sanger, 1967) based on the detection of radiolabelled partial-digestion fragments. However, the key discovery to DNA sequencing came in 1977, with the development of the 'chain-termination' technique using dNTPs (Sanger *et al.*, 1977). This became known as Sanger sequencing and became widely adopted to sequence DNA for many years (Heather & Chain, 2016). Since the 1990's great progress has been made with respect to DNA sequencing, including high throughput, Next-Generation Sequencing (NGS). NGS encompasses a number of high throughput methods such as Illumina and Roche 454 sequencing. NGS differs from previous methods in that it does not determine nucleotide sequences based on radio- or fluorescently-labelled dNTPs. Instead focus was on a newly discovered luminescent method for measuring pyro-phosphate synthesis which relies on the measureable

output of pyrophosphate released during nucleotide incorporation involving a two enzyme reaction (Ronaghi *et al.*, 1996). This method of pyrosequencing is not dissimilar to Sanger sequencing in that both are 'sequence by synthesis' approaches in which DNA polymerase is necessary to produce a discernible output.

Preceding the success of 454 pyrosequencing, a number of 'sequence by synthesis' approaches were developed. Arguably, as one of the most significant methods of sequencing was released in 2006 by Solexa, and then later purchased by Illumina. Adapter DNA molecules are passed over a lawn of complementary oligonucleotides that are bound to the surface of sequencing flowcells; bridge amplification and subsequent solid phase PCR produces localized amplification of single molecules into unique clonal, clusters (Illumina). The original Genome Analyser (GA) was followed by the HiSeq, then MiSeq, with progressively greater read lengths and depths at lower costs (Heather & Chain, 2016). With vast applications including whole genome sequencing and clinical pathology, NGS sequencing has also become an increasingly invaluable tool to answer otherwise intractable questions for molecular ecologists. The development of such culture-independent methods has allowed for sequencing of microbial communities directly from environmental samples, known as 'metagenomics' - what is there? (Bragg & Tyson, 2014). This bypasses the need to isolate and culture microbes, which is often the limiting bottleneck when it comes to exploration of microbial diversity, abundance, and composition.

Taxonomic assignment using open annotation software such as BLAST (Altschul *et al.*, 1990) and functional assignment such as parsing fungal OTUs by ecological guild (Nguyen *et al.*, 2016), makes NGS an increasingly powerful tool for generating novel findings regarding both taxonomic identity and ecological function. Such databases are becoming increasingly richer with increased feasibility associated with lower costs and increased accessibility of metagenomics approaches. Functional

assignment is even more important in a world of ecology where there is a shift towards recognising the key role microorganisms play in ecosystem functioning.

1.8 | Conclusion

One potential reason we observe highly diverse communities is density dependent mortality resulting from host-specific fungal pathogen build up. We can propose fungal pathogen build up via negative PSF contributes to overall local plant rarity as a reduction in localised recruitment, survival and fitness of common species gives advantage to juveniles of other species. Host-specific fungal pathogen loads are also maximized under high host plant densities, eventually resulting in negative feedback acting on abundant plants (Klironomos, 2002). Therefore, diversity is maintained by opposing competitive exclusion from common species, this acts as a stabilising mechanism that reduces the competitive ability of species with a higher ability of utilising resources. Furthermore, in light of the pathogen-hypothesis, differences in grassland diversity could arise from differences in the prevalence of host pathogen specificity, or, a difference in the strength of negatice PSF induced by host specific pathogens. However, neither of these mechanisms have been explored. The knowledge learned from plant-microbial interactions in nature not only has direct relevance to conservation and nature restoration, but might also help to explore scope to incorporate a diversity-microbial perspective to inform more sustainable agri-ecosystems.

1.9 | Thesis rationale

The overall aim of this project is to develop a better understanding of plant-fungal interactions in regulating grassland biodiversity and to test the two assumptions of the pathogen hypothesis. i.e. are fungal pathogens host specific, and is this a function of host density. These insights ‘learned from nature’ in this thesis has direct relevance to nature conservation and also a future of more sustainable agriculture.

This particularly concerns the diversity-productivity relationship and the role microorganisms play in ecosystem functioning. Not only this, but the ability of soils to function sustainably is becoming an increasingly more important field within ecology. With projected trends regarding climate change and increased demand for food crops as our population rises it is paramount we begin to conserve our soils and ensure sustainability is optimised thus demands of future generations are not compromised. Held intrinsically within the theme of soil sustainability is the ability of soils to support a high aboveground diversity, this is important for ecosystem productivity, stability, and resilience to environmental perturbation. Developing an understanding of soil sustainability requires a holistic approach to be adopted. This involves providing a mechanistic basis to conceptualise the multi-trophic three-way triangle between beneficial microbes (i.e. symbionts and saprotrophs), pathogenic microbes (i.e. bacteria and fungi) and antagonistic microbes. Together, the overall outcome of these interactions results in either positive, negative, or neutral PSF for members of conspecific plant species and thus has direct relevance regarding aboveground vegetation dynamics. However, to date, the majority of studies have taken a 'black-box' approach to understand PSF effects, this means the identity of the microbial players remains largely unknown. Nonetheless, widespread uptake of next-generation sequencing has revolutionised our understanding of microbial ecology and the application of this to PSF in soils is pivotal to enhance our understanding of vegetation dynamics. Consequently, the overall aim of this thesis is to understand microbial drivers of vegetation dynamics in natural grassland ecosystem and to test the broad generality of these patterns across various agricultural systems.

1.10 | Thesis structure

- In Chapter 3, to enhance our understanding of plant species coexistence I test for the two underlying assumptions of the 'pathogen hypothesis' in a hyperdiverse natural grassland ecosystem. This includes testing for plant host

specificity within the total and fungal root associated microbiome and whether this is a function plant spatial distribution.

- In Chapter 4, I examine the first assumption of the 'pathogen hypothesis' across a naturally occurring aboveground biodiversity gradient. Here, I explored the root-associated mycobiomes of grassland plants from a low (Norway) and intermediate (UK) grassland ecosystem to test for signals of host-specific plant-fungal interactions.
- In Chapter 5, I examine root-associated and soil-borne fungal and bacterial communities in two agricultural field trials addressing sustainability approaches to be applied both within and between cropping cycles. I first test for within plant species and plant species microbial host-specificity and explore for potential signaling of NDD.
- In Chapter 6, I investigate the soil-borne microbial diversity across an intrinsic aboveground biodiversity gradient implemented within the world's largest agricultural production system, oil palm. Here, I characterize the soil-borne microbial diversity and composition including bacteria, archaea, micro-eukaryotes and fungi. The overall aim of this chapter is to determine the effects of manipulating understory vegetation, serving as a biodiversity gradient, on microbial diversity and discuss subsequent implications this could have for yield and ecosystem functioning.

Chapter 2

General methods

All data chapters in this thesis (Chapter 3 – 6) have used next-generation sequencing (NGS) to profile belowground microbial communities. Outlined here are all methods that are common across all data Chapters, this includes details for library preparation, soil chemistry and bioinformatics. Chapter specific methods are outlined in the methods section of each associated Chapter.

2.1 | Molecular methods

Depending on the chapter, root-associated or soil-borne DNA were extracted for subsequent microbial profiling (Figure 2.1). Prior to root DNA extraction the entire root complex of each dried plant sample was homogenised in 15 ml reinforced tubes with screw caps and o-rings (Fisher, UK) with six stainless steel beads (QIAGEN, UK) using Precellys homogeniser (or 2 ml tubes with two beads). 0.03g of root homogenate was weighed and placed into extraction tubes. For extraction of DNA from soil cores, 0.25 g of fresh weight soil was weighed from a homogenised soil core and placed into extraction tubes on ice. In chapters 3-5, DNA extraction followed the Cetyl Trimethylammonium Bromide protocol (CTAB; Griffiths *et al.*, 2000). The resulting DNA was eluted into 50 ųL of polymerase chain reaction (PCR) grade water (Invitrogen, Waltham, Massachusetts, USA). Extraction of soil-borne DNA in Chapter 6 was performed using the DNAeasy Power Soil kit (Qiagen) according to the user manual (see Chapter 6 for specific details). The abundance of DNA in each environmental sample were quantified using the Quant-iT PicoGreen assay kit (Invitrogen, USA) and each sample was standardised to 1 ng/µL in PCR water to allow for high-throughput automated sample preparation.

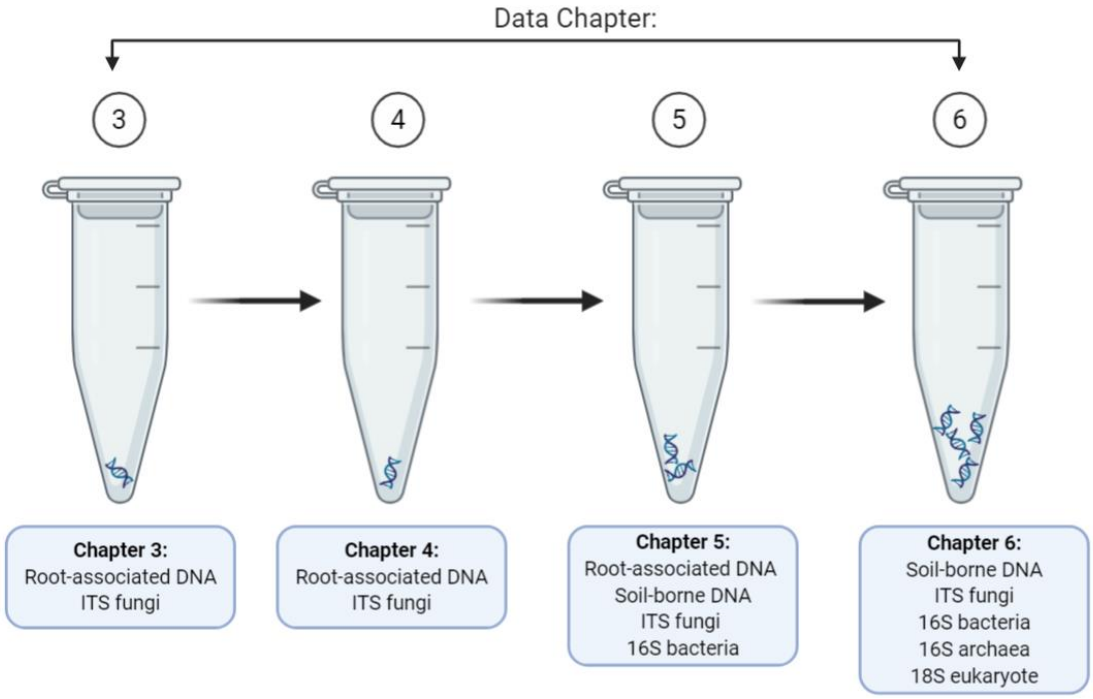


Figure 2.1. Schematic visualising the environmental source material and phylogenetic marker genes of interest explored throughout data chapters 3-6.

2.2 | Targeted amplicon sequencing

Microbial communities were characterised using 2 x 300 bp Miseq amplicon sequencing of the fungal internal transcribed spacer (ITS) region, 16S bacterial and archaeal rRNA genes and eukaryote 18S rRNA gene. Expansive research including a review of literature and laboratory analysis were performed for selection of fungal specific primers. The ITS2 region was chosen for targeted amplification based on enhanced interspecific variability and decreased PCR bias (Toju *et al.*, 2012; Tedersoo *et al.*, 2014; Taylor *et al.*, 2016). This ensures results are reflective of the true fungal richness and diversity from mixed fungal communities. The variant on ITS3, ITS3_KYO2 (Toju *et al*., 2012), was selected specifically as amplification rate was high (~ 95 %) and double banding occurred infrequently. However, due to the variable nature of the ITS region and non-specific PCR amplification (amplification of plant material), double banding was not entirely preventable. The 16S rRNA primers were chosen for their universal coverage of bacteria and archaeal communities. Similarly, 18S rRNA primers were chosen to cover a wide range of micro-eukaryote organisms, spanning multiple trophic levels. Libraries for each target gene were prepared separately using Illumina's Nextera™ XT Library Prep Kit (Illumina, Cambridge, UK) following the associated protocol 16S Metagenomic Sequencing Library Preparation Protocol (Illumina, 2013) and the primer pairs in Table 2.1. All primers were modified to include forward and reverse Illumina overhang adapters.

Table 2.1. Sequences of PCR primers for phylogenetic marker genes.

Reference	Target gene		Sequence
ITS3_KYO2 (Toju *et al*., 2012)	ITS Fungi	Forward primer	ITS3_KYO2 5' -GATGAAGAACGYAGYRAA - 3'
ITS4 (White *et al.*, 1990)		Reverse primer	ITS4 5' -TCCTCCGCTTATTGATATGC - 3'
Klindworth et al., (2013)	16S Bacteria	Forward primer	341F 5' - CCTACGGGNGGCWGCAG - 3'
Herlemann et al., (2011)		Reverse primer	805R 5' - GACTACHVGGGTATCTAATCC - 3'
Chapelle (2002)	16S Archaea	Forward primer	344F 5' - ACGGGGYGCAGCAGGCGCGA - 3'
Ohene-Adjei et al., (2007)		Reverse primer	915R 5' - GTGCTCCCCCGCCAATTCCT - 3'
Hugerth et al., (2014)	18S Eukaryote	Forward primer	574F 5' - CGGTAAYTCCAGCTCYV - 3'
Hugerth et al., (2014)		Reverse primer	1132R 5' - CCGTCAATTHCTTYAART - 3'
Illumina	Adaptors	Forward primer	5' – TCGTCGGCAGCGTCAGATGTGTATAAGAG ACAG – 3'
Illumina		Reverse primer	5' – GTCTCGTGGGCTCGGAGATGTGTATAAGA GACAG – 3'

PCR reactions were robotically processed via the Hamilton Microlab STAR Line Automated Liquid Handling workstation. This allowed for high-throughput DNA metabarcoding for a large number of samples. DNA was amplified by PCR using an Applied Biosystems Veriti 96-well thermal cycler. First stage PCRs were carried out in a 25 µl reaction volume. PCR conditions targeting the fungal ITS2 region were performed with 2 µl of DNA template, 12.5 µl Taq, 1 µl of each forward and reverse primer (4 µmol), 8 µl PCR grade water, 0.05 µl of 10 mg/ml T4 gene 32 (New England BioLabs). T4 gene 32 protein was added to all ITS targeted PCR reactions to promote removal of humic acid inhibitors. PCR conditions targeting 16S rRNA associated genes were performed using 2 ul of DNA template, 1 µl of each forward and reverse primer (4 µmol), 12.5 µl Taq (Appleton Woods Ltd, Birmingham, UK), and 8 µl PCR grade water (Invitrogen). PCR reactions for amplification of the 18S rRNA gene were carried out using 2 µl of DNA template, 1.9 µl of each forward and reverse primer (4 µmol), 12.5 µl KAPA HiFi HotStart DNA Polymerase ReadyMix (KAPA Biosystems) and 6.7 µl PCR grade water. The remainder of the Illumina NGS library preparation followed the Nextera™ XT Library Prep Kit protocol (Illumina, 2013). PCR conditions used throughout the thesis are outlined below in Figure 2.2.

After the final elongation, PCR products were stored at 4 °C. PCR products were purified using Agencourt AMPure XP PCR Purification beads (Beakman Coulter Ltd), following the manufacturer's instructions. To allow for multiplexing, Nextera XT indices (Illumina) were attached to purified PCR products through a short 8 cycle PCR reaction. The PCR were performed using 2.5 µl of the purified PCR product, 2.5 µl of Nextera i5 and i7 index, 12.5 µl of appTAQ RedMix and 5 µl of PCR water (Invitrogen). PCR conditions were as follows: initial denaturation at 95 °C for 3 min, followed by 8 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and a final extension step at 72 °C for 5 min. PCR products were purified as previously described. Purified PCR products were visualized on either 1.5 % agarose gel stained with SYBR Safe (Invitrogen, US) or 2 % 96 well E.Gel (Invitrogen, US). PCR products were quantified

using the Quant-iT dsDNA assay kit (Thermo Fisher Scientific Inc. USA) on a POLAR star Omega plate reader (BMG LABTECH GmbH, Germany). Samples for each target gene were pooled in equimolar concentration by normalizing to the highest sample and the concentration of the final library was determined using a NEBNEXT® Library Quant Kit for Illumina® (New England Biolabs). Final pooled target gene libraries were again quantified using NEBNEXT® Library Quant Kit for Illumina® (NEB) and were pooled in specific ratios in the case of mixed gene libraries (see data chapters for specific details). The final library was quality checked using a DNA 1000 kit on at 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). Next generation amplicon sequencing was conducted on an Illumina MiSeq platform (Illumina, 2013) at the University of Essex (UK) following dilution of libraries to 4nM.

ITS rRNA Fungi	**16S rRNA Bacteria**	**16S rRNA Archaea**	**18S rRNA Eukaryotes**
95 °C - 3 min	95 °C - 3 min	94 °C - 3 min	95 °C - 5 min
94 °C - 30s	95 °C - 15s	94 °C - 15s	95 °C - 45s
53 °C - 30s	55 °C - 15s	59 °C - 15s	53 °C - 15s
72 °C – 1 min	72 °C – 30s	72 °C – 30s	72 °C – 45s
72 °C - 7 min	72 °C - 7 min	72 °C - 10 min	72 °C - 10 min
x 35	x 25	x 30	x 28

Figure 2.2. First stage amplicon PCR conditions utilised throughout each data chapter. Note, not all four genes were targeted within each data chapter, see Figure 2.1 for a summary of targeted amplicon libraries for each data chapter.

2.3 | Bioinformatics

Overall sequencing quality was visualised using Illumina Sequencing Analysis Viewer. Sequence reads were processed as described by (Dumbrell *et al.*, 2017), accessed through BioLinux. Raw reads were quality trimmed using the software Sickle (Joshi & Fass, 2011) and error correction was performed using BayesHammer (*Nikolenko et al.*, 2013) implemented with default settings in SPAdes v3.7.1 (Nurk *et al.*, 2013). Forward and reverse reads were pair-end joined using the Pear algorithm (Zhang *et al.*, 2014) implemented in PANDAseq version 1.33 (Masella *et al.*, 2012). OTUs were de-replicated, sorted by abundance and clustered at the 97 % similarity level using the VSEARCH algorithm version 2.1.2 (Rognes *et al.*, 2016) and UCHIME (Edgar et al., 2011) was used was used to detect and remove any chimeras. Taxonomy was assigned using Naïve Bayesian Classifier RDP classifier (Wang et al., 2007) , against the RDP database for 16S bacteria and archaea, UNITE for fungi (Kõljalg *et al.*, 2013) and PR2 for 18S eukaryotes (Guillou *et al.*, 2013). Regarding fungal sequences, taxonomic annotation for unassigned OTUs was performed using blastn version 2.8.1 *(Zhang et al*, 2000) against NCBI nucleotide database (Morgulis *et al.*, 2008). Trophic mode and a confidence ranking describing this assignment were identified for each OTU utilising the FUNGuild database. Data tables were produced including details of OTU abundance per sample, the taxonomic classification and functional guild (for fungal ITS OTUs only) for each OTU. Separate data tables were produced to include all environmental data such as sampling site and soil variables.

2.4 | Soil chemistry

2.4.1 | Preparation

Each soil core was weighed and oven dried at 80 ^{o}C to remove moisture, percentage moisture was recorded. Large inorganic particulate was removed using a 2 mm sieve (these are removed with little concern due to their lack of contribution to TOC and their chemical inertness). A subsample of each dried core was homogenised in 15 ml reinforced tubes (Fisher, UK) with six 3 mm tungsten beads (QIAGEN, UK) using Precellys homogeniser (Bertin Technologies, France). Homogenised samples were stored at 4 ^{o}C in an airtight bag until further processing.

2.4.2 | pH

2 g of homogenised soil was mixed with 10 ml of deionised water (1:5 soil to water suspension), vortexed for 30 seconds and incubated at room temperature for two hours. Prior to pH measurements, the pH meter was calibrated using buffers at pH4, pH6 and pH8, subsequently, pH was taken according to the user manual.

2.4.3 | Nutrient analysis

Aqueous soil extracts were prepared by the extraction of a 1:10 (soil to deionized water) in an ultrasonic water bath for 30 minutes followed by ~ 15 mins of centrifugation at 3000 rpm until a clear supernatant was obtained. The supernatant was filtered through a 0.2 um PES syringe filter (Phenomenex, US), frozen and stored until analysis. Concentrations of cations and anions were determined by an Ion Chromatography System Dionex ICS-3000 RFIC. Anions were determined using a 0 – 30 mM gradient program (using KOH to get the acquired molarity) separated on a AG18 guard column with a 2 x 250 mm ionpac AS18 anion column with injection loads from a 10 ul loop. Cations were determined on the same instrument using 20 mM

methanesulphonic acid (no gradient) separated on a CG12A guard with a 4 x 250 mm CS12A CS18 cation separating column.

2.4.4 | Total carbon

Total Organic Carbon (TC) and Inorganic Carbon (IC) were determined on a SKARLAR Primacs^{MCS} Solid Sample TOC module (Skalar Analytical, Netherlands). Standard curves were constructed using $C_6H_{12}O_6$ (40 % carbon) and $CaCO_3$ (12 % carbon) as the organic carbon source and inorganic carbon source respectively (R^2 = 0.998). A representative subsample of each dried and homogenised soil core was oven dried at 80 ^oC overnight to ensure the removal of any moisture. For TOC, ~ 0.05 g (exact weight for each sample recorded) of dry soil was heated to 1000 ^oC in the presence of pure oxygen to ensure complete combustion. For IC, ~ 0.5 g of dry soil was acidified with 1 ml of 10% HCl, IC is calculated as a measurement of the increase in pressure from the production of CO_2. Total Organic Carbon (TOC) = TC – IC. It was assumed Total Carbon (TC) = Inorganic Carbon (IC) + Organic Carbon (OC), but only trace amounts of IC were recorded for a subset of samples for each data Chapter, therefore I assumed; Total Carbon (TC) = Organic Carbon (OC).

Chapter 3

Evidence for fungal host-specificity and NDD from a natural hyperdiverse grassland ecosystem.

3.1 | Abstract

Understanding how species coexist within diverse plant communities is a central aim of ecology attracting renewed interest given increasing rates of biodiversity loss and the need to sustainably manage natural systems. Although soil fungi have been putatively implied as important regulators of plant diversity, relatively little is known about plant-fungi interactions belowground, despite the fundamental ecological importance and multiple practical implications (e.g. agriculture and conservation) this information would provide. In this study, I utilised next-generation sequencing techniques to explore the two underlying assumptions of the 'pathogen hypothesis' from three natural hyperdiverse grassland ecosystems located in Slovenia. Here I show that root-associated fungal communities are host specific across all plant taxonomic ranks and that there is a positive correlation between plant spatial distribution and pathogenic diversity (NDD). Further exploration of the pathogenic fungal community revealed a strong phylogenetic host signal in which pathogenic fungal communities were driven by plant functional group (i.e. grasses versus forbs). Moreover, species of grasses that all belong to a single family shared pathogenic fungal communities whilst families within forbs were compositionally distinct. These results imply disease risk and transmission is a function of plant group that share overlap between their pathogenic consortia and as such, grasses and forbs may differ in the strength of negative PSF in which they endure. The positive correlation between plant spatial aggregation and pathogenic diversity indicates pathogens are richer when the local density of any one plant species is high, this is in align with Janzen-Connell type effects and NDD driven coexistence in which dominant plants progressively make

their environment less suitable for members of their own species. This study advocates the incorporation of a plant-microbial perspective into co-existence theory. The concept of learning from nature adopted in this observational study may have important implications for restoration management and the development of more sustainable agri-ecosystems.

3.2 | Introduction

Temperate grasslands alone occupy 8 % of the Earth's surface, occurring on almost every continent (White *et al.*, 2000) and are a reservoir of biodiversity. This makes grassland ecosystems of great importance value to both wildlife and human resources through the ecosystem services that they provide (Tilman *et al.*, 1996). Unfortunately, temperate grasslands are the most altered, but least protected ecosystems on Earth (Hoekstra *et al*., 2005). As such, it is important we begin understand ecological theories that underpin biodiversity so we can begin to conserve and maintain the extant biodiversity value across the many bioregions in the world that grasslands occupy. Despite lack of support for niche separation (Mommer *et al*, 2010), frequently the most explored theory regarding species coexistence, these biomes commonly demonstrate very high small-scale species diversity and seemingly defy the principles of competitive exclusion (Pärtel *et al.*, 1996; Wilson *et al.*, 2012). As such, research in plant ecology is underpinned by understanding the fundamental challenge of stable co-existence. Yet explaining how numerous species co-exist in the same ecological space and time despite differences in fitness abilities, remains unanswered.

Multiple theories have previously been described to explore this phenomena (Chapter 1.3). The majority of studies to date have had an aboveground bias but more recent studies suggest belowground microbial interactions have been underestimated as key drivers of stable plant co-existence (van dver Putten, 2017). This alternative hypothesis for species co-existence is proposed to operate through microbial regulated negative PSF (Mills & Bever, 1998; Petermann *et al.*, 2008; Maron *et al.*, 2011). More specifically, the pathogenic constituents of the root mycobiome (root associated fungal biota) have been recognised as the fundamental microbes driving negative PSF and its subsequent role in vegetation dynamics (Olff *et al.*, 2000). There are two key reasons why soil-borne microbes, particularly those that are pathogenic,

are underexplored in natural grasslands. First, research on plant pathogens typically focusses on aboveground pathogens with conspicuous phenotypic effects on plant health. Second, current research on soil-borne pathogens focusses on taxa relevant to agriculture because they hold a higher economic value. This may be one reason that previous theories have had limited success in explaining the co-existence of competing plant species.

The fundamental aim in plant coexistence theory is to explain why competitive exclusion does not occur despite huge fitness differences in competing plant species, this involves an understanding of how competitor traits can maintain diversity (Bever et al., 2010). Petermann *et al.*, (2008) proposes that such negative PSF effects could be a type of Janzen-Connell effect (Janzen, 1970; Connell, 1971) with the capacity to promote species coexistence by preventing superior competitors repeatedly occupying the same site. The theory of the Janzen-Connell hypothesis, commonly applied to explain species rich ecosystems in the tropics (Bagchi *et al*., 2014; Liu *et al*, 2015; Liang *et al*., 2016), is analogous to the more modern term, the 'pathogen hypothesis', which is receiving increasing recognition with respect to grassland diversity (Mommer *et al.*, 2018; Ampt *et al.*, 2019). The two assumptions of the 'pathogen hypothesis' are host specificity and NDD (see 1.3.3). Host specificity of fungal pathogens is a requisite, because if absent, there will be no differential effects of PSF and thus will have no overall effect on plant species diversity or composition. Secondly, if pathogens attack irrespective of plant density the net effect on plant diversity will be relative small. This is very similar to a study of rainforest diversity in which specific fungal pathogens and insectivores had an overall significant effect on diversity and composition (Bagchi *et al.*, 2014a). However, insectivores attacked irrespective of density and therefore their contribution to overall plant diversity was low.

Historically, exploration of plant-microbe interactions in grassland ecosystems have taken a 'black box' approach. Mills & Bever (1998), Packer & Clay (2000), and Klironomos (2002) have recorded plant performance and compared plant species grown on intact 'home' soils and those grown on 'away' soils which assumedly harbour a shift in microbial composition. Collectively, these studies reveal a reduction in plant biomass when plants are grown on 'home' soils. Similarly, sterilisation of 'home' soils with fungicide also revealed complete removal of NDD (Petermann *et al.*, 2008) and soil sterilisation at low plant diversity revealed the greatest increase in biomass (Schnitzer *et al.*, 2011). These studies point towards, but have no means to confirm microbial host specificity and NDD. Coupled with technological advancement and enhanced interest, more recent work has taken the biggest stepping stone with regards to identifying the main 'players' in PSF and opening the 'black-box' approach. Mommer et al., (2018) arguably provides the strongest evidence to suggest fungal pathogens play a pivotal role in vegetation dynamics. Mommer utilises next-generation sequencing techniques to determine the taxonomic and functional identity of soil borne fungal communities from a ten-year temperate grassland biodiversity experiment (Ruijven & Berendse, 2009). Data showed plant species were colonised by distinct fungal pathogen communities (inferring host specificity) and importantly, 57 % of pathogens were lost when plant species were mixed. In align with Janzen-Connell theory and the 'pathogen hypothesis' this implies pathogen pressure is reduced at low plant densities (high diversity ecosystems) and as such more diverse ecosystems are typically more productive.

So far, as terrestrial ecologists, we have empirical evidence of PSF through plant growth bioassays and have begun to explore the taxonomy of the fungal players involved through next-generation sequencing. Studies to date have predominantly taken an experimental approach but the exact identity of potential host-specific mycobiomes in late successional natural field settings has not yet been explored. To understand how plant diversity can dampen transmission and accumulation of soil-

borne fungal pathogens, we must first confirm host specificity and NDD in natural ecosystems and use advanced molecular tools to taxonomically identify the microbial players involved. Such a molecular level approach not only has applications from a diversity-productivity viewpoint, but will also undoubtedly help to inform practices for conservation management.

In this study, I concentrated on root-associated fungi as these were hypothesised to host the main pathogenic constituents involved in structuring plant communities. Utilising next-generation sequencing approaches I determined the taxonomic and functional identity of root-associated fungal communities of 19 temperate plant species across three species rich natural field sites in Slovenia. These are considered 'late-successional' grasslands and have been untouched by external inputs for decades. These responsive grasslands make an optimum study site to infer potential legacy effects from years of soil conditioning by soil microbes. Spatial data of above-ground plant distribution was taken into account as it is known host plant density shares a relationship with fungal community composition. Edaphic properties were also quantified as nutrient availability is a key factor influencing soil fertility and with that the associated microbial constituents (Wardle *et al.*, 2004). The aim of this study is to identify potential drivers of temperate grassland plant diversity and the role they play in structuring plant community composition and, as such, their ability to maintain or promote diversity.

The following hypotheses were tested:

H1: Different plant species accumulate species-specific fungal microbiota (total and pathogenic) communities under natural field conditions. This confers host specificity.

H2: Functional groups of plants (grass and forbs) accumulate different species-specific fungal microbiota.

H3: Plant distribution and frequency is a function of belowground fungal microbiota and is therefore a reflection of NDD type effects.

The information learned from this study will widen our knowledge and facilitate our understanding in the microbial ecology of the rhizosphere associated with non-cultivated plant species in natural ecosystems. This knowledge is directly relevant to conservation management and will develop insights that might help to inform more sustainable agri-ecosystems.

3.3 | Methods

3.3.1 | Study site

Fieldwork was undertaken at three natural grassland areas in the Karst region of Slovenia (shallow soil, rocky, carbonate bed rock, neutral pH). The Karst region is a limestone plateau extending across the border of south-western Slovenia and north-eastern Italy (Figure 3.1). These grasslands are highly diverse with upto 80 vascular species plant species identified across the three sampling plots. This makes it an ideal place to study the role of fungal pathogens in negative PSF through evaluating host specific fungal relationships and characterising how this varies with aboveground plant distribution.

3.3.2 | Sampling

In each ~ 1 hectare sampling site, five singular plants of each plant species were randomly selected and carefully extracted from the soil at each site (roots intact), giving a total of 15 plant species replicates across sampling sites (sampling period; 13th-15th June 2016). Roots from each plant were washed using tap water to remove all debris and organic matter and dried before storing. Plant spatial data and cores for belowground soil analysis were collected using 50 randomly placed quadrats in approximately 1 hectare sampling area per site. Each quadrat (0.25 m^2, gridded into 25 cells) were placed at random and the presence and absence of each plant species were recorded in each cell. Soil cores (diameter 5 cm, depth 10 cm) were taken from the top left, bottom left and bottom right corners (sampling period; 2nd-13th October 2017). Soil corers were sterilised with 70 % ethanol between samples. Roots and soil cores were dried at 60°C until a constant weight was achieved to ensure removal of all moisture. This method of drying for the preservation and long-term storage of DNA is advocated by Clark & Hirsch, (2008) and Wang *et al.*, (2017). Samples were transported at room temperature in sterile and airtight plastic bags to the University of

Essex, UK, for molecular processing. A total of 317 root samples covering 29 grassland species were sampled, plant species were chosen for sampling based on their presence across all three sampling sites to ensure true biological replication. These included both grasses and flowering plants (hereafter referred to as 'forbs'), the genus' of which are particularly common across temperate grasslands in Europe.

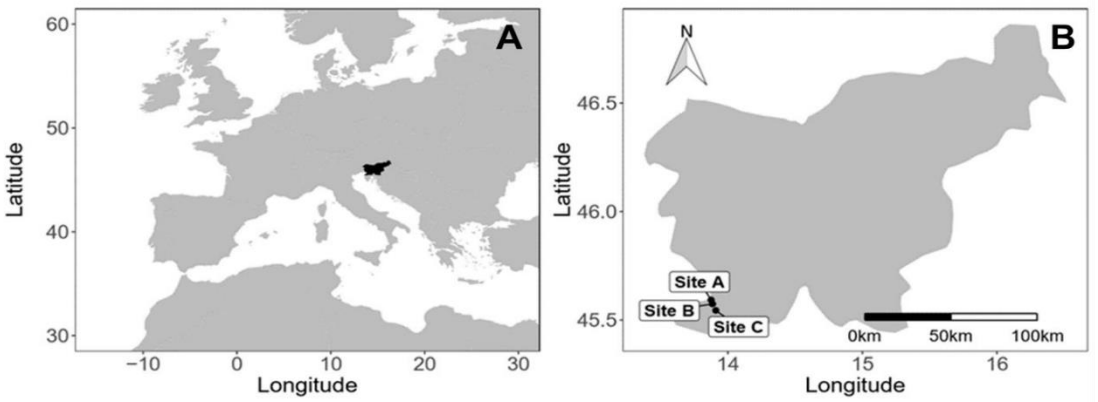


Figure 3.1. Location of sampling sites. A) Location of Slovenia in Europe. B) Magnified view of Slovenia showing location of each sampling site (Site A: 45°35'33.90"N, 13°52'29.89"E. Site B: 45°34'26.35"N, 13°53'7.26"E. Site C: 45°32'39.20"N, 13°54'35.85"E).

3.3.3 | Root DNA Extraction

DNA was extracted from the entire root complex of each plant sample following the Cetyl Trimethylammonium Bromide protocol (CTAB; Griffiths *et al.*, 2000). Details for DNA extraction are outlined in the general methods.

3.3.4 | Molecular analysis

Root associated fungal communities were characterised using 2 x 300 bp Miseq amplicon sequencing of ~ 350 bp fragment targeting the ITS2 region of the Small Subunit (SSU). Library preparation methods are outlined in the general methods (Chapter 2). Sequencing was conducted at The University of Essex on an Illumina Miseq (providing 2 x 300 bp sequences).

3.2.5 | Soil chemistry

All edaphic properties from each soil core were measured as outlined in the general methods.

3.2.6 | Statistical analysis

Bioinformatic analyses were conducted using the methods outlined in the general methods (Chapter 2). Samples were removed from the OTU table that were not replicated across the three sampling sites, this left 19 plant species from 267 root samples. Those samples with > 3 replicates within each biological site were included in the analyses. The OTU table was filtered to remove negative controls, after confirmation that contamination was negligible, non-target amplicons (i.e. plant material belonging to the phylum Streptophyta and Chlorophyta; 813 OTUs) and low abundance OTUs (< 3) as these are more likely to be non-biological (Flynn *et al.*, 2015). Data was pooled by site for each plant species to account for random effects caused by within site differences. Further community OTU subsets were created for multiple fungal phyla and functional groups of fungi (i.e. potential plant pathogens,

saprotrophs and symbiotrophs). Frequency and Variance:Mean Ratios (VMR) were calculated for each plant species from aboveground spatial data (hereafter referred to as 'frequency' and 'VMR' respectively). Plant frequency was determined by calculating the number of times each plant was present in each of the 25 cells of a gridded quadrat and converted to an overall percentage. An average was taken of the frequency across all three sampling sites. Variance was calculated by first obtaining the mean number of times each plant was present in each of the 25 cells of a gridded quadrat, from this standard deviation was calculated and subsequently squared to obtain the variance. The VMR gives a measure of the dispersion index of each plant species above ground (< 1 = uniform; 1 = random; > 2 clumped) respectively.

Linear-models (LMs) were used to determine any differences in abiotic factors between sampling sites. To determine if plant species (including plant order, genus, family and plant functional group), frequency, or VMR had an effect on fungal OTU richness a linear-model (LM) or negative binomial generalised model (GLM) within the package 'MASS' (Ripley, 2011) was implemented. A negative binomial GLM was used to account for over dispersion of OTU richness, a common feature of count data. Differences in library size were controlled for by including log(number of sequences) as the first (fixed) term in the model. This approach to dealing with differences in sequencing depth is encouraged by Warton *et al.*, (2015) as it avoids many of the unfavourable aspects introduced by alternatives such as rarefraction (McMurdie & Holmes, 2014). This is particularly important within the context of this study as rarefraction frequently removed biologically important pathogenic taxa. The effects of plant species, frequency and VMR on community diversity indices (diversity and evenness) were explored using linear models with a Gaussian distribution. Community diversity and evenness were quantified using Shannon's diversity and Simpson's evenness index respectively (see Morris et al., 2014). All community evenness values were logit-transformed to meet linear modelling assumptions (Warton & Hui, 2011).

For the purpose of visualisation, small sample sizes were removed and data were rarefied to the smallest library size prior to producing figures. The total fungal community, Ascomycota, Basidiomycota and all fungal guilds were rarefied to 1000 reads per sample for compositional analysis, whilst the remaining fungal subsets were rarefied to 100 OTUs per sample due to the lower relative abundance within each sample. Differences in overall fungal community composition performed on rarefied data and were visualized using nonmetric multidimensional scaling (NMDS) on Bray–Curtis distances. PERMANOVA was performed to determine how much variance in fungal community clustering can be explained by plant species, plant order and plant functional group (permutations = 10,000).

To investigate how the abundance of OTUs changed between plant functional group multivariate generalized linear models (MV-GLMs) were used (Wang *et al*., 2012). A negative binomial mean-variance error family was used to account for over dispersion, a common property of count data. Heterogeneity in library size was accounted for by incorporating an offset term as the first fixed term in the model. Multivariate and unadjusted univariate P values were obtained by Wald tests, both using 10,000 Monte Carlo permutations.

All statistical, diversity and community analyses were conducted using the R statistical language version 4.0.2 with standard R libraries (R Core Team, 2020) ; the community analysis specific package “vegan” (Oksanen *et al*., 2018), “MASS” (Ripley, 2011), “mvabund” (Wang *et al.*, 2012) and ‘ggplot2’ (Wickham, 2009).

3.4 | Results

3.4.1 | Soil variables

The following soil variables were significantly different between sites: moisture (Table 3.1. LM; $F_{2,142} = 5.94$, $P < 0.05$) and total carbon (Table 3.2. LM; $F_{2,142} = 4.16$, $P < 0.05$); cations sodium (Table 3.2. LM; $F_{2,143} = 4.87$, $P < 0.01$) and ammonium (Table 3.2. LM; $F_{2,143} = 12.69$, $P < 0.001$); and anions butyrate (Table 3.3. LM; $F_{2,143} = 20.71$, $P < 0.001$), formate (Table 3.3. LM; $F_{2,141} = 7.14$, $P < 0.01$) and acetate (Table 3.3. LM; $F_{2,143} = 3.17$, $P < 0.05$).

Table 3.1. Mean and standard error of pH, moisture and total carbon across three sampling sites in Slovenia. Significant differences between sites are highlighted in the column header by an asterisk (* <0.05; ** <0.01, *** <0.001).

Site		**pH**	**Moisture (%) ****	**Total Carbon (mg/g) ***
A	Mean	6.48	24.73	97.39
	SE	0.07	0.33	4.59
B	Mean	6.56	29.37	115.23
	SE	0.07	1.44	5.28
C	Mean	6.10	28.19	107.60
	SE	0.07	0.73	2.97

Table 3.2. Mean and standard error of cations across three sampling sites in Slovenia. Significant differences between sites are highlighted in the column header by an asterisk (* <0.05; ** <0.01, *** <0.001).

Cations (umol/l)						
Site		**Na ***	**NH_4^+ *****	**K**	**Mg**	**Ca**
A	Mean	62.60	66.93	588.91	83.02	573.88
	SE	7.20	7.86	227.28	8.89	62.63
B	Mean	105.23	81.11	692.28	90.27	675.88
	SE	24.11	8.52	274.47	11.00	75.62
C	Mean	164.62	31.57	484.14	72.26	531.48
	SE	33.04	4.13	193.94	9.23	80.58

Table 3.3. Mean and standard error of anions across three sampling sites in Slovenia. Significant differences between sites are highlighted in the column header by an asterisk (* <0.05; ** <0.01, *** <0.001).

Anions (umol/l)							
Site		**P**	**SO_4^{2-}**	**NO_3^-**	**NO_2^-**	**Cl-**	**Butyrate *** **
A	Mean	10.76	21.30	10.86	18.27	613.40	3.50
	SE	9.92	1.63	2.71	2.86	295.50	0.53
B	Mean	20.02	22.13	9.69	18.58	1025.12	4.57
	SE	12.09	1.23	1.63	3.78	418.87	0.44
C	Mean	22.60	18.33	15.68	10.09	1407.44	0.90
	SE	14.64	1.46	3.06	2.36	432.13	0.17
Site		**Propionate**	**Formate ** **	**Lactate**	**Acetate * **	**Fluoride**	
A	Mean	0.78	19.16	10.49	121.55	0.78	
	SE	0.26	6.05	3.55	21.49	0.47	
B	Mean	0.99	44.73	16.97	141.38	1.10	
	SE	0.31	10.54	5.99	21.89	0.51	
C	Mean	0.41	6.40	3.39	75.00	0.23	
	SE	0.10	2.96	1.49	12.99	0.07	

3.4.2 | Root-associated fungi

3.4.2.1 | *Summary*

The analysis of 267 root samples across the three biological sites yielded 2232 OTUs. The Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota, and Zygomycota make up 60.54, 31.29, 0.78, 0.79 and 0.51 % of all fungal OTUs from all samples, respectively. Taxonomic annotation for 'unassigned fungi' was performed using blastn, version 2.8.1 (Zhang *et al.*, 2000). Any sequences that returned plant lineages i.e. Streptophyta and Chlorophyta were removed from the analysis (813 OTUs). Subsequently, 6.08 % of fungal OTUs were unable to be assigned a taxonomic classification to the level of phyla. In total, 1520 (68.1 %) fungal OTUs were assigned a functional guild. In this study, there is strong focus on plant pathogens, in particular, root associated pathogens. In total, 217 OTUs were characterised as pathotrophs only whilst 691 OTUS were deemed to have mixed trophic modes (i.e. Pathotroph - Saprotroph, Symbiotroph) – of all potential pathotrophs, 372 OTUs were described as potential plant pathogens, this contributes to approximately 15% of the full data set. In addition, 596 OTUs were characterised as potential symbiotrophs (241 symbiotrophs only). The most abundant ten OTUs represented a total of 31.14% of all detected ITS fungal sequences. Among these, OTU.16, OTU.25 and OTU.15 are the three most abundant fungal OTUs across all sites, belonging to the phyla Basidiomycota (species; *Mycena albidolilacea*), unidentified fungal sp., and Ascomycota *(*species; *Anthostomella sepelibilis)* respectively.

3.4.3 | Alpha-diversity

3.4.3.1 | Total fungal community

Total root-associated fungal community richness (Figure 3.2. GLM; $LR_{37,55} = 29.38$, $P < 0.05$) significantly differed across plant species but diversity (Figure 3.2. LM; $F_{37,55} = 1.07$, $P = 0.41$) and evenness did not (Figure 3.2. LM; $F_{37,55} = 1.47$, $P = 0.16$). Basidiomycota and Chytridiomycota were the only fungal phyla to differ between plant species (Table 3.4).

Fungal OTU richness was not related to plant functional group i.e. forbs and grasses (GLM; $F_{54,55} = 0.01$, $P = 0.94$) but diversity and evenness were significantly different between forbs and grasses (diversity LM; $F_{54,55} = 4.33$, $P < 0.05$, evenness LM; $F_{54,55} = 7.29$, $P < 0.01$). Overall, the total richness of root-associated fungi were significantly highest in grasses.

Despite significant differences in total fungal richness across plant species, alpha-diversity measures were not a function of average plant frequency (richness GLM; $LR_{54,55} = 1.11$, $P = 0.29$, diversity LM; $F_{55,56} = 0.02$, $P = 0.88$, evenness LM; $F_{54,55} = 0.42$, $P = 0.52$) or V:M ratio (richness GLM; $LR_{54,55} = 0.2$, $P = 0.65$, diversity LM; $F_{54,55} = 2.66$, $P = 0.11$, evenness LM; $F_{54,55} = 3.31$, $P = 0.07$) of each plant species.

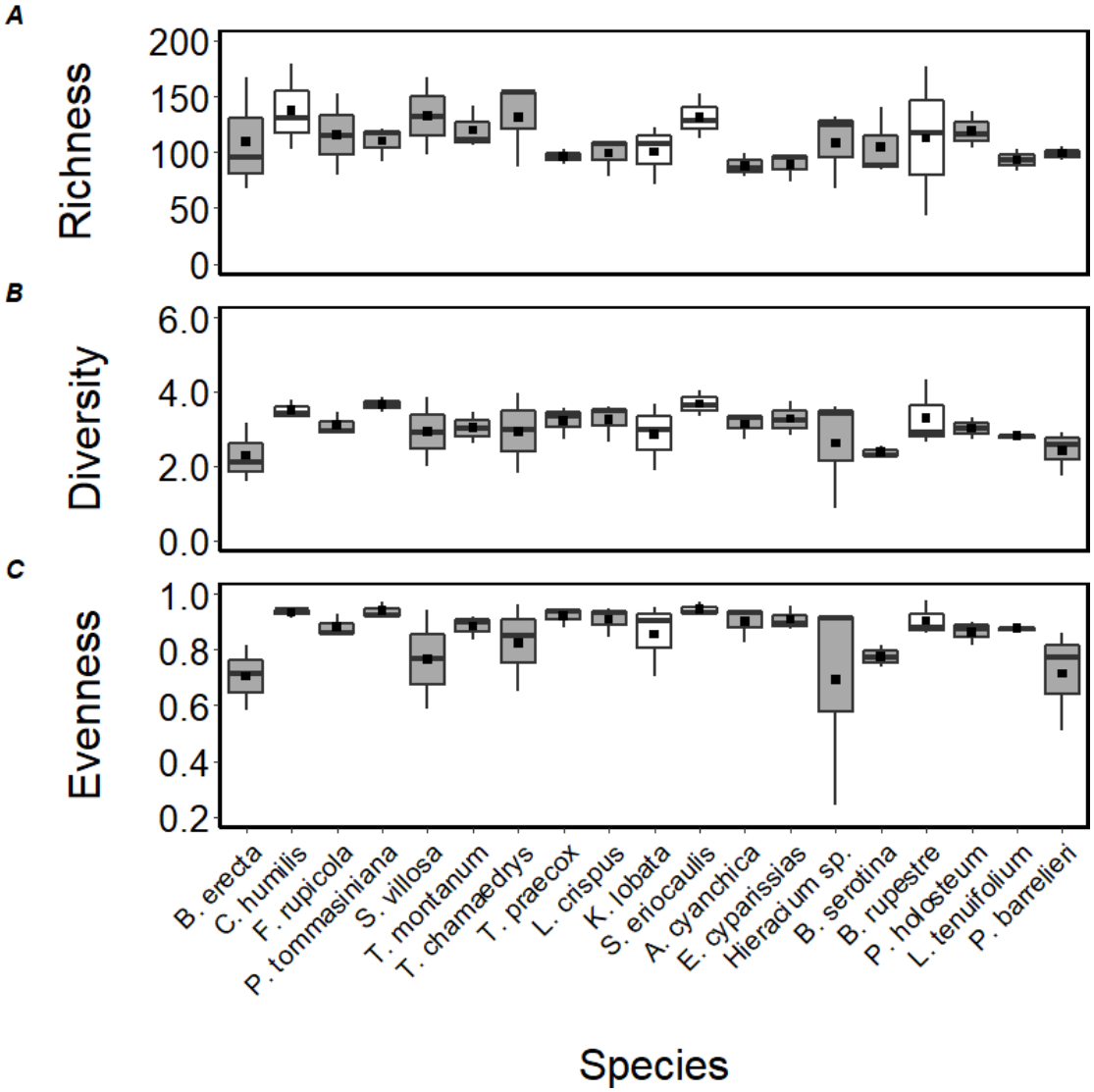


Figure 3.2. Total fungal; A) OTU richness; B) OTU Shannon's Diversity Index; C) OTU Simpson's Diversity Index across plant species across each 3 natural grassland sites in Slovenia. Plants are ordered in ascending order relative to VMR. Each box plot represents the median (horizontal line), mean (solid black square) and 25th to 75th percentiles, bars. Grasses; white box plots, forbs; grey box plots.

Table 3.4. Linear-modelled analysis of fungal richness, diversity and evenness as an effect of plant species and plant functional group (i.e. forbs and grasses). Significant results are highlighted in bold.

Plant species effects			
	Richness	**Diversity**	**Evenness**
Total	**$LR_{37,55}$ = 29.38, P < 0.05**	$F_{37,55}$ = 1.07, P = 0.41	$F_{37,55}$ = 1.47, P = 0.16
Ascomytota	$LR_{37,55}$ = 71.26, P = 0.08	$F_{37,55}$ = 0.95 P = 0.53	$F_{37,55}$ = 1.16, P = 0.33
Basidiomycota	**$LR_{36,54}$ = 35.64, P < 0.01**	$F_{36,54}$ = 0.82, P = 0.66	$F_{36,54}$ = 0.93, P = 0.55
Chytridiomycota	**$LR_{35,53}$ = 45.66, P < 0.001**	**$F_{29,47}$ = 2.49, P < 0.05**	**$F_{29,47}$ = 2.81, P < 0.01**
Zygomycota	$LR_{35,53}$ = 6.89, P = 0.99	$F_{33,51}$ = 1.51, P = 0.15	$F_{32,50}$ = 0.42, P = 0.97
Glomeromycota	$LR_{36,54}$ = 18.89, P = 0.39	$F_{33,51}$ = 1.22, P = 0.3	$F_{33,51}$ = 1.1, P = 0.39
Plant functional group			
Total	$F_{54,55}$ = 0.01, P = 0.94	**$F_{54,55}$ = 4.33, P < 0.05**	**$F_{54,55}$ = 7.29, P < 0.01**
Ascomytota	$LR_{54,55}$ = 0.97, P = 0.32	$F_{54,55}$ = 0.06, P = 0.81	$F_{54,55}$ = 0.21, P = 0.65
Basidiomycota	$LR_{53,54}$ = 0.02, P = 0.89	$F_{53,54}$ = 1.47, P = 0.23	$F_{53,54}$ = 2.46, P = 0.12
Chytridiomycota	$LR_{52,53}$ = 0.27, P = 0.6	$F_{46,47}$ = 0.96, P = 0.33	$F_{46,47}$ = 1.07, P = 0.3
Zygomycota	$LR_{52,53}$ = 0.61, P = 0.43	$F_{49,50}$ = 1.87, P = 0.17	$F_{49,50}$ = 1.33, P = 0.25
Glomeromycota	$LR_{53,54}$ = 1.85, P = 0.17	$F_{50,51}$ = 2.38, P = 0.13	$F_{50,51}$ = 2.04, P = 0.16

3.4.3.2 | Fungal functional guilds

All guilds of fungi (pathogens, symbiotrophs and saprotrophs) significantly differed in OTU richness across plant species (Table 3.5). Diversity and evenness values regarding pathogens, symbiotrophs and saprotrophs were not significantly different between plant species, except from saprotrophs that differed in OTU evenness.

OTU richness was not related to plant functional group across any fungal guild (Table 3.6). However, diversity and evenness were significantly different between grass and forbs for all fungal guilds, except diversity measures that were approaching significance for saprotrophs. Overall, the diversity and evenness of pathogenic and symbiotrophic fungi were significantly higher in grasses.

Table 3.5. Linear-modelled analysis of fungal richness, diversity and evenness as an effect of plant species and plant functional group (i.e. forbs and grasses). Significant results are highlighted in bold.

Plant species effects			
	Richness	Diversity	Evenness
Pathogens	**$LR_{37,55}$ = 41.94, P < 0.01**	LM; $F_{37,55}$ = 0.86, P = 0.62	LM; $F_{37,55}$ = 0.87, P = 0.6
Symbiotrophs	**$LR_{37,55}$ = 30.44, P < 0.05**	$F_{37,55}$ = 1.6, P = 0.11	$F_{36,54}$ = 1.82, P = 0.06
Saprotrophs	**$LR_{37,55}$ = 34.04, P < 0.05**	**$F_{37,55}$ = 1.18, P < 0.05**	$F_{37,55}$ = 1.56, P = 0.13
Plant functional group			
Pathogens	$LR_{54,55}$ = 0.05, P = 0.87	**$F_{54,55}$ = 4.34, P < 0.05**	**$F_{54,55}$ = 4.81, P < 0.05**
Symbiotrophs	$LR_{54,55}$ = 2.08, P = 0.15	**$F_{54,55}$ = 4.13, P < 0.05**	**$F_{53,54}$ = 4.34, P < 0.05**
Saprotrophs	$LR_{54,55}$ = 0.01, P = 0.94	$F_{54,55}$ = 3.25, P = 0.07	**$F_{54,55}$ = 4.15, P < 0.05**

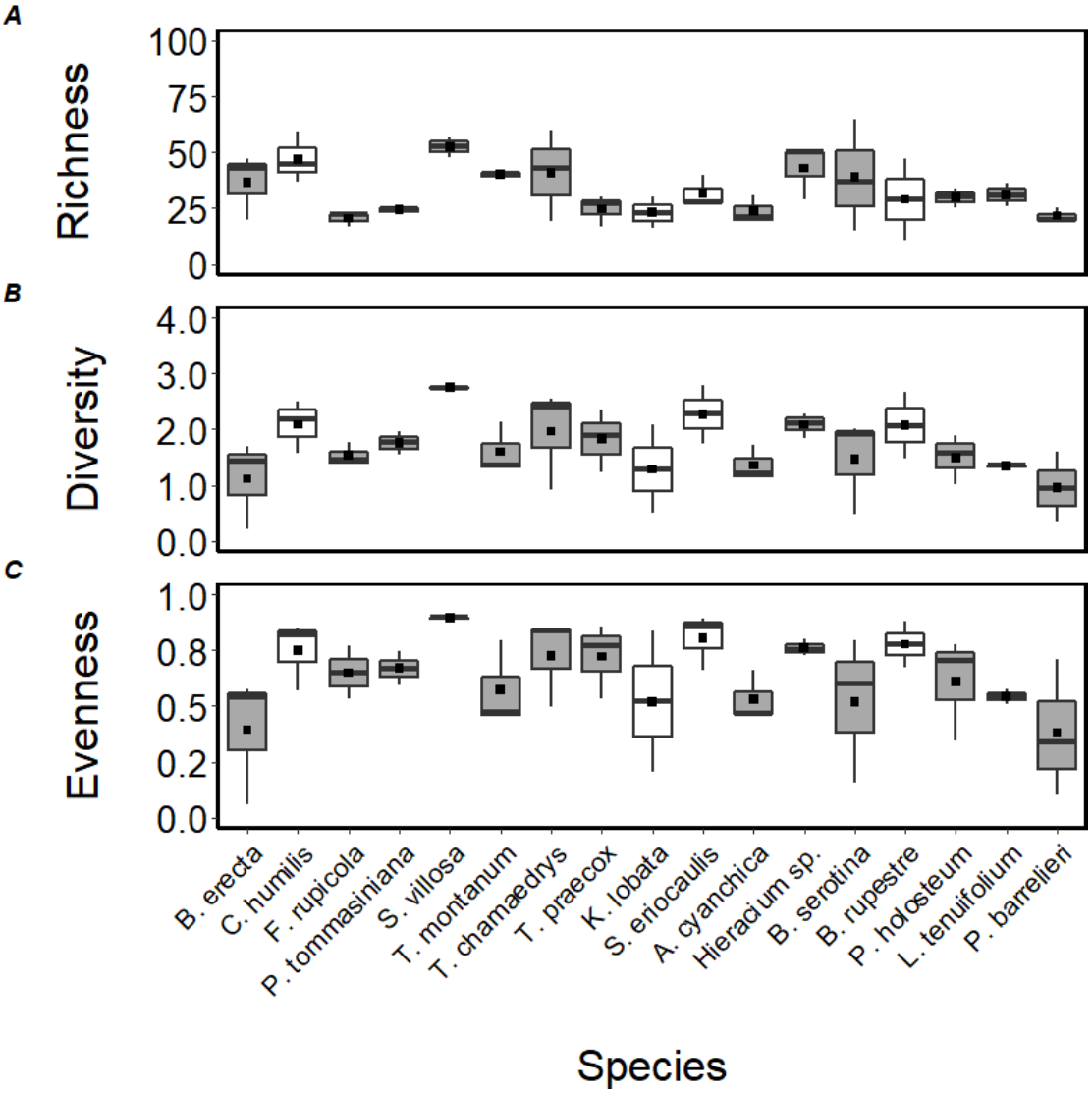


Figure 3.3 Pathogenic fungal; A) OTU richness; B) OTU Shannon's Diversity Index; C) OTU Simpson's Diversity Index across plant species across each 3 natural grassland sites in Slovenia. Plants are ordered in ascending order relative to VMR. Each box plot represents the median (horizontal line), mean (solid black square) and 25th to 75th percentiles, bars. Grasses; white box plots, forbs; grey box plots.

Similar to the total fungal community, pathogenic fungal communities were not a function of plant frequency (richness GLM; $LR_{54,55} = 1.02$, $P = 0.31$, diversity LM; $F_{54,55} = 0.15$, $P = 0.69$, evenness LM; $F_{54,55} = 0.76$, $P = 0.76$) but VMR was significantly correlated with pathogenic diversity (Figure 3.4. LM; $F_{54,55} = 7.72$, $P < 0.01$) and evenness (LM; $F_{54,55} = 7.48$, $P < 0.001$).

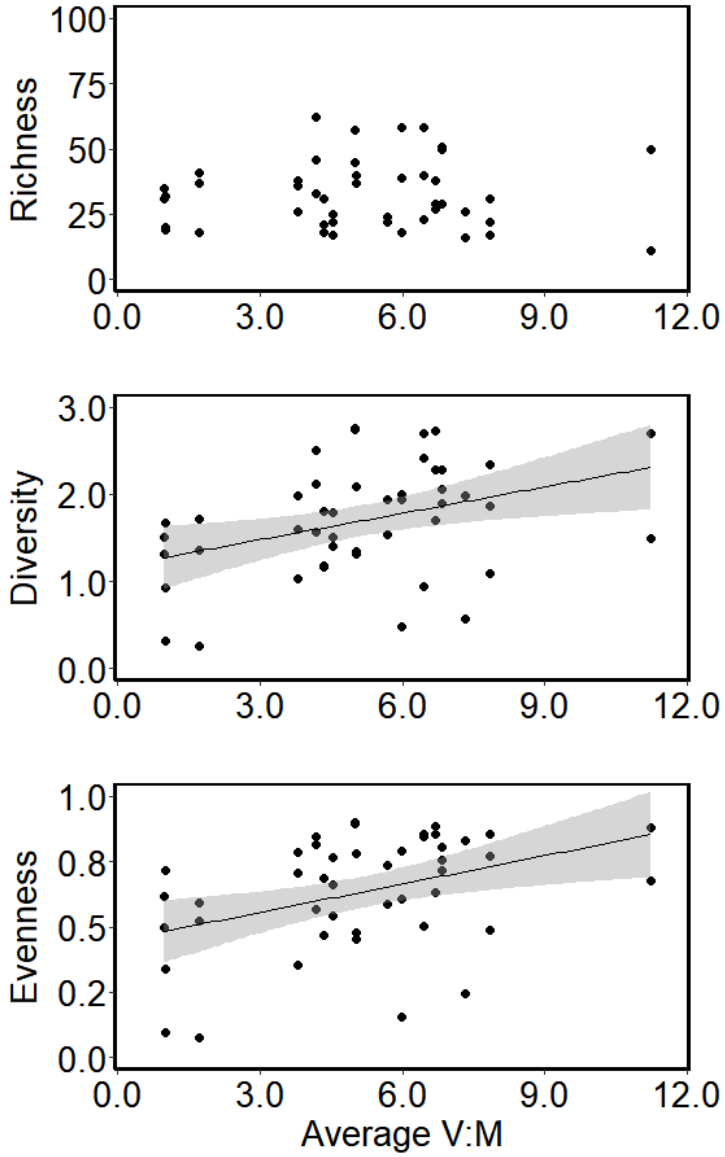


Figure 3.4. Linear-modelled results of pathogenic alpha diversity with average VMR. A regression line is fitted where results are significant. <1 uniform, 1 random, >1 aggregated.

3.4.4 | Beta diversity

3.4.4.1 | Total fungal community

Root associated fungal communities, i.e. all fungal OTUs, were specific to plant species (Figure 3.5. PERMANOVA; $F_{18,54}$ = 1.62 , P < 0.001), plant genus (PERMANOVA; $F_{17,54}$ = 1.65, P < 0.001), family (PERMANOVA; $F_{13,54}$ = 1.78 , P < 0.001), order (Figure 3.5. PERMANOVA; $F_{5,54}$ = 1.57 , P < 0.001), and between plant functional group (Figure 3.5. PERMANOVA; $F_{1,54}$ = 1.73, P < 0.05).

Similar patterns of plant species-specific communities were found for communities of Ascomycota, and Basidiomycota, but not for Chytridiomycota, Zygomycota or Glomeromycota (Table 3.6). However, communities of Glomeromycota and Zygomycota were specific at the plant family and order level.

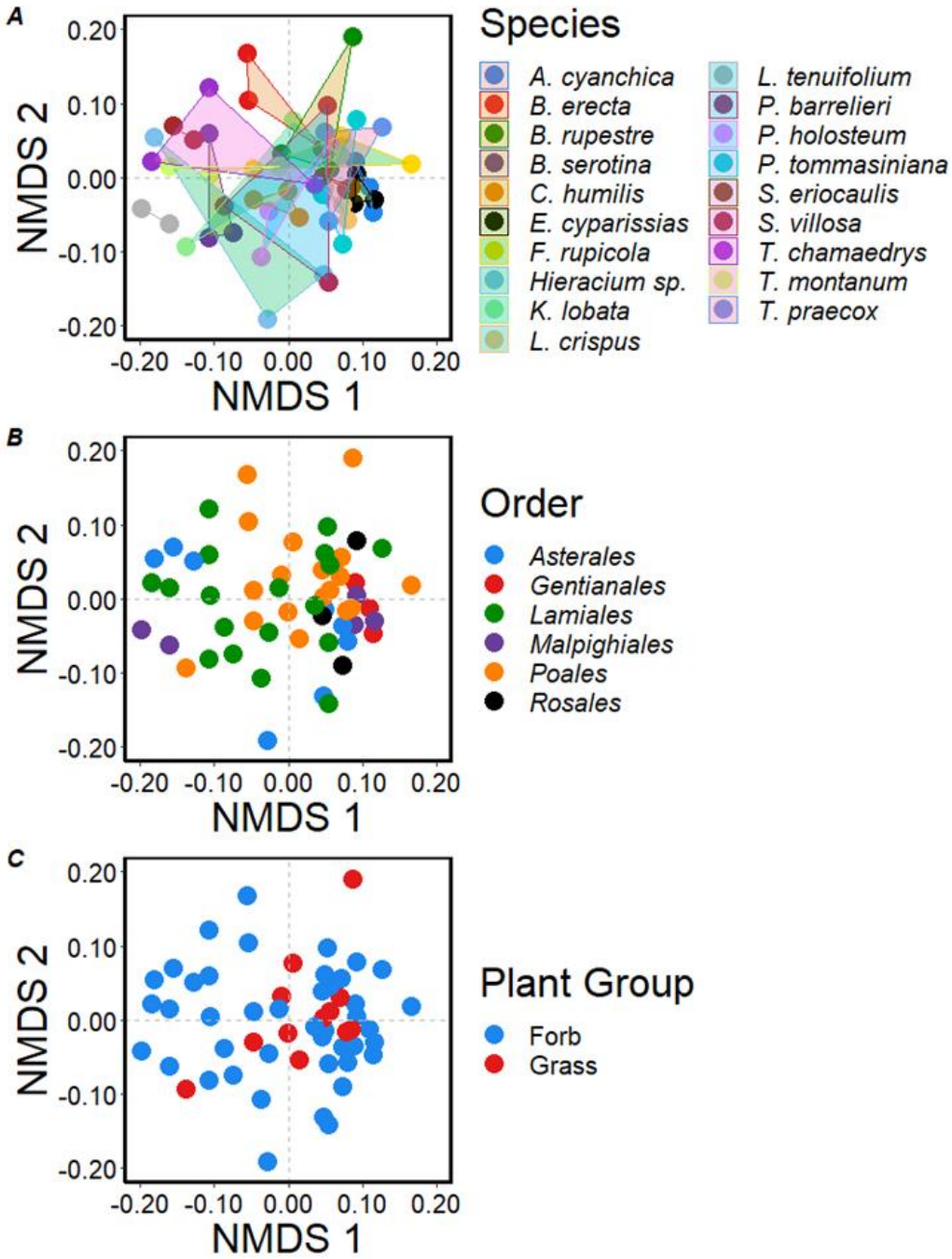


Figure 3.5. Plant-species specific root-associated totl fungal communities across three natural grassland sites in Slovenia. Nonmetric multidimensional scaling (NMDS) ordinations, based on Bray's distance matrix of the total fungal community. A) plant species effects, B) plant effects at the plant Order level, C) plant effects at the plant functional group level. Stress = 0.2098.

Table 3.6. PERMANOVA analysis of assemblies of fungal OTUS (including; Ascomycota, Basidiomycota, Chytridiomycota, Zygomycota and Glomeromycota) across differing plant taxonomic levels and between plant functional group. Significant results are highlighted in bold.

	Ascomy-cota	**Basidiomy-cota**	**Chytridiomy-cota**	**Zygomy-cota**	**Glomeromy-cota**
Plant sp.	**$F_{18,54}$ = 1.62, P <0.001**	**$F_{18,47}$ = 1.21, P <0.05**	$F_{1,22}$ = 1.04, P = 0.29	$F_{10,18}$ = 0.97, P = 0.29	$F_{15,30}$ = 1.09, P = 0.53
Plant genus	**$F_{17,54}$ = 1.66, P <0.001**	**$F_{17,47}$ = 1.22, P <0.05**	$F_{13,22}$ = 1.04, P = 0.29	$F_{10,18}$ = 0.98, P = 0.52	$F_{17,30}$ = 1.11, P = 0.17
Plant family	**$F_{9,54}$ = 1.78, P <0.001**	**$F_{9,47}$ = 1.34, P <0.01**	$F_{7,22}$ = 1.15, P = 0.07	**$F_{6,18}$ = 1.36, P < 0.05**	**$F_{7,30}$ = 1.35, P <0.05**
Plant order	**$F_{5,54}$ = 1.56, P <0.001**	**$F_{5,47}$ = 1.26, P <0.05**	$F_{4,22}$ = 1.11, P = 0.13	**$F_{3,18}$ = 1.78, P < 0.05**	**$F_{4,30}$ = 1.29, P <0.05**
Plant group	**$F_{1,54}$ = 1.81, P <0.01**	**$F_{1,47}$ = 1.78, P <0.05**	$F_{1,22}$ = 0.94, P = 0.55	$F_{1,18}$ = 1.44, P = 0.13	$F_{1,30}$ = 1.06, P = 0.37

3.4.4.2 | Fungal guilds

Assemblages of plant fungal pathogens were specific to plant species, genus, family, order, and between plant functional group (Figure 3.6; Table 3.7). Pathogens within forbs were significant at the plant family (PERMANOVA; $F_{6,35} = 1.28$, $P < 0.05$) and plant order (PERMANOVA; $F_{5,35} = 1.4$, $P < 0.05$), but not plant species level. Pathogens within grasses were not specific at any plant taxonomic level.

Host specificity was also found for plant-specific symbiotrophs, and saprotrophs between plant species, genus, family and order; but not between plant functional groups (Table 3.7).

Table 3.7. PERMANOVA analysis of assemblies of pathogenic, symbiotic and saprotroph fungal OTUs across differing plant taxonomic levels and between plant functional group. Significant results highlighted in bold. (Grasses belong to a singular order so PERMANOVA not possible).

	Pathogens	**Pathogens within forbs**	**Pathogens within grasses**	**Symbiotr-ophs**	**Saprotr-ophs**
Plant sp.	**$F_{16,45} = 1.21$, $P < 0.05$**	$F_{12,35} = 1.13$, $P = 0.14$	$F_{3,9} = 1.21$, $P = 0.21$	**$F_{17,46} = 1.27$, $P < 0.05$**	**$F_{18,52} = 1.33$, $P < 0.001$**
Plant genus	**$F_{15,45} = 1.22$, $P < 0.05$**	$F_{11,35} = 1.42$, $P = 0.12$	$F_{3,9} = 1.21$, $P = 0.2$	**$F_{16,46} = 1.29$, $P < 0.01$**	**$F_{17,52} = 1.36$, $P < 0.01$**
Plant family	**$F_{8,45} = 1.44$, $P < 0.01$**	**$F_{6,35} = 1.28$, $P < 0.05$**	$F_{2,9} = 1.12$, $P = 0.3$	**$F_{8,46} = 1.45$, $P < 0.01$**	**$F9_{,52} = 1.39$, $P < 0.01$**
Plant order	**$F_{5,45} = 1.49$, $P < 0.01$**	**$F_{5,35} = 1.4$, $P < 0.05$**	-	**$F_{5,46} = 1.48$, $P < 0.01$**	**$F_{5,52} = 1.42$, $P < 0.01$**
Plant group	**$F_{1,45} = 1.94$, $P < 0.05$**	NA	NA	$F_{1,46} = 1.59$, $P = 0.06$	$F_{1,52} = 1.46$, $P = 0.06$

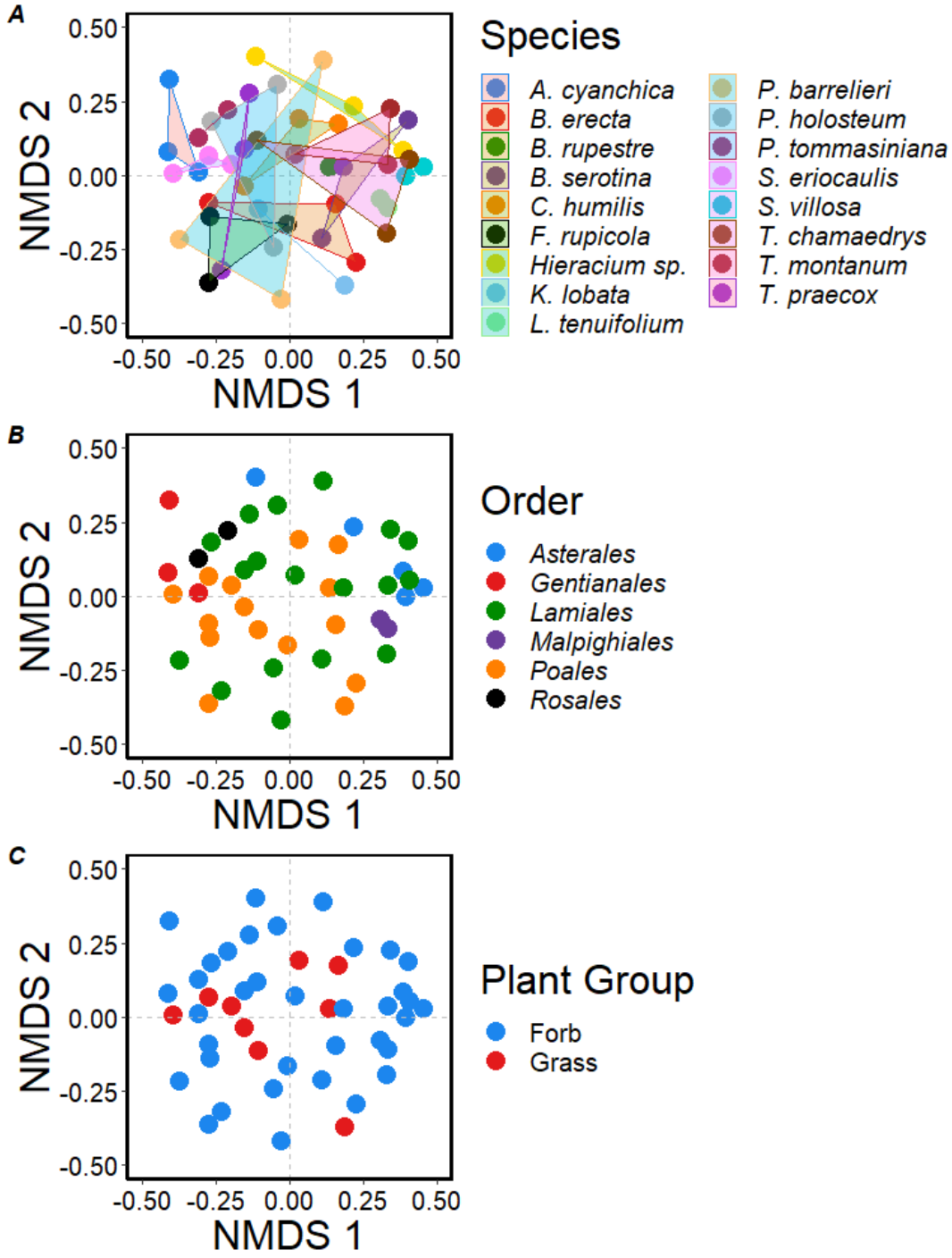


Figure 3.6. Plant-species specific root-associated pathogenic fungal communities across 3 natural grassland sites in Slovenia. Nonmetric multidimensional scaling (NMDS) ordinations, based on Bray's distance matrix of the total fungal community. A) plant effects, B) plant effects at the Order level, C) plant effects at the group level (stress = 0.2499).

3.4.5 | MV-GLM. Multivariate species analysis between grasses and forbs.

3.4.5.1 | Total fungal community

Plant functional group was shown to be related to specific fungal communities, therefore I performed a MV-GLM to determine what OTUs are likely driving this difference. Because grasses demonstrated significantly higher OTU richness, forbs were set as the intercept to determine what OTUs were driving this result. A total of 435 OTUs occurred > 1 % in the overall dataset. Multivariate modelling revealed that plant functional group significantly affected fungal OTU abundances (154 OTUs; Wald = 33.63, $P < 0.001$), after controlling for unequal sequencing depths. Of all the OTUs affected by plant functional group, 34 OTUs were significantly more abundant and 120 OTUs were significantly less abundant in grasses in comparison to forbs (Figure 3.7).

3.4.5.2 | Pathogenic fungal community

Multivariate modelling revealed that plant functional group also significantly affected pathogenic fungal OTU abundances (65 OTUs; Wald = 16.24, $P < 0.001$) within grasses in comparison to forbs, after controlling for unequal sequencing depths. A total of 159 OTUs occurred > 1% in the overall dataset. Of all the OTUs affected by plant functional group, 12 OTUs were significantly more abundant (Table 3.8) and 53 OTUs were significantly less abundant in grasses in comparison to forbs (Figure 3.7). For pathogenic taxa (OTUs) that occurred in significantly greater abundance in forbs in comparison to grasses, see Appendix 3.2.

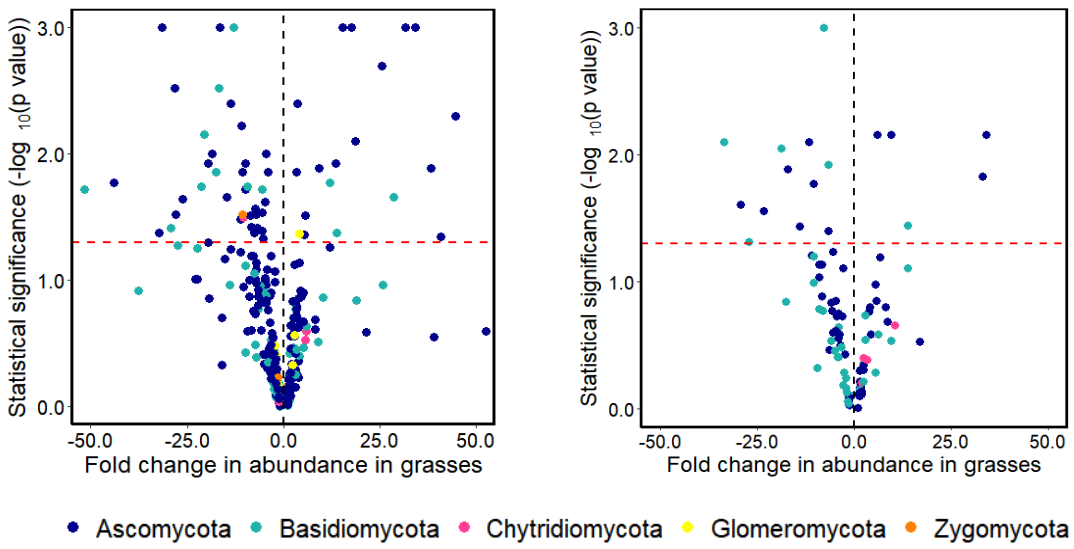


Figure 3.7. Volcano plot of MV-GLM modelled shifts in total fungal phyla (left) and pathogenic fungal phyla (right) abundances in grasses when compared to forbs. Coloured points above the horizontal dotted line represent phyla that showed statistically significant shifts in abundance ($P < 0.01$), whereas points below the line represent phyla that did not show statistically significant shifts ($P > 0.05$). Phyla further to the left were statistically less abundant while phyla to the right were statistically more abundant when compared to forbs.

Table 3.8. MV-GLM modelled shifts of pathogenic taxa occurring in significantly higher abundance in grasses when compared to forbs.

OTU.ID	**Phylum**	**Species**
OTU.413	Ascomycota	*Microdochium bolleyi*
OTU.2856	Ascomycota	*Aureobasidium pullulans*
OTU.404	Ascomycota	*Diaporthaceae sp*
OTU.22922	Ascomycota	*Periconia sp CY137*
OTU.11554	Ascomycota	*Volutella sp*
OTU.20185	Ascomycota	*Leptosphaeria proteicola*
OTU.507	Basidiomycota	*Hydnopolyporus fimbriatus*
OTU.402	Ascomycota	*Microdiplodia sp*
OTU.10978	Basidiomycota	*Grifola frondosa*
OTU.11620	Basidiomycota	*Rhodotorula lamellibrachiae*
OTU.2894	Basidiomycota	*Mycena dura*
OTU.84757	Basidiomycota	*Rhodotorula philyla*

3.5 | Discussion

This study characterises fungal communities from late successional hyperdiverse natural grassland species in Slovenia. The role of fungal pathogens in the regulation of grassland diversity is based on three main lines of evidence; 1) NGS shows grassland plants are associated with a diverse range of fungal taxa, 2) fungal isolates have been shown to reduce plant fitness in growth assays and 3) the relationship between plant diversity and productivity is diminished by fungicide application *(*Bonanomi *et al*., 2005; Petermann *et al*., 2008; Mangan *et al*., 2010). However, all of these studies fail to characterise the true taxonomic identity of the fungal communities involved. Through characterisation of the root-associated mycobiome across nineteen grassland species (common to all three sampling sites), this study begins to satisfy the main assumptions regarding the 'pathogen-hypothesis'. This opens up the 'black-box' regarding the identity of fungal microbes, particularly the role of fungal pathogens (Bever *et al.*, 2015), and supplements the results seen from experiments investigating the empirical biodiversity-productivity relationship (Mommer *et al*., 2010, 2018; Maron *et al*., 2011; Kulmatiski *et al*., 2012) and predictions for vegetation dynamics (Heinen *et al.*, 2020).

3.5.1 | Host-specificity of fungal mycobiomes

H1: Different plant species accumulate species-specific fungal microbiota (total and pathogenic) communities under natural field conditions. This confers host specificity.

Firstly, I observed support for host specificity because plant species differed in their total fungal richness (Figure 3.2). This pattern in richness was also conserved across the second and third most abundant phyla, Basidiomycota and Chytridiomycota. This suggests plant species vary in their accumulation of different fungal taxa. Analysis of fungal composition revealed plant species harboured

significantly different fungal communities across all plant taxonomic ranks, including plant functional group (Figure 3.5). This was consistent across the Ascomycota and Basidiomycota. These results indicate plants accumulate distinct fungal communities and thus the first assumption of the 'pathogen-hypothesis' can be strongly backed with evidence. In agreement with Mommer *et al.*, (2018), host-specificity of the two most abundant phyla Ascomycota and Basidiomycota largely drives this relationship, whilst Glomeromycota do not show host-specificity at any plant taxonomic rank. This is not surprising because Glomeromycota, a dominant fungal phyla of soils largely comprised of beneficial AMF, demonstrate a cosmopolitan distribution and wide host range (Smith & Read, 2008; Dumbrell *et al*., 2010). A wide host range implies AMF are able to colonise plants of different species, genera and family. Due to this lack of host specificity this large network of hyphae interconnecting contiguous plants can aid in signalling and nutrient acquisition between plants (Chiariello *et al.*, 1982; Francis & Read, 1984). Despite this, Mommer et al., (2018) suggests evidence for host ranges of AMF should be validated with more specific fungal primers, this is likely to avoid PCR bias and increase preferential taxonomic coverage of the Glomeromycota.

3.5.2 | Pathogenic fungi

H1: Different plant species accumulate different species-specific fungal microbiota (total and pathogenic) communities under natural field conditions. This confers host specificity.

H2: Functional groups of plants (grass and forbs) accumulate different species-specific fungal microbiota.

Given the potential role PSFs and fungal pathogens play in vegetation dynamics, it was important to investigate host specificity of functional fungal guilds (i.e. potential plant pathogens, symbiotrophs and saprotrophs). This study implies pathogenic fungi are host specific as revealed by significantly different OTU richness

across plant species (Figure 3.3). More importantly, distinct pathogenic fungal communities were found across all plant taxonomic ranks and plant functional group (Figure 3.6, Table 3.8). Despite this, separation of grass and forb associated pathogens, giving a more detailed exploration of fungal pathogens, revealed pathogens within forb species and pathogens within grass species were not compositionally distinct (Table 3.8). This suggests distinct pathogenic communities found at the plant species level are largely driven by functional group differences between grasses and forbs. Nonetheless, pathogenic fungal communities demonstrated host specificity within forb species when explored at the plant family level, and upwards towards higher taxonomic classification. This implies there is a phylogenetic host signal in which host specific effects begin to arise at higher taxonomic classifications where the phylogenetic distance between families of forbs is greater. Forbs are a broad taxonomic group (belonging to many families), much more so than grasses that all belong to a single family, Poaceae. This is in align with work by Gilbert and Webb (2007) and Liu et al., (2012) in which a phylogenetic relationship between disease risk and genetic similarity is implied. This means forb pathogens are unlikely to simultaneously infect all forb families. This was also reflected by multivariate modelling of shifts in OTU abundance in which 53 pathogenic fungi were more abundant in forbs, but only 12 taxa were more abundant in grasses. This in turn has knock on effects implying grasses, with shared pathogenic communities associated with their single plant family, would experience a larger overall effect of negative PSF on grasses as a functional group as a whole. This finding may explain why plants in conditioning studies reveal stronger negative PSF, implemented through legacy effects, in soils previously occupied by their own functional group (Petermann *et al*., 2008; Hendriks *et al*., 2015; Heinen *et al*., 2020). More specifically, stronger negative PSF is commonly found in grasses as opposed to forbs (Heinen *et al*., 2020). Moreover, overlap in pathogenic suites across different plant species begins to challenge of the quantitative assessment of plant density, for example, the data from

this study implies ecologists should modify the way plant density is calculated. Plant density should be a sum of species that share microbial pathogenic suites as opposed to a simple function of plant species.

3.5.3 | Frequency and VMR - NDD effects

H3: Plant distribution and frequency is a function of belowground fungal microbiota and is therefore a reflection of NDD type effects.

In context with this study, I explored the effect of plant frequency and aboveground spatial distribution (reflected by the calculated VMR) on fungal richness, diversity and evenness, again, with emphasis on fungal pathogens as these were highlighted as the key mediators of negative PSF. This study did not reveal a relationship between plant frequency and total or pathogenic fungal community richness, diversity or evenness. This may have been because this chapter looks only at 'local' rarity but it is possible the mechanisms driving plant rarity might differ between spatial scales (Kempel *et al*., 2018). For example, a plant may demonstrate a local high conspecific density, but the dense aggregation of this plant may occur infrequently. Therefore, the overall frequency of this plant species would be considered rare.

However, the pathogenic fungal community did respond to the spatial distribution of plant species as represented by the Variance to Mean Ratio (VMR - Figure 3.4). To my knowledge, past studies exploring plant-microbial interactions and their role in vegetation dynamics, only focus on overall plant diversity (i.e. monoculture, 2 – 4 – 8 - 16 species mixes) and fail to consider the potential role that plant spatial distribution might play in driving fungal community dynamics. This study therefore closes this knowledge gap by incorporating aboveground plant distribution.. In brief, the greater the VMR, the closer the spatial aggregation and 'clumpiness' of an individual plant population, and as such, a loose level of local host density can be

inferred. As can be expected from a natural grassland ecosystem, the majority of plants exhibit varying degrees of an aggregated distribution. Pathogenic fungal diversity and evenness was positively correlated with average VMR (i.e. pathogenic diversity/evenness increased when plants occurred in more dense aggregation), conversely, there was no significant correlation found between the total fungal community and average VMR. This speculation around greater NDD and increase pathogenic diversity is in line with Semchenko *et al.*, (2018) in which negative PSF was stronger for plant species that had accumulated more diverse communities of fungal pathogens. This has vital implications for aboveground vegetation dynamics and supports pathogen regulation of high local densities in order to prevent the proliferation of conspecific plant species which may ultimately lead to competitive exclusion of inferior plant species. However, VMR data must be interpreted with caution as it is only representative of the average distribution and microbial sequencing from root samples was not directly paired with aboveground spatial data. This means, randomly selected root systems might not have been sampled from a local population of the 'typical' plant distribution as implied by the average VMR. To assess this in the future, above and belowground samples would have to be taken in parallel with accurate measurements of host plant density. As such, these results show spatial distribution as opposed to plant frequency might play a more significant role in natural ecosystems by where plant community dynamics are long established and are not affected by the homogenising force of experimental approaches. Therefore, incorporating the spatially and temporally dynamic nature of aboveground plant distribution might have more success in predicting the mechanistic basis of pathogen-mediated vegetation dynamics.

3.6 | Spatial and temporal dynamics within grassland ecosystems

In literature, there remains ambiguity surrounding the mechanistic basis of pathogen mediated regulation of plant diversity. Negative PSF can affect plant communities by (1) supressing dominant plant communities – 'rare species advantage' (Putten *et al*., 1993; Mommer *et al*., 2018); (2) disproportionate suppression of rare communities (Klironomos, 2002; Mangan *et al.*, 2010), or an intermediate (3) plant soil backs effect the competitive ability of all plant species (Reinhart, 2012). I suggest it is possible that any 'snap shot' of a given ecological system could provide evidence for either viewpoint, with this being dependent on the transitioning period from dominancy to plant rarity in an ecosystem. This spatio-temporal feature is unique to grassland ecosystems and this could be why it has not yet been explored because current PSF theory is centred on tropical forests that are predominantly static for long periods of time. I have portrayed this in Figure 3.8 in which all three mechanisms are acting simultaneously causing population growth rates to decrease when a species is locally abundant and increase when a species is locally rare (Comita *et al.*, 2010). To confirm this, the questions I would ask are - does species-specific pathogen load remain high and thus maintain plant species rarity? Or, does decreasing host abundance, resultant of increased pathogen load, see a decrease in species-specific pathogen load overtime? Both of these theories have solid experimental support documented in literature and thus stresses the need for further investigation because the driving factors behind plant species commonness and rarity remains poorly understood.

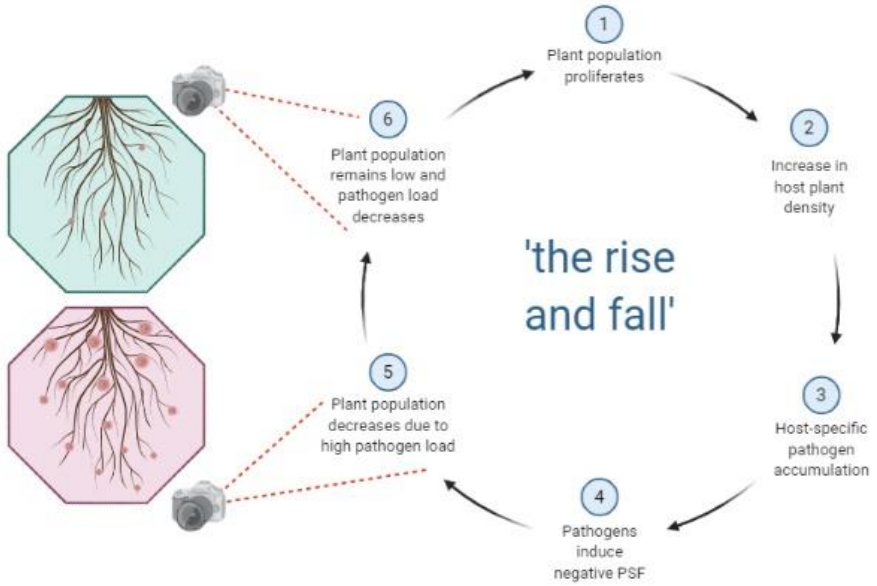


Figure 3.8. The cycle following both above and belowground community dynamics over time. Snapshots taken at Step.5 and Step.6 highlights a potential reason why we see inconsistencies in theories regarding pathogen mediated regulation of grassland diversity.

Another key area that stands out is pathogenic host range. Whilst this study and support in literature documents strong host-specific effects of fungal pathogens, it is commonly neglected that pathogens may be able to survive and proliferate on members of other plant species but often these plant species may remain asymptomatic. In grassland systems, with very few areas of bare ground, it is likely neighbour identity could facilitate the spread of disease to contiguous plants if they are characterised as ‘carriers’, or non-host plants could act as a potential barrier for the spread of fungal pathogen disease. The importance of considering neighbouring identity is stressed by the phylogenetic host signal in this study in which grass species are shown to share pathogenic microbial suites. As such, disease transmission in grass dominated ecosystems could be enhanced, if true, this holds implications for both nature and agricultural management. Furthermore, multivariate modelling between species of grasses and forbs revealed distinct pathogenic communities are unlikely driven by a complete shift in pathogenic identity, but subtle responses of

specific pathogenic taxa (OTUs). This stresses that pathogens can be hosted by a number of plant species. Therefore, infection risk and plant rarity is not merely a function of species density, but the combined density of closely related species and asymptomatic neighbouring density. Neighbouring identity, where an asymptomatic host, or non-host has the ability to facilitate or delay pathogen accumulation, could also provide an area with vast potential for optimum crop selection in more sustainable intercropping systems.

3.7 | Positive PSF and vegetation dynamics?

Coupled with plant-microbial interactions regarding the pathogenic community, PSF induced by other microbial functional groups must not be dismissed. The overall outcome of PSF is a sum of both the positive and negative PSF operating at any given time. Interestingly, symbiotic and saprotrophic taxa demonstrated distinct communities between plant species. This has interesting potential as some saprotrophs have been shown to portray a 'home-field' advantage in which plant species accumulate saprotrophs that preferentially decompose their own plant material (Ayres *et al*., 2009; Miki, 2012), this could be important for those species that may rely on nutrients released from host decomposition. However, unlike fungal pathogens, neither fungal symbionts nor saprotrophs differed between plant functional group. This suggests any potential effects of positive PSF induced by saprotrophs or symbionts are more sensitive because they act upon the host plant at the species level. Interestingly, saprotrophs were richer in grasses in comparison to forbs. However, because saprotrophic community composition was not a function of plant functional group, this suggests increased richness in grasses is a result of accumulation of rare OTUs that have minimal effect on the overall composition. The fine balance between positive and negative PSF is something that undoubtedly effects the overall outcome of PSF and therefore population dynamics of aboveground diversity.

3.8 | Conclusion

In conclusion, this study satisfies the two assumptions underlying the 'pathogen-hypothesis'. First, host specificity was met for both the total root associated fungal mycobiome and fungal pathogens, with a prominent phylogenetic host signal between plant functional group as a potential key driver of community dynamics natural grassland ecosystems. Importantly, species of grasses share pathogenic suites whereas families within forbs are associated with distinct pathogenic communities. Second, there was a significant relationship between pathogenic fungal diversity and plant distribution. This showed pathogen diversity increased as local plant distribution becomes more spatially aggregated. This has vital implications for plant vegetation dynamics suggesting pathogens accumulate on plants occurring in high local densities. This prevents their proliferation and resists the forces of competitive exclusion. The data from this study suggests ecologists should discontinue their view of density and pathogen accumulation as a fixed process in which plant rarity is maintained. Rather, populations of plants rise and fall in alliance with their associated pathogenic load. Therefore, long term studies that allow for natural population dynamics to exist would allow thorough exploration for potential analogous shifts in both plant and microbial populations. In the next chapter I explore signals for fungal host-specificity across an aboveground diversity gradient to see if plant-fungal host specificity is conserved.

3.9 | Limitations

Despite the aforementioned evidence for distinct total fungal communities explained by plant species, and pathogenic fungal communities explained by plant functional group, this can only suggest, but not confirm, host specificity. More specifically, the descriptive data generated in this study followed by guild assignment to pathogen cannot confirm a correlation between the presence of the detected fungal

pathogen with disease. This is because many fungal microbes will demonstrate mixed trophic modes which is dependent on the host-fungus interaction or environmental context (Gilbert & Parker, 2016). To further this, OTU presence is not necessarily an indication of metabolic activity and so it cannot be confirmed if fungal pathogens are active/dormant at the time of sampling. To overcome this, identification followed by sequencing and/or quantification of functional genes involved in pathogenicity would be required. Nonetheless, the data I show in this study offers valuable insights into the mediation of plant species coexistence by fungal microbes as it is one of the first to adopt high throughput NGS technology to reveal the taxonomic identity of the microbial suite that may be involved in the feedback between plants and their soil in a natural field setting. As such, this data will facilitate a more targeted experimental approach to the design of follow-up studies to explore single fungus and plant interactions, but perhaps more importantly, will benefit the exploration of more complex interactions between multiple plants and multiple fungal species. These phytopathology studies would require 1) isolation of fungal strains, 2) inoculation of plant hosts, and 3) quantitative assessment of plant phenotypic traits.

As an alternative, more recent papers utilising NGS Illumina technology have adopted a more stringent approach for the assignment of fungal trophic mode (Francioli *et al.*, 2020). This involves a two step approach where (1) a rough assignment to trophic mode is made based on selecting taxa classified as 'highly probable' or 'probable' and (2) the potential pathogenicity of taxa is refined further by only retaining ASVs (amplicon sequence variant) that were characterised as pathogenic to the species level in literature. While this *modus operandi* is advocated to overcome the uncertainty regarding the species-specific virulence of certain fungal taxa (Nilsson *et al.*, 2019), it is limited in that the exclusion of many fungal ASVs limits the analysis of the 'whole' pathogenic population. To conclude, while databases are becoming increasingly more reliable in predicating ecological guild, to fully understand

the role of pathogenic fungal communities in the spatiotemporal dynamics of soil-borne disease in natural ecosystems, it still remains pertinent that NGS technology to identify fungal pathogens is followed by species-specific phytopathology studies.

Chapter 4

Characterisation of belowground plant species-specific fungal communities across an aboveground grassland diversity gradient.

4.1 | Abstract

Plant-Soil-Feedbacks (PSFs) and host-specific fungal pathogens provide a powerful framework for understanding plant vegetation dynamics and continue to receive increasing recognition across a number of biomes. However, we lack a comprehensive view of these mechanisms from natural grassland ecosystems, and in particular, how these mechanisms might vary within grasslands differing in aboveground plant diversity. Here, I determine the relative prevalence and direction of host-specificity for plant species coexistence and diversity by utilising next-generation sequencing techniques to explore host plant specific root-fungal interactions from two European ecosystems providing a low (Norway) and intermediate (UK) level of aboveground diversity. No relationship between plant diversity and total or pathogenic fungal richness was evident at a global scale. Moreover, I reveal a breakdown in the presence of host-specific fungal and pathogenic mycobiomes, and an increase in host-specificity of saprotrophic communities from low diversity ecosystems. These results could imply the strength and direction of host-specificity are important predictors of vegetation dynamics in which positive PSF could drive low plant diversity by promoting the proliferation of few plants. Moreover, in align with diverse grassland ecosystems, host-specificity of pathogens in intermediate grasslands showed a clear discrimination between plant functional group, i.e. grasses and forbs. This suggests there is a phylogenetic host signal of disease susceptibility and thus has implications for broad scale shifts in vegetation dynamics. This work substantiates the hypothesis that root-

fungal-pathogens are an important driver of plant species co-existence. However, the requirement to adopt a fully encompassed approach that enables exploration of all potential PSF effects and the microbial team players involved in plant species coexistence is a necessity for future work. Identifying the relative importance of each PSF response across a diversity gradient will allow ecologists to make more reliable predictions regarding vegetation dynamics and thus has applications for conservation management.

4.2 | Introduction

Early frameworks of species coexistence have been able to offer an insight into plant population dynamics (i.e. Pickett, 1980; Pärtel *et al.*, 1996; Chase *et al.*, 2002). However, some of the empirical evidence supporting these theories, particularly in natural field conditions, remains mixed. Negative density dependence (NDD) mediated through Janzen-Connell type effects has explained vegetation dynamics across a number of terrestrial ecosystems (Petermann *et al.*, 2008; Bagchi *et al.*, 2010; Liang *et al.*, 2016), with fungal pathogens identified as the main players of this interaction (Bagchi *et al.*, 2014; Liang *et al.*, 2016). More recently, similar theories are gaining increased recognition in temperate ecosystems (Hille Ris Lambers et al., 2002), including grassland ecosystems in which host-specific soil microbes govern plant species diversity by dampening fitness levels of superiorly competitive plant species (Petermann *et al*., 2008; Mommer *et al*., 2018).

To satisfy NDD and Janzen-Connell type responses, differential effects on plant species is required to cause long term differences in the overall community composition, and for this to take place, it relies on the assumption that fungal pathogens driving this relationship must be host-specific. The increasingly rich body of evidence implying host specificity in grassland plants in literature (Bever, 1994; Mommer *et al.*, 2018), coupled with host-specificity of total and pathogenic fungal communities evidenced in Chapter 3, provides encouraging evidence to support this theory. However, the maintenance of host-specificity is unsubstantiated i) at a large geographical scale, or ii) across a natural aboveground grassland biodiversity gradient. Defining the universality of this relationship could have important implications for vegetation dynamics and may offer a more reliable prediction for broad scale global aboveground diversity patterns.

On a small and experimental scale, increasing plant species richness is shown to have a positive effect on plant productivity (Tilman *et al*., 2001; Hooper *et al*., 2005). This has been confirmed by a number of 'black box' conditioning experiments (Klironomos, 2002; Petermann *et al.*, 2008; Maron *et al.*, 2011), and more recently through advanced molecular techniques in which increased biomass is attributed to a loss of pathogenic fungal diversity with increased plant species richness (Mommer *et al.*, 2018). Both of such studies elucidate to an increase in negative PSF at high plant densities (i.e. low plant diversity), and reduced negative PSF at greater plant diversity. Furthermore, an earlier but relevant study suggests the mechanisms underpinning the diversity-productivity relationship could be robust at multiple geographical locations across Europe (Hector *et al.*, 1999). In this study eight European grasslands were explored to synthesise the impact of a loss of plant diversity on productivity, it was revealed grasslands with a fewer number of functional groups were less productive. These field observations imply host plant specificity, particularly of pathogenic fungal groups, are maintained on large geographical scales and across natural aboveground diversity gradients.

To complement previous findings, the work in this chapter is analogous to Chapter 3. In Chapter 3, next-generation sequencing approaches were utilised to characterise the root-associated fungal mycobiome of grassland plants from a hyperdiverse ecosystem. In align with previous experimental work, I confirmed host-specificity of pathogenic fungal communities. The composition of these communities were distinct between families of forbs and grasses thus inferring a phylogenetic relationship of host-specificity as also supported by Hector *et al.*, (1999), Gilbert & Webb, (2007) and Liu *et al.*, (2012). While there have been a number of experimental studies that manipulate aboveground plant diversity (i.e. Weisser *et al.*, 2017; Mommer *et al.*, 2018), to my knowledge, no previous studies in 'true' natural ecosystems have directly measured the strength of PSF or maintenance of host-specificity across

grasslands offering an aboveground plant diversity gradient, or, a test of these measures across a large geographical scale. A review by Philippot *et al.*, (2013) suggests the more diverse the plant community, the more diverse its associated rhizodeposits, and consequently microbial diversity will be. However, reflective of the role fungal pathogens play in plant-species coexistence, this relationship becomes more complex when exploring diversity of root-associated pathogens. Experimental work by Mommer *et al.*, (2018) failed to demonstrate an increase in fungal diversity associated with an increase in plant species richness, but the linear relationship was dampened by the loss of pathogenic OTUs found with increasing diversity. This implies grasslands with higher levels of plant diversity will acquire less fungal pathogens and hence pathogen pressure will be lessened. These inconsistencies highlight the need for further investigation. For these two reasons, there are two possible outcomes (1) fungal diversity will increase with plant diversity due to the accumulation of host-specific mycobiomes, or (2) fungal diversity will decrease as fungal pathogenic load decreases with high plant diversity. It also must not be overlooked that grasslands with low diversity (and therefore enhanced local density of any given plant population) might be an outcome of weakened (or absent) host-specific plant-fungal relationships, this is a phenomena that has not previously been explored but will be tested in this study. The absence of host-specific pathogens and negative PSF would mean fitness differences would remain unequal which could ultimately lead to the competitive exclusion of inferior species. As such, the absence of host-specific NDD might provide a mechanistic basis that sheds light on low diversity ecosystems. Alternatively, if host-specificity is conserved, a difference in the strength of NDD may explain why temperate grasslands frequently differ in their aboveground diversity. If this holds true, this provides a further avenue for research.

In this study, root-associated fungal microbiomes of plants sampled from Europrean grasslands offering intrinsically medium (UK) and low (Norway) levels of

plant diversity were characterised utilising next-generation sequencing approaches. The aim of this study was to identify if host-specificity of total and pathogenic fungi were conserved geographically, and across an aboveground diversity gradient of plants. As such, the following hypotheses were tested;

H1: Total fungal richness will increase with plant species richness.

H2: Fungal pathogens will decrease in diversity with increasing plant diversity.

H3: Total and pathogenic fungi will demonstrate host-specificity at all sites.

H4: Families of grasses and forbs accumulate compositionally distinct total and pathogenic fungal communities across a gradient of aboveground diversity.

4.3 | Methods

COVID-19: results in this chapter were impacted by the COVID-19 lockdown beginning March 2020. Data were unable to be recovered as it was stored servers that were inaccessible for the duration of lockdown. For publication purposes, phylogenetic data from Chapters 3 and 4 will be integrated and taxonomic OTUs will be reassigned so direct comparisons can be made across the three field sites located in Europe.

4.3.1 | Study site

Akin to Chapter 3, fieldwork was repeated in two temperate regions; UK (South East, Colchester. Sampling period – June 2018) and Norway (South West, Oppland. Sampling period – October 2018). This supplements data collected from Slovenia in Chapter 3 and as such provides a natural aboveground diversity gradient (Table 4.1). These natural grasslands offer a gradient of host plant diversity, 5-10 species, 10-20 species, 30 species + respectively. Collectively, these sites provide an ideal place to study the role of fungal pathogens in negative soil feedback through evaluating host specific fungal relationships and characterising how the strength of this varies with plant diversity. Three sites of approximately 1 hectare were selected for biological site replicates within each region. The field sampling protocol was replicated within each country.

Table 4.1. Summary of diversity patterns and locations in three geographical field sites.

	Norway			**UK**			**Slovenia**
Avrg. species richness	10			15			30
Site	1	2	3	1	2	3	See details from previous chapter
Latitude	51.960	51.905	51.903	60.996	60.961	61.082	
Longitude	1.000	0.910	0.917	9.117	9.317	9.019	

4.3.2 | Sample collection

Five singular plants of each species were randomly selected and carefully extracted from the soil at each site (roots intact), giving a total of five plant species replicates where possible per site. Roots complexes were washed using tap water to remove all debris and organic matter. Soil cores for belowground soil analysis were collected using 25 randomly placed quadrats in approximately 1 hectare sampling area per site. Soil cores (diameter 5 cm, depth 10 cm) were taken from the bottom left corner of each quadrat for analysis of belowground edaphic properties, sterilised with 70 % ethanol between samples. Roots and soil cores were dried at 60 °C until a constant weight was achieved to ensure removal of all moisture. Moisture of soil cores were recorded. Samples were transported at room temperature in sterile and airtight plastic bags to The University of Essex, UK, for molecular processing

4.3.3 | Root DNA Extraction

DNA was extracted from the entire root complex of each plant sample following the Cetyl Trimethylammonium Bromide protocol (CTAB; Griffiths *et al.*, 2000). Details for DNA extraction are outlined in the general methods. Each sample was standardised to 1 ng/µL in PCR water to allow for high-throughput automated sample preparation.

4.3.4 | Molecular analysis

Root associated fungal communities were characterised using 2 x 300 bp Miseq amplicon sequencing of ~ 350 bp fragment targeting the ITS2 region of the Small Subunit (SSU). Library preparation methods are outlined in the general methods (Chapter 2). Sequencing was conducted at The University of Essex on an Illumina Miseq (providing 2 x 300 bp sequences).

4.3.5 | Soil chemistry

All edaphic properties from each soil core were measured as outlined in the general methods (Chapter 2).

4.4.6 | Statistical analysis

Bioinformatic analyses were conducted using the methods outlined in the general methods (Chapter 2). The OTU table was filtered to remove negative controls, after confirmation that contamination was negligible, non-target amplicons and low abundance OTUs (< 3) as these are likely to be non-biological (Flynn *et al.*, 2015). 'Unidentified' OTUs classified by the UNITE database were explored further for their phylogeny using their awarded a 'species hypothesis (SH)' number (Nilss*on et al*., 2019) , those belonging to the phyla Streptophyta and Chlorophyta were removed from the data set. Data were pooled by site for each plant species to account for random effects caused by within site differences. Further community OTU subsets were created for multiple fungal phyla and functional guilds of fungi (i.e. plant pathogens, saprotrophs and symbionts).

Linear-models (LMs) were used to determine any differences in abiotic factors between sampling sites. To determine if plant species (including plant order, genus, family and plant functional group) had an effect on fungal OTU richness a linear-model (LM) or negative binomial generalised model were applied if data were not normal (GLM) within the package 'MASS' (Ripley, 2011). Differences in library size were controlled for by including log(number of sequences) as the first (fixed) term in the model. This approach to dealing with differences in sequencing depth is encouraged by (Warton *et al.*, 2015) as it avoids many of the unfavourable aspects introduced by alternatives such as rarefraction (McMurdie & Holmes, 2014). This is particularly important within the context of this study as rarefraction frequently removed biologically important pathogenic taxa. The effects of plant species on community diversity indices (diversity and evenness) were explored using linear models with a Gaussian distribution. All community evenness values were logit-transformed to meet linear modelling assumptions (Warton & Hui, 2011). For the purpose of visualisation, small sample sizes were removed and data were rarefied to the smallest library size

prior to producing figures. Differences in overall fungal community composition were visualized using nonmetric multidimensional scaling (NMDS) on Bray–Curtis distances and a PERMANOVA was performed to determine how much variance in fungal community clustering can be explained by plant species, plant order and plant functional group (permutations = 10,000).

To investigate how the abundance of OTUs changed between plant functional group multivariate generalized linear models (MV-GLMs) were used (Wang *et al.*, 2012). A negative binomial mean-variance error family was used to account for over dispersion, a common property of count data. As described above heterogeneity in library sizes was accounted for by incorporating an offset term as the first fixed term in the model. Multivariate and unadjusted univariate P values were obtained by Wald tests, both using 10,000 Monte Carlo permutations.

All statistical, diversity and community analyses were conducted using the R statistical language version 3.3.1 with standard R libraries (R Core Team, 2020); the community analysis specific package “vegan” (Oksanen *et al.*, 2018) and statistical packages “stats” (R Team, 2016), “mvabund” (Wang *et al.*, 2012), and “MASS” (Ripley, 2011).

4.4 | Results

4.4.1 | Soil variables

4.4.1.1 | Norway

A total of 25 soil cores were taken from each site. Total Carbon (Table 4.2. LM; $F_{1,67}$ = 28.25, P < 0.001); cations sodium (Table 4.3. LM; $F_{1,71}$ = 9.42, P < 0.01), ammonium (LM; $F_{1,71}$ = 43.56, P < 0.001), magnesium (LM; $F_{1,71}$ = 17.97, P < 0.001) and calcium (LM; $F_{1,71}$ = 13.47, P < 0.001) and anions iodate (Table 4.4. LM; $F_{1,24}$ = 4.95, P < 0.05), lactate (LM; $F_{1,64}$ = 6.72, P < 0.05), chlorite (LM; $F_{1,70}$ = 10.99, P < 0.01), sulphate (LM; $F_{1,70}$ = 22.28, P < 0.001), phosphate (LM; $F_{1,70}$ = 18.98, P < 0.001) and gluconate (LM; $F_{1,50}$ = 4.09, P < 0.05) significantly differed between sites.

Table 4.2. Mean and standard error of pH, moisture and total carbon across three sampling sites in Norway. Significant differences between sites are highlighted in the column header by an asterisk (* <0.05; ** <0.01, *** <0.001).

Site		**pH**	**Total Carbon (mg/g) *****
A	**Mean**	5.17	28.01
	SE	0.12	3.50
B	**Mean**	4.91	31.66
	SE	0.08	2.65
C	**Mean**	4.93	60.72
	SE	0.11	5.77

Table 4.3. Mean and standard error of cations across three sampling sites in Norway. Significant differences between sites are highlighted in the column header by an asterisk (* <0.05; ** <0.01, *** <0.001).

Cations (umol/l)							
Site		**Na ****	**NH_4^+ *****	**K**	**Mg *****	**Mn**	**Ca *****
A	**Mean**	267.39	33.67	409.17	156.16	5.60	441.56
	SE	5.82	5.51	143.24	10.56	1.97	27.24
B	**Mean**	249.33	53.21	425.55	108.44	4.19	416.79
	SE	6.79	6.19	152.43	11.86	1.37	30.80
C	**Mean**	307.47	88.10	529.70	253.45	3.42	633.61
	SE	11.86	5.85	147.83	17.39	1.16	45.70

Table 4.4. Mean and standard error of anions across three sampling sites in Norway. Significant differences between sites are highlighted in the column header by an asterisk (* <0.05; ** <0.01, *** <0.001).

Anions (umol/l)					
Site		**NO_3-**	**NO_2−**	**P *****	**PO_3− 3**
A	Mean	48.08	6.1	1.45	1.99
	SE	4	3.75	0.31	0.3
B	Mean	47.36	4.05	2.11	0.69
	SE	3.58	2.38	1.28	0.19
C	Mean	42.78	5.15	22.77	2.25
	SE	4.95	3.07	5.6	0.35
Site		**SO_4^{2-} *****	**Cl-**	**Chlorite ****	**Acetate**
A	Mean	65.1	672.04	2.04	89.32
	SE	4.46	226.84	0.9	44.34
B	Mean	43.39	659.16	1.86	62.1
	SE	3.06	211.24	0.85	12.17
C	Mean	104.47	816.39	7.27	105.62
	SE	5.27	251.5	1.42	23.04
Site		**Fluoride**	**Lactate ***	**Formate**	**Butyrate**
A	Mean	18.64	33.55	94.28	1.37
	SE	3.02	6.25	18.9	0.18
B	Mean	11.17	32.82	127.42	1.09
	SE	1.35	6.25	25.44	0.13
C	Mean	16.66	59.98	159.06	1.18
	SE	1.1	8.3	32.31	0.2
Site		**Iodate ***	**Gluconate***	**Bromide**	**Succinate**
A	Mean	0.12	1.76	0.2	1.24
	SE	0.12	0.42	0.06	0.82
B	Mean	0.74	2.79	0.1	1.57
	SE	0.4	0.85	0.02	1.13
C	Mean	2.1	3.96	0.33	1.69
	SE	0.71	1.08	0.08	0.93

4.4.1.2 | UK

A total of 25 soil cores were taken from each site. Total Carbon (Table 4.5. LM; $F_{1,67}$ = 47.27, P < 0.001), cations sodium (Table 4.6. LM; $F_{1,68}$ = 31.93, P < 0.001), ammonium (LM; $F_{1,68}$ = 8.72, P < 0.01) and calcium (LM; $F_{1,68}$ = 53.38, P < 0.001) and anions fluoride (Table 4.7. LM; $F_{1,67}$ = 16.37, P < 0.001), acetate (LM; $F_{1,67}$ = 16.47, P < 0.001), lactate (LM; $F_{1,29}$ = 20.23, P < 0.001), phosphite (LM; $F_{1,67}$ = 14.91, P < 0.001), formate (LM; $F_{1,67}$ = 8.04, P < 0.01), chlorite (LM; $F_{1,67}$ = 12.37, P < 0.001), nitrite (LM; $F_{1,67}$ = 17.23, P < 0.001), sulphate (LM; $F_{1,67}$ = 45.57, P < 0.001), and phosphate (LM; $F_{1,67}$ = 10.22, P < 0.01) significantly differed between sites.

Table 4.5. Mean and standard error of pH, moisture and total carbon across three sampling sites in the UK. Significant differences between sites are highlighted in the column header by an asterisk (* <0.05; ** <0.01, *** <0.001).

Site		**pH**	**Moisture (%)**	**Total Carbon (mg/g) *****
A	**Mean**	6.27	30.55	52.07
	SE	0.07	1.77	3.28
B	**Mean**	5.80	25.33	32.11
	SE	0.10	0.65	2.20
C	**Mean**	5.43	20.15	26.46
	SE	0.10	0.73	2.11

Table 4.6. Mean and standard error of cations across three sampling sites in the UK. Significant differences between sites are highlighted in the column header by an asterisk (* <0.05; ** <0.01, *** <0.001).

Cations (umol/l)								
Site		**Na *****	**NH_4^+ ****	**K**	**Mg**	**Mn**	**Ca *****	**Li**
A	**Mean**	362.46	9.51	257.49	143.93	0.00	1631.73	2.01
	SE	14.63	3.87	138.31	14.57	0.00	140.73	0.46
B	**Mean**	316.36	24.43	635.08	271.66	0.00	914.39	3.59
	SE	8.83	4.08	153.47	26.84	0.00	73.54	0.57
C	**Mean**	277.05	32.54	826.17	141.05	1.93	631.01	0.86
	SE	8.46	7.34	422.11	17.54	1.30	58.66	0.28

Table 4.7. Mean and standard error of anions across three sampling sites in the UK. Significant differences between sites are highlighted in the column header by an asterisk (* <0.05; ** <0.01, *** <0.001).

Anions (umol/l)						
Site		**NO_3-**	**NO_2− *****	**P ****	**PO_{3-3} ***	**SO_4^{2-} *****
A	**Mean**	47.47	21.31	4.63	2.11	122.07
	SE	11.25	5.69	0.85	0.38	7.52
B	**Mean**	42.87	3.42	0.65	0.46	70.13
	SE	4.12	1.34	0.09	0.17	6.22
C	**Mean**	38.56	1.59	1.85	0.61	60.79
	SE	4.32	0.78	0.42	0.17	4.48
Site		**Cl-**	**Chlorite *****	**Acetate *****	**Fluoride *****	**Iodate**
A	**Mean**	692.62	30.09	129.06	16.47	0.70
	SE	345.22	9.55	23.99	2.51	0.29
B	**Mean**	1018.39	8.83	101.66	7.90	3.04
	SE	307.11	2.74	16.67	0.66	0.60
C	**Mean**	1021.71	2.59	34.46	7.46	0.56
	SE	529.09	0.91	7.49	0.83	0.23
Site		**Lactate *****	**Formate**	**Butyrate**	**Gluconate**	
A	**Mean**	138.13	168.05	0.31	1.99	
	SE	12.28	34.07	0.14	0.66	
B	**Mean**	93.86	175.80	0.58	1.36	
	SE	23.99	34.89	0.25	0.78	
C	**Mean**	42.13	54.41	0.48	1.41	
	SE	11.51	16.30	0.18	0.65	

4.4.2 | Root-associated fungi

The analysis of 80 roots samples from Norway (n = 3) and 107 root samples from the UK (n = 3) yielded 3972 fungal OTUs (4082 unrarefied), 2212 and 3030 OTUs were recovered from Norway and the UK respectively.

4.4.2.1 | Norway

In Norway, the Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota, and Zygomycota make up 66.87, 28.87, 0.51, 0.86 and 2.87 % of all fungal OTUs from all samples, respectively. The most abundant ten OTUs represented a total of 44.91 % of all detected ITS fungal sequences. Among these, OTU.999, OTU.1026, OTU.636, OTU.11 and OTU.1 are the five most abundant fungal OTUs across all sites. OTUs .999, .1026, .636 and .11 belong to the phylum Ascomycota, species *Pleosporales sp, Davidiella tassiana, Gnomonia comari* and *Penicillium novae-zeelandiae* respectively. OTU.1 was classified as *Trichosporon gracile,* belonging to the phylum Basidiomycota.

A total of 707 (31.96 %) fungal OTUs were assigned to a broad functional category. In this study there is strong focus on plant pathogens, in particular, root associated pathogens. In total, 58 OTUs were characterised as pathotrophs only whilst 423 OTUS were deemed to have mixed trophic modes (i.e. Pathotroph - Saprotroph, Symbiotroph) – of all potential pathotrophs, 199 OTUs were described as potential plant pathogens. In addition, 542 OTUs were characterised as potential symbiotrophs (145 symbiotrophs only) and 814 OTUs were characterised as potential saprotrophs (526 OTUs saprotrophs only).

4.4.2.2 | UK

In the UK, The Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota, and Zygomycota make up 72.26, 24.03, 0.71, 1.23 and 1.76 % of all fungal OTUs from all samples, respectively. The most abundant ten OTUs represented a total of 33.16 % of all detected ITS fungal sequences. Among these, OTU.1026, OTU.2104, OTU.74, OTU.2585 and OTU.776 are the five most abundant fungal OTUs across all sites. OTUs .1026, .2104, .74 and .2585 belong to the phylum Ascomycota, species *Davidiella tassiana, Diaporthaceae sp, Gaeumannomyces cylindrosporus* and unclassified (genus *Sordariales*). OUT.776 was classified as *Hydropisphaera sp* belonging to the Basidiomycota.

A total of 885 (29.2%) fungal OTUs were assigned to a broad functional category. In total, 83 OTUs were characterised as pathotrophs only whilst 423 OTUS were deemed to have mixed trophic modes (i.e. Pathotroph - Saprotroph, Symbiotroph) – of all potential pathotrophs, 199 OTUs were described as potential plant pathogens. In addition, 542 OTUs were characterised as potential symbiotrophs (135 symbiotrophs only) and 814 OTUs were characterised as potential saprotrophs (337 OTUs saprotrophs only).

4.4.3 | Alpha-diversity between Norway and the UK.

4.4.3.1 | Total fungal community

There were no significant differences between countries in total fungal richness (GLM; LR $_{72.73}$ = 0.3, P = 0.58. Norway; M = 104.04, SE = 3.49, UK; M = 107.31, SE = 6.8), diversity (LM; F $_{72.73}$ = 0.15, P = 0.7. Norway; M = 3.09, SE = 0.09, UK; M = 3.04, SE = 0.15) or evenness (LM; LR $_{72.73}$ = 0.03, P = 0.84. Norway; M = 0.87, SE = 0.01, UK; M = 0.84, SE = 0.03), (Figure 4.1).

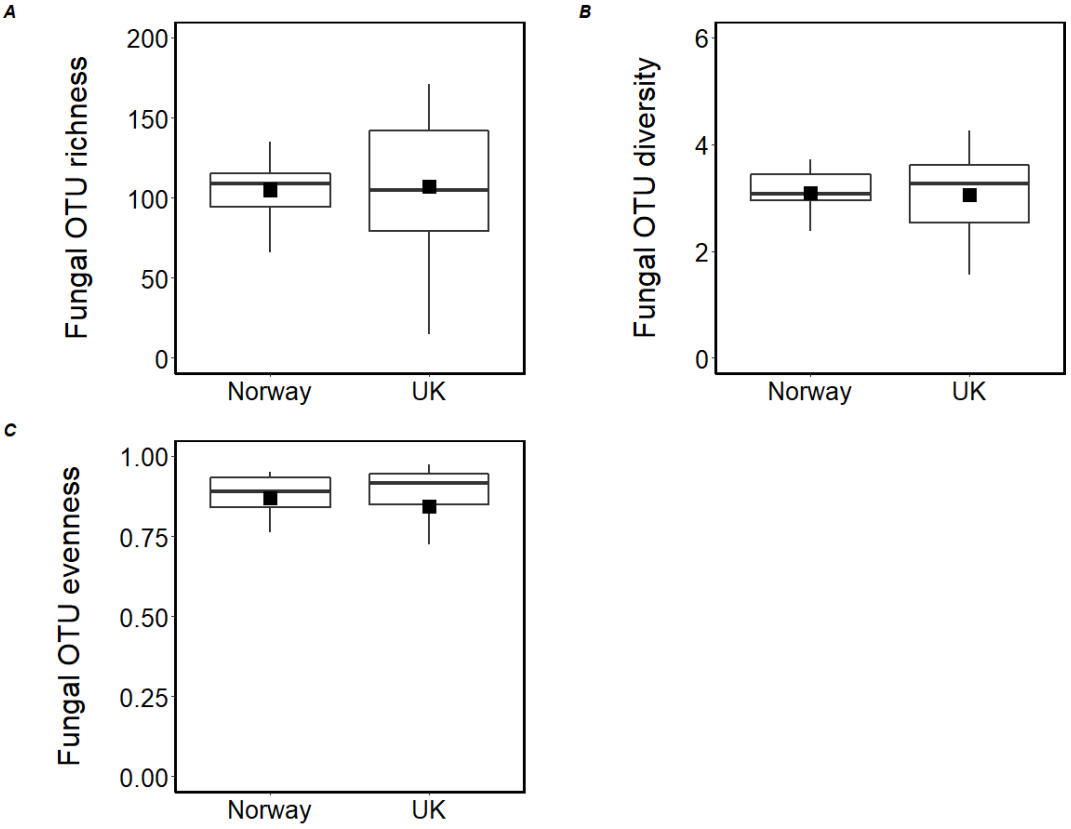


Figure 4.1. Root associated fungal community assemblies; A) OTU richness, B) OTU Shannon's Diversity Index, C) OTU Simpson's Diversity Index between Norway and the UK. Each box plot represents the median (horizontal line), mean (solid black square) and 25th to 75th percentiles, bars.

4.4.3.2 | Fungal guilds - richness

There were no significant differences between countries in pathogenic fungal richness (GLM; $LR_{72.73} = 0.2$, P = 0.65. Norway; M = 20.77, SE = 1.26, UK; M = 23.77, SE = 1.79) or saprotroph richness (LM; $LR_{72.73} = 0.33$, P = 0.57. Norway; M = 50.79, SE = 2.23, UK; M = 54.56, SE = 3.55). Symbiotrophs were significantly richer in Norway (LM; $LR_{72.73} = 4.74$, $P < 0.05$. Norway; M = 41.21, SE = 2.9, UK; M = 41.12, SE = 3.51) than the UK (Figure 4.2).

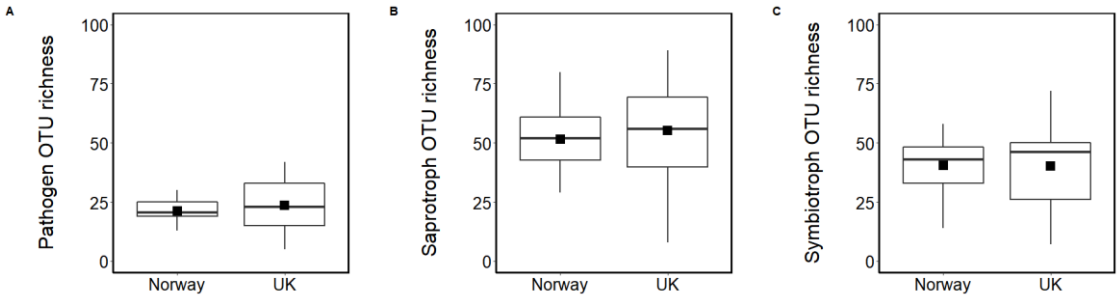


Figure 4.2. Root associated fungal taxonomic richness; A) Pathogens, B) Saprotrophs, C) Symbiotrophs between Norway and the UK. Each box plot represents the median (horizontal line), mean (solid black square) and 25th to 75th percentiles bars.

4.4.3.3 | Fungal guilds - diversity

There were no significant differences between countries in pathogenic fungal diversity (GLM; $LR_{72,73} = 0.97$, P = 0.33. Norway; M = 1.51, SE = 0.12, UK; M = 1.7, SE = 0.14), saprotrophic diversity (LM; $F_{72,73} = 0.29$, P = 0.59. Norway; M = 2.53, SE = 0.11, UK; M = 2.44, SE = 0.16) or symbiotroph diversity (LM; $F_{72,73} = 0.99$, P = 0.32. Norway; M = 2.22, SE = 0.16, UK; M = 2.16, SE = 0.15).

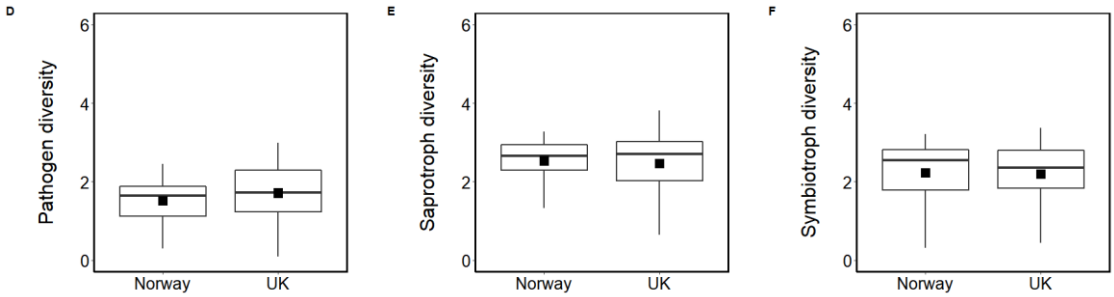


Figure 4.3. Root associated fungal taxonomic diversity; D) Pathogens, E) Saprotrophs, F) Symbiotrophs between Norway and the UK. Each box plot represents the median (horizontal line), mean (solid black square) and 25th to 75th percentiles bars.

4.4.3.4 | Fungal guilds - evenness

There were no significant differences between countries in the evenness between countries for fungal pathogens (GLM; $LR_{70,71} = 1.31$, P = 0.25. Norway; M = 0.61, SE = 0.04, UK; M = 0.65, SE = 0.04), saprotrophs (LM; $F_{72,73} = 0.39$, P = 0.54. Norway; M = 0.81, SE = 0.02, UK; M = 0.76, SE = 0.04) or symbiotrophs (LM; $F_{73,74} = 0.56$, P = 0.46. Norway; M = 0.74, SE = 0.04, UK; M = 0.72, SE = 0.04).

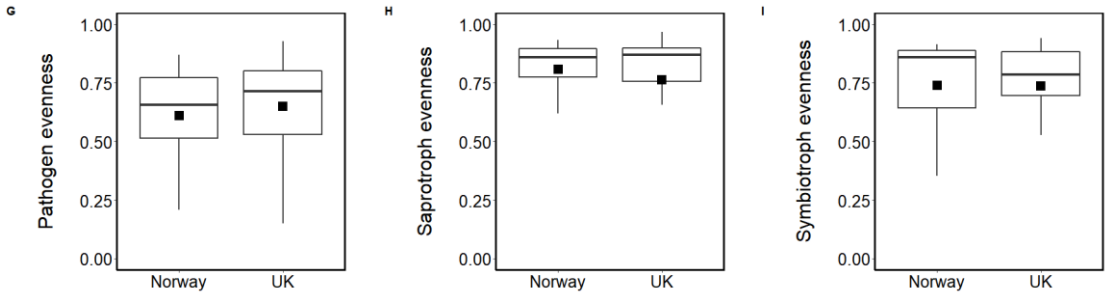


Figure 4.4. Root associated fungal taxonomic evenness; G) Pathogens, H) Saprotrophs, I) Symbiotrophs between Norway and the UK. Each box plot represents the median (horizontal line), mean (solid black square) and 25th to 75th percentiles bars.

4.4.4 | Alpha-diversity

4.4.4.1 | Norway - plant species effects

Total root-associated fungal community richness (GLM; $LR_{23,37}$ = 30.79, P < 0.01) significantly differed across plant species whilst diversity and evenness did not (Table 4.8. Figure 4.5). Only the Basidiomycota maintained differences in richness across plant species. Glomeromycota alpha-diversity measures did not differ across plant species. Regarding fungal functional guilds, only saprotrophic richness and evenness significantly differed across plant species.

Table 4.8. Linear-modelled results for plant species effects on fungal communities split by phyla and fungal guild for Norway. Plant pathogens refer to potential plant pathogens only. Significant results are highlighted in bold.

	Fungal phyla		
	Richness	Diversity	Evenness
Total	**$LR_{22,36}$ = 32.58, P < 0.01**	$F_{22,36}$ = 0.75, P = 0.7	$F_{22,36}$ = 0.57, P = 0.86
Ascomytota	$LR_{22,36}$ = 15.64, P = 0.33	$F_{22,36}$ = 0.37, P = 0.97	$F_{22,36}$ = 0.34, P = 0.97
Basidiomycota	**$LR_{22,36}$ = 38.03, P < 0.01**	$F_{22,36}$ = 1.72, P = 0.12	$F_{22,36}$ = 1.08, P = 0.42
Chytridiomycota	$LR_{18,31}$ = 12.26, P = 0.51	$F_{18,31}$ = 0.97, P = 0.51	$F_{14,27}$ = 1.01, P = 0.49
Zygomycota	$LR_{20,33}$ = 21.85, P = 0.06	$F_{20,33}$ = 1.17, P = 0.36	$F_{19,32}$ = 1.31, P = 0.29
Glomeromycota	$LR_{18,30}$ = 13.29, P = 0.34	$F_{18,30}$ = 0.83, P = 0.62	$F_{18,30}$ = 0.82, P = 0.63
Fungal guilds			
Pathogens	$LR_{22,36}$ = 14.36, P = 0.42	$F_{22,36}$ = 1.04, P = 0.45	$F_{21,35}$ = 1.05, P = 0.44
Symbiotrophs	$LR_{22,36}$ = 12.32, P = 0.58	$F_{22,36}$ = 0.58, P = 0.84	$F_{22,36}$ = 0.59, P = 0.84
Saprotrophs	**$LR_{22,36}$ = 35.76, P < 0.01**	$F_{22,36}$ = 1.56, P = 0.17	**$F_{22,36}$ = 2.27, P < 0.05**

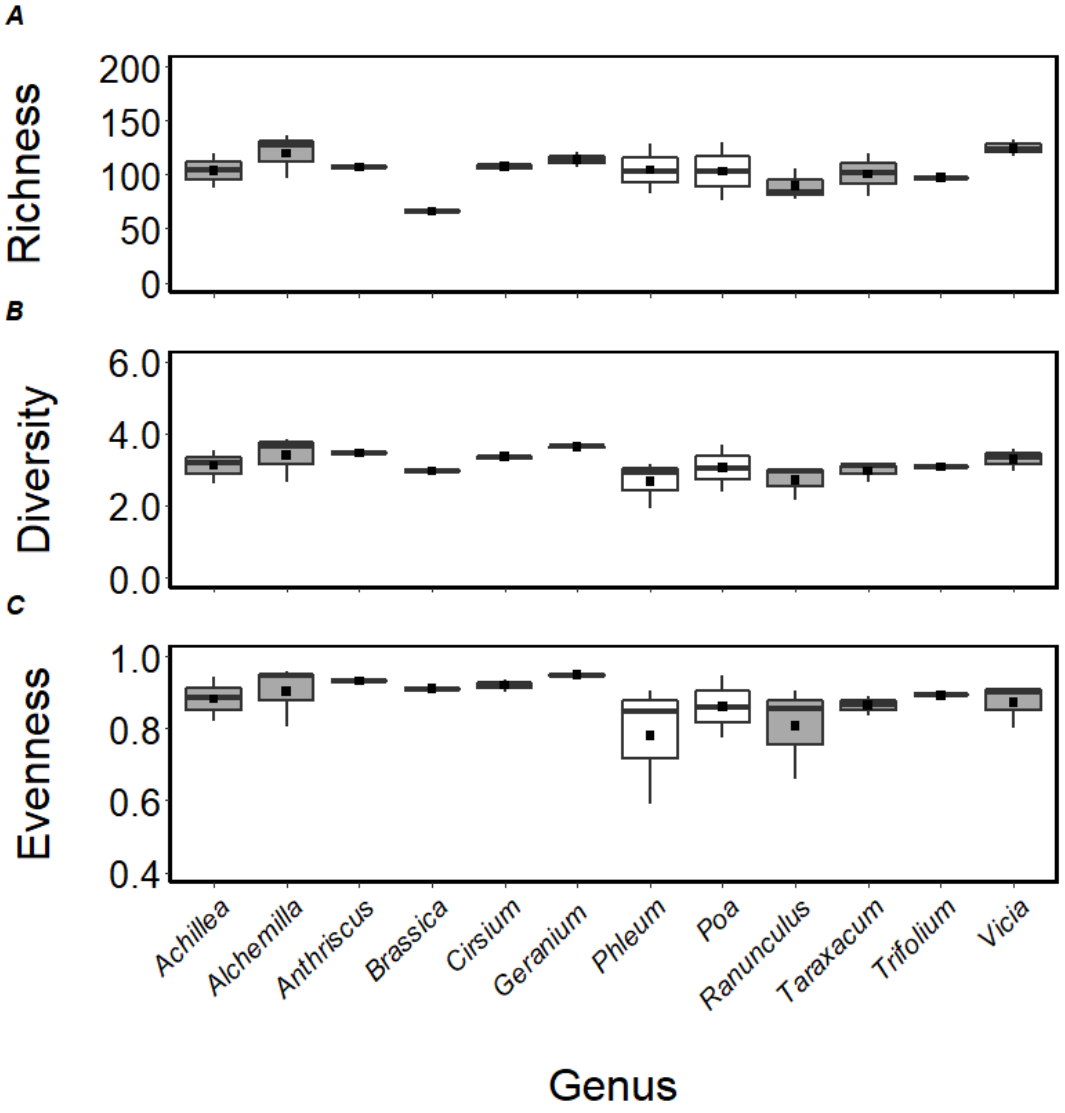


Figure 4.5. Plant-species specific total fungal communities assemblies; A) OTU richness, B) OTU Shannon's Diversity Index, C) OTU Simpson's Diversity Index across plant species across each 3 natural grassland sites in Norway. Each box plot represents the median (horizontal line), mean (solid black square) and 25th to 75th percentiles, bars. Grey boxplots; forbs, white boxplots; grasses.

4.4.4.2 | Norway - plant functional group effects

When OTUs were modelled with plant functional group the total fungal community differed across OTU diversity only ($F_{35,36} = 5.49$, $P < 0.05$). Saprotrophs were the only functional guild to differ between forbs and grasses. Overall, the richness, diversity and evenness of the total fungal community and saprotrophs were highest in grasses (Table 4.9).

Table 4.9. Linear-modelled results for plant functional group effects on fungal communities split by phyla and fungal guild for Norway. Plant pathogens refer to potential plant pathogens only. Significant results are highlighted in bold.

	Fungal phyla		
	Richness	**Diversity**	**Evenness**
Total	$LR_{35,36} = 3.57$, P = 0.06	**$F_{35,36} = 5.49$, P < 0.05**	$F_{35,36} = 3.13$, P = 0.08
Ascomytota	$LR_{35,36} = 0.54$, P = 0.46	$F_{35,36} = 1.97$, P = 1.17	$F_{35,36} = 1.91$, P = 0.17
Basidiomycota	$LR_{35,36} = 1.84$, P = 0.17	$F_{35,36} = 2.13$, P = 0.15	$F_{35,36} = 0.99$, P = 0.32
Chytridiomycota	$LR_{30,31} = 0.04$, P = 0.84	$F_{30,31} = 0.01$, P = 0.93	$F_{26,27} = 0.25$, P = 0.61
Zygomycota	$LR_{32,33} = 1.56$, P = 0.21	$F_{32,33} = 1.92$, P = 0.17	$F_{31,32} = 0.8$, P = 0.37
Glomeromycota	$LR_{30,29} = 0.61$, P = 0.43	$F_{29,30} = 0.05$, P = 0.82	$F_{29,30} = 0.19$, P = 0.66
	Fungal guilds		
Pathogens	$LR_{35,36} = 0.57$, P = 0.44	$F_{35,36} = 0.18$, P = 0.67	$F_{34,35} = 0.00$, P = 0.95
Symbiotrophs	$LR_{35,36} = 0.12$, P = 0.73	$F_{35,36} = 0.00$, P = 0.94	$F_{35,36} = 0.03$, P = 0.86
Saprotrophs	**$LR_{35,36} = 5.59$, P < 0.05**	**$F_{35,36} = 9.84$, P < 0.01**	**$F_{35,36} = 10.42$, P < 0.01**

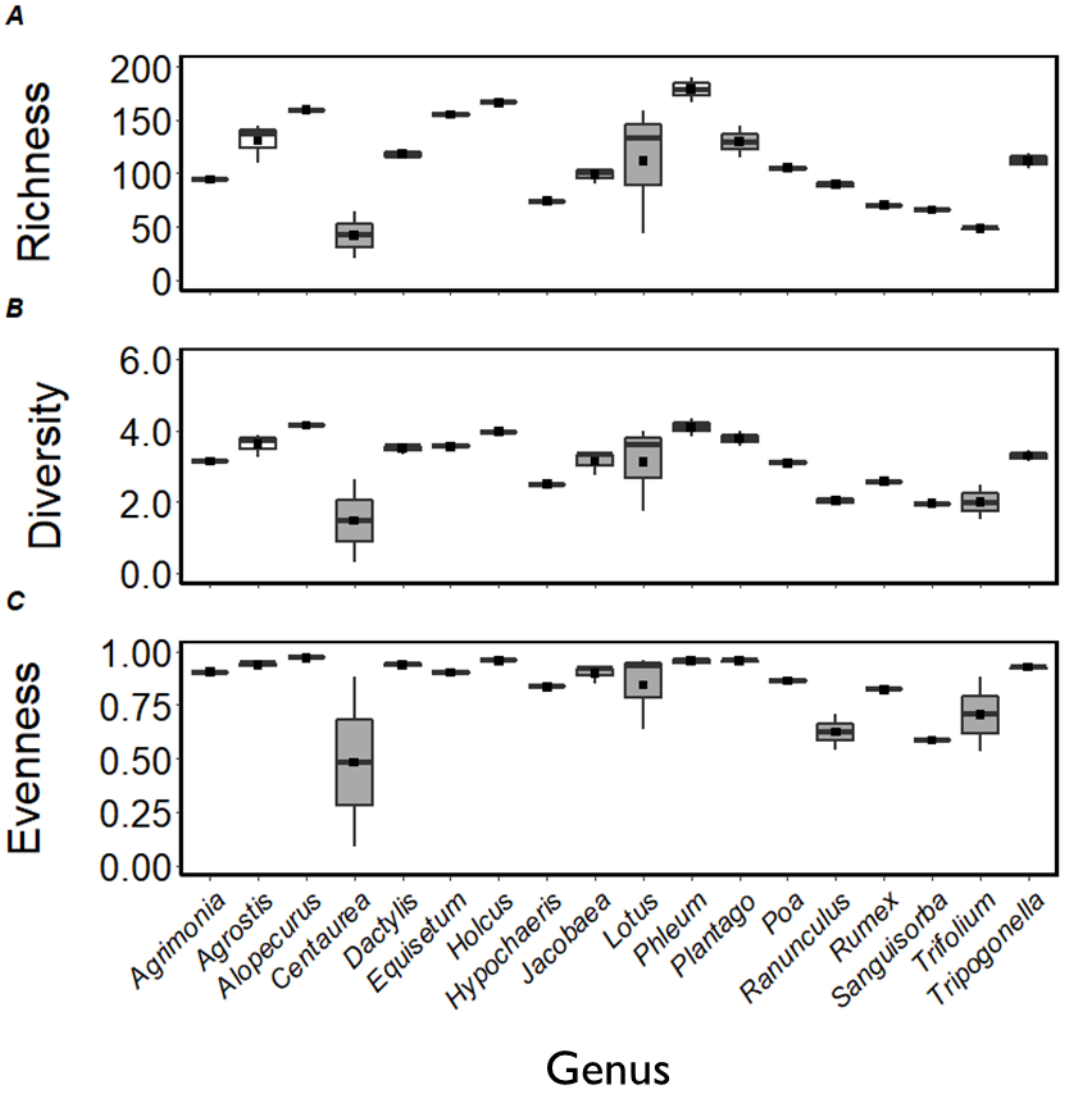
4.4.4.3 | UK - plant species effects

Total root-associated fungal community richness and evenness significantly differed across plant species whilst diversity met marginal significance (Table 4.10. Figure 4.6). Differences in richness were maintained across the Ascomycota and Basidiomycota. The Glomeromycota did not differ in alpha-diversity across plant species. Regarding fungal functional guilds, OTU richness differed across plant species for all fungal guilds (Table 4.10), but no differences were found for measures of diversity or evenness.

Table 4.10. Linear-modelled results for plant species effects on fungal communities split by phyla and fungal guild for the UK. Plant pathogens refer to potential plant pathogens only. Significant results are highlighted in bold.

UK	**Fungal phyla**		
	Richness	**Diversity**	**Evenness**
Total	**$LR_{11,35}$ = 64.58, P < 0.001**	$F_{11,35}$ = 2.41, P = 0.06	**$F_{11,35}$ = 2.74, P < 0.05**
Ascomytota	**$LR_{11,35}$ = 60.27, P < 0.001**	**$F_{11,35}$ = 24.21, P < 0.001**	**$F_{11,35}$ = 3.39, P < 0.05**
Basidiomycota	**$LR_{11,35}$ = 72.2, P < 0.001**	**$F_{11,36}$ = 38.81, P < 0.001**	**$F_{11,35}$ = 2.88, P < 0.05**
Chytridiomycota	$LR_{10,31}$ = 31.88, P = 0.06	$F_{10,31}$ = 2.34, P = 0.08	$F_{8,27}$ = 2.64, P = 0.08
Zygomycota	$LR_{11,33}$ = 3.59, P = 0.08	$F_{11,34}$ = 1.45, P = 0.26	$F_{10,31}$ = 1.19, P = 0.4
Glomeromycota	$LR_{9,32}$ = 22.76, P = 0.47	$F_{9,32}$ = 0.34, P = 0.98	$F_{8,29}$ = 0.77, P = 0.7
	Functional guilds		
Pathogens	**$LR_{11,35}$ = 48.46, P < 0.01**	$F_{11,35}$ = 1.19, P = 0.39	$F_{11,34}$ = 1.22, P = 0.37
Symbiotrophs	**$LR_{11,35}$ = 79.47, P < 0.001**	$F_{11,35}$ = 2.21, P = 0.08	$F_{11,35}$ = 2.59, P = 0.051
Saptrotrophs	**$LR_{11,35}$ = 60.27, P < 0.001**	$F_{11,35}$ = 1.36, P = 0.31	$F_{11,35}$ = 1.48, P = 0.25

Figure 4.6. Plant-species specific total fungal communities assemblies; A) OTU richness, B) OTU Shannon's Diversity Index, C) OTU Simpson's Diversity Index across plant species across each 3 natural grassland sites in the UK. Each box plot represent. Each box plot represents the median (horizontal line), mean (solid black square) and 25th to 75th percentiles, bars. Grey boxplots; forbs, white boxplots; grasses.



4.4.4.4 | UK - plant functional group effects

When OTUs were modelled between plant functional group the total fungal community differed across diversity (LM; $F_{34,35} = 9.13$, $P < 0.01$) and evenness (LM; $F_{34,35} = 9.63$, $P < 0.01$) only. This relationship was maintained across the phylum Ascomycota and Basidiomycota, the Basidiomycota differed in OTU richness also (GLM; $LR_{34,35} = 4.72$, $P < 0.05$). Overall, all measures of alpha-diversity were higher in grasses than forbs. Regarding fungal functional guilds, all measures of alpha diversity significantly differed between plant functional group symbiotrophs and saprotrophs. In contrast, evenness was the only alpha diversity measure for pathogenic communities that differed between plant functional group. Again, grasses yielded significantly higher measures of alpha-diversity.

Table 4.11. Linear-modelled results for plant functional group effects on fungal communities split by phyla and fungal guild for the UK. Plant pathogens refer to potential plant pathogens only. Significant results are highlighted in bold.

	Fungal phyla		
	Richness	Diversity	Evenness
Total	$LR_{34,35}$ = 2.58, P = 0.11	**$F_{34,35}$ = 9.13, P < 0.01**	**$F_{34,35}$ = 9.63, P < 0.01**
Ascomycota	$LR_{34,35}$ = 1.83, P = 0.18	**$F_{34,35}$ = 6.14, P < 0.05**	**$F_{34,35}$ = 6.09, P < 0.05**
Basidiomycota	**$LR_{34,35}$ = 4.72, P < 0.05**	**$F_{34,35}$ = 5.97, P < 0.05**	**$F_{34,35}$ = 5.52, P < 0.05**
Chytridiomycota	$LR_{30,31}$ = 2.96, P = 0.08	$F_{30,31}$ = 1.1, P = 0.3	$F_{26,27}$ = 2.23, P = 0.15
Zygomycota	$LR_{33,34}$ = 0.00, P = 0.95	$F_{33,34}$ = 0.29, P = 0.59	$F_{30,31}$ = 0.00, P = 0.98
Glomeromycota	$LR_{31,32}$ = 0.51, P = 0.47	$F_{31,32}$ = 0.75, P = 0.39	$F_{28,29}$ = 0.01, P = 0.92
	Fungal guilds		
Pathogens	$LR_{34,35}$ = 3.21, P = 0.07	$F_{34,35}$ = 3.83, P = 0.06	**$F_{33,34}$ = 8.29, P < 0.01**
Symbiotrophs	**$LR_{34,35}$ = 9.43, P < 0.01**	**$F_{34,35}$ = 10.31, P < 0.01**	**$F_{34,35}$ = 6.57, P < 0.05**
Saprotrophs	**$LR_{34,35}$ = 6.07, P < 0.05**	**$F_{34,35}$ = 12.6, P < 0.01**	**$F_{34,35}$ = 11.83, P < 0.01**

4.4.5 | Beta-diversity: composition

4.4.5.1 | Total fungal composition - plant species effects

In Norway the total fungal composition did not differ significantly across plant species or any higher taxonomic level (Figure 4.7 - A). Zygomycota demonstrated distinct fungal communities when explored for plant family (PERMANOVA; $F_{7,22} = 1.27$, $P < 0.05$) and plant order (PERMANOVA ; $F_{7,22} = 1.27$, $P < 0.05$) effects (Appendix 4.1).

In the UK, the total fungal composition differed significantly across all plant taxonomic ranks (Figure 4.7 – B), these results were conserved across Ascomycota. Distinct fungal compositions were found for; Basidiomycota (genus PERMANOVA; $F_{17,29} = 1.09$, $P < 0.05$, family PERMANOVA; $F_{7,29} = 1.19$, $P < 0.01$, order PERMANOVA; $F_{7,29} = 1.19$, $P < 0.01$), Chytridiomycota (family PERMANOVA; $F_{5,19} = 1.5$, $P < 0.01$, genus; $F_{5,19} = 1.5$, $P < 0.01$) and Glomeromycota (family PERMANOVA; $F_{5,18} = 1.33$, $P < 0.05$, order PERMANOVA; $F_{5,18} = 1.33$, $P < 0.05$), (Appendix 4.2.)

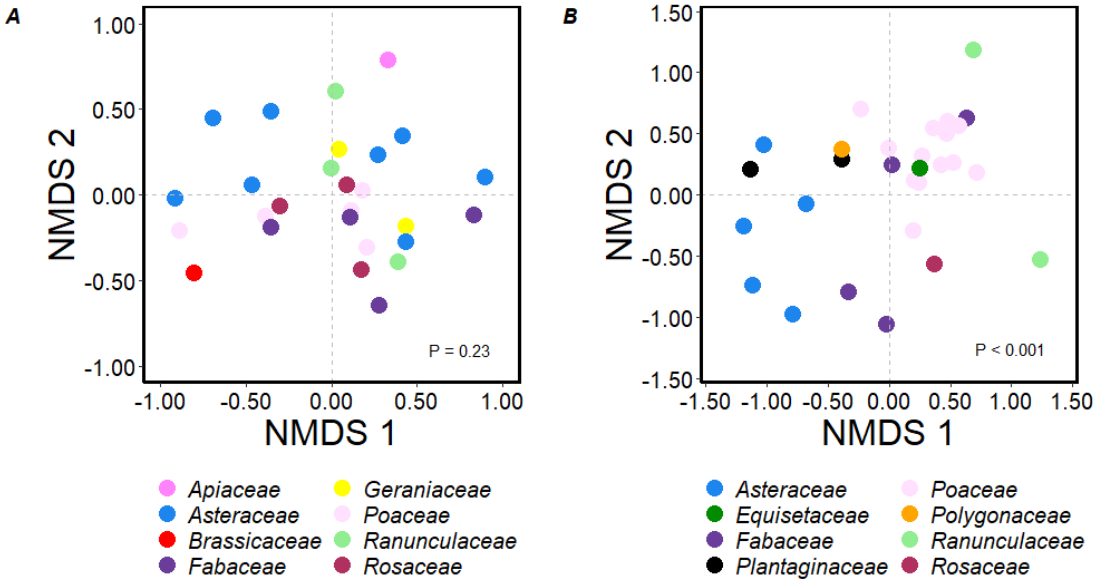


Figure 4.7. Plant family effects for plant-species specific root-associated total fungal communities across 3 natural grassland sites in A) Norway (stress = 0.1790) and B) the UK (stress = 0.1857). Nonmetric multidimensional scaling (NMDS) ordinations, based on Bray's distance matrix of the total fungal community.

4.4.5.2 | Total fungal composition; plant functional group effects

In Norway, differences in fungal communities between plant functional group were approaching significance only for the total fungal community (Figure 4.8; A - PERMANOVA; $F_{1,26} = 1.27$, P = 0.06) and the Basidiomycota (PERMANOVA; $F_{1,29} = 1.29$, P = 0.058).

In the UK, distinct fungal communities between plant functional groups were found for the total fungal community (Figure 4.8; B - PERMANOVA; $F_{1,31} = 3.44$, P <0.001) and the Ascomycota (PERMANOVA; $F_{1,33} = 4.39$, P <0.001), Basidiomycota (PERMANOVA; $F_{1,29} = 1.72$, P < 0.001), Zygomycota (PERMANOVA; $F_{1,20} = 2.54$, P <0.01) and Glomeromycota (PERMANOVA; $F_{1,18} = 1.78$, P <0.05).

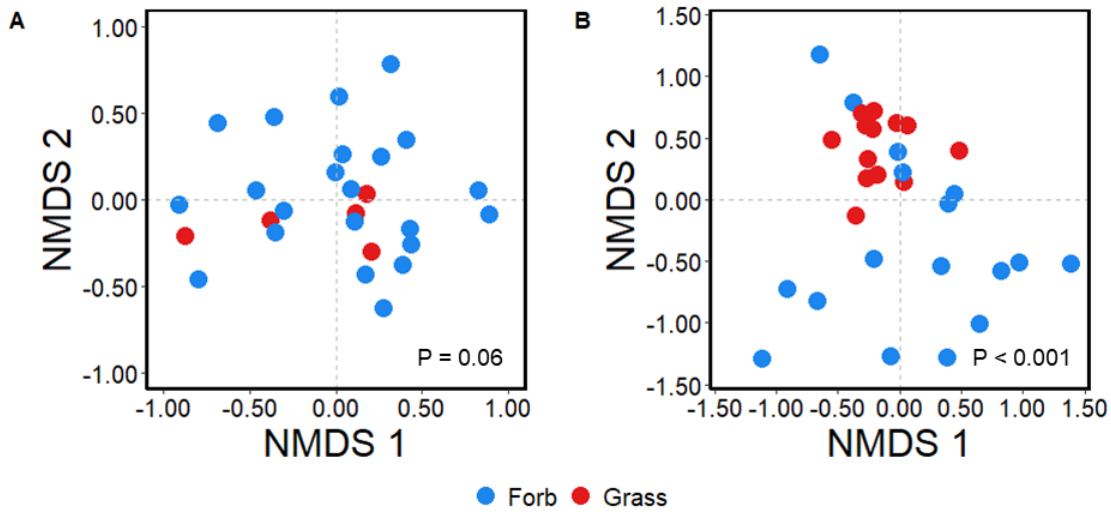


Figure 4.8. Plant functional group effects for plant-species specific root-associated total fungal communities across 3 natural grassland sites in A) Norway (stress: 0.1857) and B) the UK (stress: 0.1790). Nonmetric multidimensional scaling (NMDS) ordinations, based on Bray's distance matrix of the total fungal community.

4.4.6 | Fungal composition by guild.

4.4.6.1 | Norway

In Norway, there were no distinct fungal communities of pathogens (Figure 4.9 - A) or symbiotrophs across any plant taxonomic level, but saprotrophs were compositionally distinct between grasses and forbs (Table 4.12. Figure 4.9). Compositional analysis of pathogenic fungal between plant functional group are depicted in Figure 4.10 – A.

Table 4.12. PERMANOVA analysis of Norway for assemblies of pathogenic, symbiotic and saprotrophic fungal OTUS across differing plant taxonomic levels and between plant functional group. Significant results are highlighted in bold. (Grasses belong to a singular family so PERMANOVA not possible).

	Pathogens	**Pathogens within forbs**	**Pathogens within grasses**	**Symbiotro-phs**	**Saprotro-phs**
Plant sp.	$F_{10,21} = 1.02$, P = 0.38	$F_{8,17} = 1.15$, P = 0.09	$F_{1,3} = 1.1$, P = 0.33	$F_{11,23} = 1.01$, P = 0.41	$F_{12,27} = 1.06$, P = 0.19
Plant genus	$F_{9,21} = 1.02$, P = 0.41	$F_{7,17} = 1.14$, P = 0.11	$F_{1,3} = 1.1$, P = 0.33	$F_{10,23} = 1.01$, P = 0.43	$F_{11,27} = 1.09$, P = 0.13
Plant family	$F_{6,21} = 1$, P = 0.45	$F_{5,17} = 1.11$, P = 0.19	-	$F_{7,23} = 1.11$, P = 0.11	$F_{7,27} = 1.9$, P = 0.12
Plant order	$F_{6,21} = 1$, P = 0.45	$F_{5,17} = 1.11$, P = 0.19	-	$F_{7,23} = 1.11$, P = 0.11	$F_{7,27} = 1.9$, P = 0.12
Plant group	$F_{1,21} = 1.1$, P = 0.26	-	-	$F_{1,23} = 1.23$, P = 0.12	**$F_{1,27} = 1.42$, P < 0.05**

4.4.6.2 | UK

In the UK, species-specific pathogenic communities were found when data were explored for plant family (Figure 4.9 – B. PERMANOVA; $F_{7,25} = 1.28$, $P < 0.01$) and plant order (PERMANOVA; $F_{7,25} = 1.28$, $P < 0.01$). Though, pathogens within species of forbs only were distinct at the plant species (PERMANOVA; $F_{7,12} = 1.74$, $P < 0.05$) and plant genus (PERMANOVA; $F_{8,12} = 1.4$, $P < 0.05$) level. Pathogens within grasses were not plant species-specific at any taxonomic level. Symbiotrophs and saprotrophs differed across all plant taxonomic levels, and all guilds of fungi (pathogens, symbiotrophs and saprotrophs) differed between plant functional group (Table 4.13, Figure 4.10 – B).

Table 4.13. PERMANOVA analysis of the UK for assemblies of pathogenic, symbiotic and saprotrophic fungal OTUS across differing plant taxonomic levels and between plant functional group. Significant results are highlighted in bold. (Grasses belong to a single family so PERMANOVA not possible).

	Pathogens	**Pathogens within forbs**	**Pathogens within grasses**	**Symbiotrophs**	**Saprotrophs**
Plant species	$F_{17,25}$ = 1.06, P = 0.23	**$F_{7,12}$ = 1.74, P < 0.05**	$F_{7,12}$ = 0.71, P = 0.98	**$F_{19,26}$ = 1.26, P < 0.05**	**$F_{21,31}$ = 1.18, P < 0.05**
Plant genus	$F_{15,25}$ = 1.08, P = 0.18	**$F_{8,12}$ = 1.4, P < 0.05**	$F_{6,12}$ = 71, P = 0.98	**$F_{16,26}$ = 1.21, P < 0.05**	**$F_{17,31}$ = 1.2, P < 0.05**
Plant family	**$F_{7,25}$ = 1.28, P < 0.01**	$F_{6,12}$ = 1.15, P = 0.13	-	**$F_{7,26}$ = 1.37, P < 0.001**	**$F_{7,31}$ = 1.51, P < 0.001**
Plant order	**$F_{7,25}$ = 1.28, P < 0.01**	$F_{6,12}$ = 1.15, P = 0.13	-	**$F_{7,26}$ = 1.37, P < 0.001**	**$F_{7,31}$ = 1.51, P < 0.001**
Plant group	**$F_{1,25}$ = 1.95, P < 0.01**	-	-	**$F_{1,26}$ = 2.06, P < 0.001**	**$F_{1,31}$ = 2.76, P < 0.001**

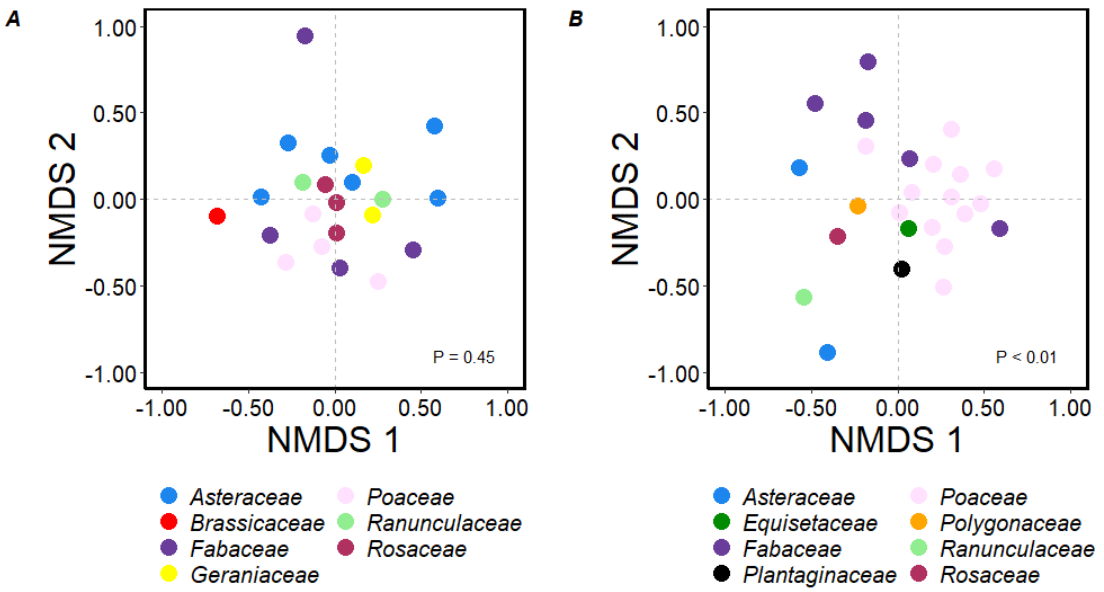


Figure 4.9. Plant family effects for plant-species specific root-associated pathogenic fungal communities across three natural grassland sites in A) Norway (stress: 0.1972) and B) the UK (stress: 0.2175). Nonmetric multidimensional scaling (NMDS) ordinations, based on Bray's distance matrix of the pathogenic fungal community.

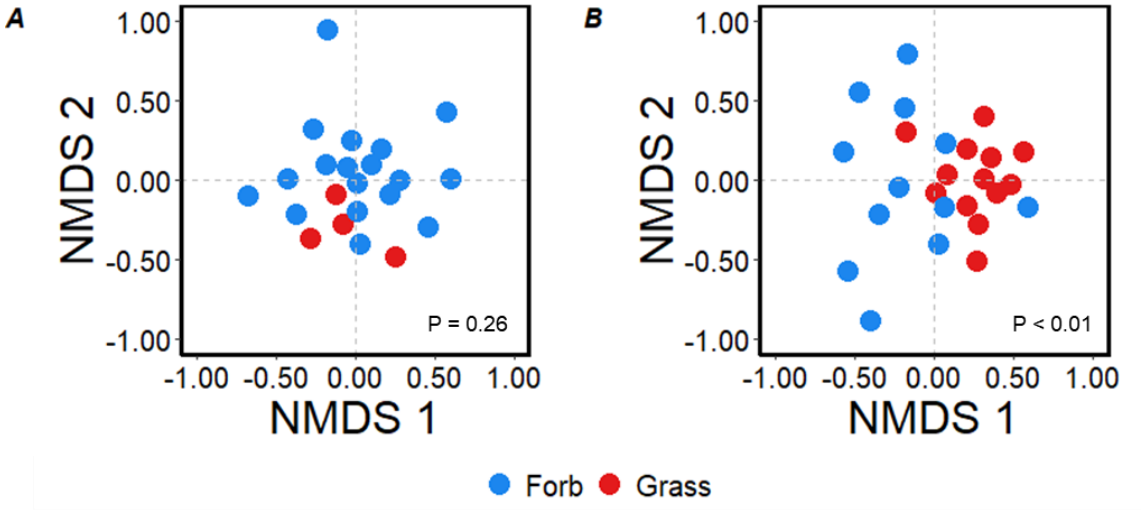


Figure 4.10. Plant functional group effects for plant-species specific root-associated pathogenic fungal communities across three natural grassland sites in A) Norway (0.1972) and B) the UK (0.2175). Nonmetric multidimensional scaling (NMDS) ordinations, based on Bray's distance matrix of the pathogenic fungal community.

4.4.7 | MV-GLM results

For this analysis, Norway was set as the intercept to reveal differences in single pathogenic taxa as Norway demonstrated lack of host specificity within the pathogenic fungal community. Multivariate modelling revealed pathogenic fungal OTU abundances significantly differed between Norway (low plant diversity) and the UK (intermediate plant diversity) after controlling for unequal sequencing depths (103 OTUs; Wald = 32.99, $P < 0.001$). A total of 138 OTUs occurred > 1 % in the overall dataset. Of all the OTUs affected by country, 54 OTUs were significantly more abundant (Appendix 4.3) and 49 OTUs were significantly less abundant in the UK in comparison to Norway.

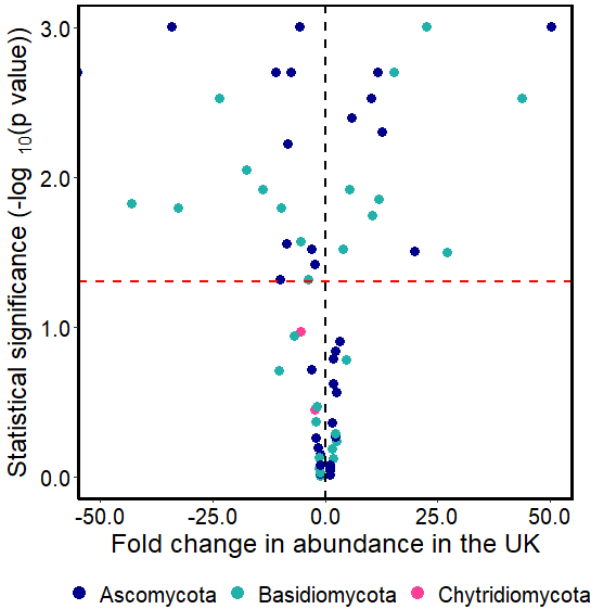


Figure 4.11. Volcano plot of MV-GLM modelled shifts in pathogenic fungal phyla relative abundances in the UK when compared to Norway. Coloured points above the horizontal dotted line represent phyla that showed statistically significant shifts in abundance ($P < 0.01$), whereas points below the line represent phyla that did not show statistically significant shifts ($P > 0.05$). Phyla further to the left were statistically less abundant while phyla to the right were statistically more abundant in the UK than when compared to Norway.

4.4.8 | Chapter 3 and 4 results summary

Below I have provided a summary of both alpha diversity and beta diversity measures across the three European field sites in Slovenia (Chapter 3), Norway and the UK.

4.4.8.1 | Alpha diversity

Table 4.14. Summary of significant plant species specific and plant functional group specific fungal communities across Norway (N), the United Kingdom (UK) and Slovenia (S). Countries are listed from the least diverse, to the most diverse. Significant results are indicated by a tick (✓).

	Alpha diversity								
	Richness			**Diversity**			**Evenness**		
	N	**UK**	**S**	**N**	**UK**	**S**	**N**	**UK**	**S**
	Total								
Plant sp.	✓	✓	✓					✓	
Functional Group				✓	✓	✓		✓	✓
	Pathogens								
Plant sp.		✓	✓						
Functional Group						✓		✓	✓
	Saprotrophs								
Plant sp.	✓	✓	✓			✓	✓		
Functional Group	✓	✓		✓	✓		✓	✓	✓
	Symbiotrophs								
Plant sp.		✓	✓						
Functional Group		✓			✓	✓		✓	✓

4.4.8.1 | Beta diversity

Table 4.15. Summary of distinct fungal communities for Norway (N), the United Kingdom (UK) and Slovenia (S) across all plant taxonomic levels. Countries are listed from the least diverse, to the most diverse. Significant results are indicated by a tick (✓).

	Beta diversity			**Beta diversity**		
	N	**UK**	**S**	**N**	**UK**	**S**
	Total			**Saprotrophs**		
Plant sp.		✓	✓		✓	✓
Plant Genus		✓	✓		✓	✓
Plant Family		✓	✓		✓	✓
Plant Order		✓	✓		✓	✓
Functional Group		✓	✓	✓	✓	
	Pathogens			**Symbiotrophs**		
Plant sp.			✓		✓	✓
Plant Genus			✓		✓	✓
Plant Family		✓	✓		✓	✓
Plant Order		✓	✓		✓	✓
Functional Group		✓	✓		✓	
	Pathogens within forbs					
Plant sp.		✓				
Plant Genus		✓				
Plant Family			✓			
Plant Order			✓			
Functional Group		NA	NA			
	Pathogens within grasses					
Plant sp.						
Plant Genus						
Plant Family						
Plant Order		NA	NA			
Functional Group		NA	NA			

4.5 | Discussion

Within this study, plant-fungal interactions and host-specific relationships across a gradient of aboveground diversity in Europe were explored. To do this, direct comparisons were made between two grassland ecosystems, Norway and the UK, and differences between patterns and trends from a hyperdiverse grassland ecosystem as studied in Chapter 3 were also inferred. Comparing results from Chapter 3 (a hyperdiverse grassland ecosystem) and this chapter, Chapter 4 (offering an intermediate and low diversity ecosystem), revealed the presence of pathogenic host-specificity differs across an aboveground diversity gradient. Because host specificity of pathogenic fungi were absent from a low diversity ecosystem, this could suggest host-specific pathogenic fungi are a pre-requisite for diversity and coexistence. This finding provides evidence to support the theory that plant diversity is maintained by disproportionate suppression of rare communities (Klironomos, 2002; Mangan *et al.*, 2010). In contrast, while host-specificity of fungal pathogens, indicative of negative PSF, are absent in low diversity ecosystems, distinct saprotrophic communities between plant functional groups could be driving enhanced dominance of few species through plant responses to positive PSF. This study enhances our capacity to predict the strength and direction of PSF and vegetation dynamics by revealing fundamental differences of host-specific fungal guilds across an aboveground diversity gradient.

4.5.1 | Fungal diversity across a plant diversity gradient and large geographical scale

H1: Total fungal richness will increase with plant species richness.

H2: Fungal pathogens will decrease in diversity with increasing plant diversity.

As described in 4.2, it is sensible to predict a greater fungal diversity will occur in the UK because grassland ecosystems that are more diverse are also more diverse

in their available rhizodeposits (Philippot *et al.*, 2013). This relationship has been consolidated in alpine grasslands by Yang *et al.*, (2017). However, analysis of fungal alpha diversity measures between Norway (low plant diversity) and the UK (intermediate plant diversity) revealed no significant difference in overall fungal or pathogenic alpha-diversity. Nonetheless, multivariate modelling revealed a greater number of pathogenic taxa (OTUs) that occurred in significantly greater abundance in the UK, from a higher plant diversity, as compared to Norway, a lower plant diversity (Figure 4.11). However, this opposes the theory implied by Mommer *et al.*, (2018) underpinning both the 'pathogen-hypothesis' and 'biodiversity-productivity' relationship in which fungal diversity is lower in more diverse ecosystems due to the dilution of pathogens. As such, the data from this study shows the loss of pathogenic OTUs in more diverse grasslands is unlikely to be applicable universally to natural grassland ecosystems across a large geographical scale. It is probable the richer pathogenic diversity and abundance in the UK is from an increase in the diversity of species-specific rhizodeposits available. Moreover, symbiotrophs were marginally richer in Norway. Although this effect was only minimal, this could imply the importance of different fungal guilds could vary from grassland to grassland.

4.5.2 | Alpha-diversity

4.5.2.1 | Alpha-diversity: *Plant species effects*

H3: Total and pathogenic fungi will demonstrate host-specificity at all sites.

In both Norway and the UK, plant species was a significant driver of total fungal OTU richness. This pattern is consistent with results from a hyperdiverse grassland ecosystem as in Chapter 3. This implies plant species assimilate distinct root-associated fungal communities and importantly, this finding is consistent across all geographical locations suggesting this pattern is conserved both at large scales and across diversity gradients. Again, the Glomeromycota do not differ between plant

species at either field location and thus satisfies their ecologically accepted ubiquitous distribution (Smith & Read, 2008; Dumbrell *et al*., 2010). At this stage it is important to consider effects of edaphic properties on microbial composition, however, soil variables were analysed from soil cores taken from bulk soil, though it is empirical that bulk and rhizosphere soils offer unique environments (Buée *et al.*, 2009; Churchland & Grayston, 2014). Within context of this study, fungi were amplified from the rhizosphere meaning fungi are buffered from differences in the abiotic properties of the bulk soil. This implies plant identity, and differences in quality and quantities of root exudates, is a larger driver of rhizosphere associated fungal microbes than edaphic properties.

More importantly, and in context with this study, results concerning plant species effects on fungal guilds in the UK closely resembled those from a hyperdiverse grassland ecosystem. In both Slovenia (Chapter 3) and the UK, plant species effects were maintained across pathogens, saprotrophs and symbionts. This is in contrast to a low diversity ecosystem (Norway), where plant species effects were only apparent for OTU richness and evenness for saprotrophs. Interestingly, saprotrophs have also been documented for their host-specific effects in which there is an evidenced 'home-field advantage'. This implies plants recruit saprotrophs that preferentially decompose OM from their own species, and thus is beneficial for the survival and fitness members of their own host plant species (Ayres *et al.*, 2009; Miki, 2012; Asplund *et al.*, 2018). The overall outcome of this interaction is positive PSF. It is plausible to suggest saprotrophs may play a role in maintaining low-intermediate aboveground diversity by mediating enhanced positive PSF resulting in conspecific proliferation of plant populations. These results begin to suggest the importance of fungal guilds and the strength and direction of PSF may vary across a diversity gradient of grassland ecosystems. Whilst all fungal guilds likely share a role of significance in hyperdiverse ecosystems, the data here suggests saprotrophs and symbionts, microbes generally

considered to result in positive PSF (Ehrenfeld *et al.*, 2005), may play a more prominent role in less diverse ecosystems and thus govern the proliferation and dominance of a few plant species.

4.5.2.1 | Alpha-diversity: plant functional group effects

H4: Families of grasses and forbs accumulate compositionally distinct total and pathogenic fungal communities across a gradient of aboveground diversity.

The reason for investigating plant functional group effects is twofold. First, I have evidenced an effect of plant functional group in hyperdiverse grassland ecosystems, and second, it is important to investigate plant functional group effects because if present, these can have broad scale implications for shifts in taxonomic diversity at the plant group level. In Norway, plant functional group drove significant differences only in the saprotrophic community with all measures of saprotrophic alpha-diversity being significantly richer in grasses. This too aligns with saprotrophs being the only fungal guild to show plant species effects. This finding indicates species belonging to the order Poales (i.e. grasses) will endure more broad taxonomic shifts in vegetation diversity as a result of enhanced PSF. This could be why some grassland ecosystems are dominated by grasses. In comparison, in the UK all functional guilds significantly differed between grasses and forbs, again, all measures were highest in grasses. Whilst it is difficult to ascertain a general trend for plant functional group across a gradient of aboveground diversity, these results provide further support to imply there is a difference in the ecological importance of fungal guilds and their functional outcome operating within grasslands from differing aboveground diversity. Data from this study implies saprotrophs and the effects of positive PSF may play a greater role in driving vegetation dynamics in low diversity ecosystems. On the other hand, both negative and positive PSF are operating in tandem within intermediate grasslands. The presence of negative PSF in intermediate grassland ecosystems

might be the distinction between low and intermediate levels of aboveground plant diversity.

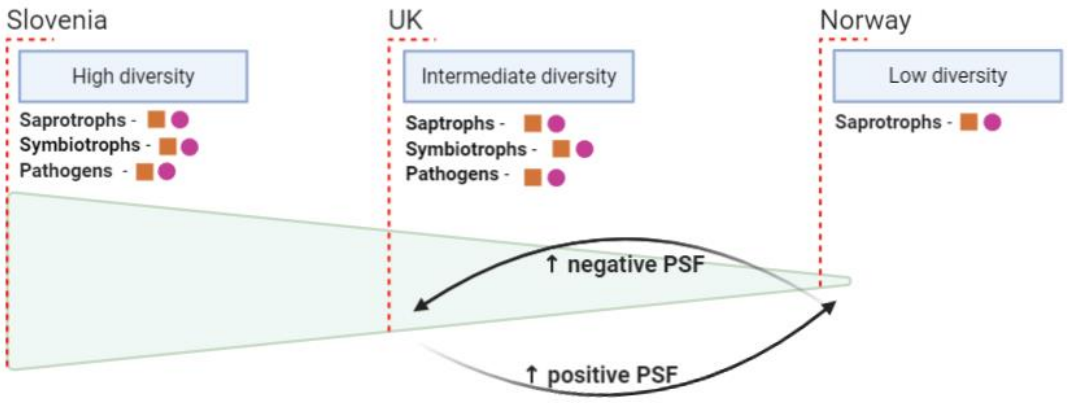


Figure 4.12. Schematic to illustrate the role of plant species and plant functional group effects on measures of alpha diversity for fungal guilds in the regulation of aboveground diversity. Combined results from this study including a hyperdiverse (Chapter 3), intermediate (UK) and low (Norway) diversity grassland ecosystem suggest increased negative PSF mediated by pathogens (an effect of plant functional group) potentially drives the difference between intermediate and low grasslands, whilst increased positive PSF driven by saprotrophs (determined by plant species effects) drives the difference between low and intermediate diversity grassland. As described in Chapter 3, an equal importance of both positive and negative feedback simultaneously drives ecosystems of high aboveground diversity. Orange squares indicate plant species effects, pink dots indicate plant functional group effects. This is important as it has consequences for either delicate species level shifts in vegetation dynamics, or broad scale shifts on plant taxonomic diversity between grasses and forbs.

4.5.3 | Beta diversity

H3: Total and pathogenic fungi will demonstrate host-specificity at all sites.

H4: Families of grasses and forbs accumulate compositionally distinct total and pathogenic fungal communities across a gradient of aboveground diversity.

In Norway, compositional differences in the total fungal community were only marginally significant between plant functional groups (Figure 4.8 - A). This was consistent, again at marginal significance, for members of the Basidiomycota. In align with this result, saprotrophs that largely belong to the phylum Basidiomycota, were the only functional guild to differ in composition between species of grasses and forbs. This too, aligns with alpha diversity measures in which the only fungal phyla and guild to differ between plant functional group and plant species in Norway were Basidiomycota and saprotrophs respectively. This provides further support to document saprotrophs, considered beneficial for ecosystem functioning for their role in decomposition (Bani *et al.*, 2018), might also be important for vegetation dynamics for their beneficial species-specific effects. However, because beta-diversity analysis showed saprotrophs are specific at plant functional group only, and not plant species, this could imply positive PSF is less sensitive to plant species and as such predicts grasses as a group will experience stronger effects of PSF regarding vegetation dynamics. This finding highlights the importance of host-specificity at different plant taxonomic ranks and the implications this has for vegetation dynamics. For example, it is plausible to predict shifts in vegetation dynamics will be more sensitive where host-specificity is present at the species level, as opposed to plant functional group. The results from this study begin to imply host-specificity of saprotrophs may also be relevant to grassland ecosystems. However, to substantiate this theory, plant growth bioassays that involve the isolation, inoculation and measurements of plant biomass would be required. Further exploration of fungal pathogens in Norway revealed no distinct composition between plant species, order, genus or family, nor between plant

functional group. Again, this aligns with measures of alpha diversity in which there were no host-specific effects for pathogens. Collectively, these results affirm the minimal role of fungal pathogens in low diversity ecosystems. However, replicated studies are required to validate this.

Compositional differences in the UK begin to more closely resemble those results from Chapter 3. The total fungal community and Ascomycota show distinct fungal communities at the plant species, genus, family, order level and plant functional group. Whilst members belonging to other fungal phyla harbour distinct fungal communities at taxonomic levels beyond plant species. More importantly, and in context with this study, distinct pathogenic communities are found at the taxonomic level of plant family, order and functional group. Akin to pathogenic composition in hyperdiverse grasslands, pathogens within grasses were not a function of any plant taxonomic level, whilst forbs harboured distinct fungal communities at the plant species and genus level. This provides further evidence to imply there is a phylogenetic signal for host specificity of fungal pathogens. Evidence for the importance of plant functional group for vegetation dynamics and productivity has been established since the 1990's (Hector *et al.*, 1999). This was an observational study, similar to such design used in this study, in which the aboveground biomass was quantified in eight European grassland sites. Hector showed ecosystems with fewer functional groups were less productive, and this was a function of both aboveground composition and geographical location. Whilst the taxonomic identity of the microbial players involved were not resolved at this stage, this early work supports broad scale shifts in plant composition are governed by shared species-specific microbial suites between plant functional groups. Furthermore, Cortois *et al.*, (2016) suggests differences in fungal communities within plant functional groups could be explained by plant functional traits such as competitive ability and dispersal strategy, though this would warrant further investigation. In contrast to Chapter 3, the UK also

held distinct fungal communities between plant functional group for symbiotrophic and saprotrophic taxa. This suggests the importance of positive PSF mediated by host-specificity of symbiotrophs and saprotrophs may be greater in grasslands from a low-intermediate diversity.

4.6 | Conclusion

Through the comparison of plant-fungal relationships across an aboveground plant diversity gradient, I have shown there is a difference in the prevalence of plant-fungal host specificity and the likely direction of PSF across an aboveground diversity gradient. The absence of fungal pathogen host specificity could explain why some grasslands demonstrate low levels of aboveground diversity. The lack of host-specificity from low diversity grasslands could be an outcome of abiotic constraints on pathogenic populations, such as precipitation, seasonality, soil infertility and unfavourable rooting conditions. In addition, these suboptimal conditions could drive enhanced allocation to antagonist defences, thus survival and subsequent attack by insects has little effect on plant fitness. The data from this study, and Chapter 3, strongly suggests there is phylogenetic signal of pathogenic fungi driven by functional group in high and intermediate diversity grasslands. This finding is critical to understanding and predicting plant vegetation dynamics because host specificity is underpinned by the taxonomic rank at which species-specific communities occur. Moreover, data from this study highlights the importance of quantifying all fungal groups to develop a fully encompassed understanding of grassland vegetation dynamics. Not only are pathogens important, but beneficial microbes should not be neglected for their role in the regulation of aboveground vegetation dynamics, specifically in low diversity ecosystems. This is further substantiated by Kardol et al., (2006) whom documents the retardation of plant succession when net response of PSF is positive. This could be why previous studies exploring fungal mediated regulation of grassland dynamics fails to see consistent results across a diversity

gradient. The findings from this study offer huge potential in predicting distributions of plant diversity within European temperate grasslands.

4.7 | Limitations

The reliability of guild assignment to fungal taxa and the requirement for species-specific testing remains an ongoing limitation throughout my thesis, see 3.9 for a more detailed description.

More specific to Chapter 4, the ability to draw reliable conclusions was limited as each data set, i.e. per country, were modelled independently from one another. This limitation was a product of COVID-19, commencing March 2020 *("results in this chapter were impacted by the COVID-19 lockdown beginning March 2020. Data were unable to be recovered as it was stored on servers that were inaccessible for the duration of lockdown").* Due to the size of the study (N = 650), ITS gene libraries were sequenced via Illumina Miseq technology on two separate occasions (Slovenia single run, UK and Norway sequenced in parallel). Whilst this is common practice to ensure sufficient read depth is attributed to each sample, under normal circumstances, the resultant raw sequences would be merged, dereplicated, clustered at 97 % similarity and finally, taxonomy would be assigned. However, this bioinformatic pipeline was confounded as raw sequence data could not be retrieved from local servers. For this reason, data was unamenable to reintegration because it carried a risk of overestimating phylogenetic diversity based on the presence of OTUs belonging to a single taxa occurring multiple times in the data set. Whilst I was able to confidently interpret differences within a region in fungal communities as determined by plant species through permutational Analysis of Variance (permANOVA), the potential power of this statistical approach was limited because I was not able to quantify the influence at a global scale, i.e. between region effects. The merging of raw OTU reads from Slovenia, the UK and Norway, followed by clustering and reassignment of OTUs

would improve the statistical power of subsequent permANOVA because it would enable the exploration of host-specific fungal communities and how this may or may not be conserved across a regional scale. The influence of region on microbial community composition is an important factor to consider as a predictor of fungal community composition as it is possible root-dwelling microbial suites may be a function of geographical location such as latitude or altitude. Although, studies exploring the relationship between geographic scale and microbial diversity have shown inconsistent results (Mueller *et al.*, 2007; Fierer *et al.*, 2012; Shi *et al.*, 2014; Tedersoo *et al.*, 2014). Nonetheless, the ability to address such ecological questions would help to broaden our knowledge concerning the ecological distribution of fungal pathogens and whether pathogenic host-specificity is conserved regionally across phylogenetically similar plant species.

Nonetheless, the challenges in this chapter did raise the interesting debate surrounding 'ASVs or OTUs?' The implementation of ASVs (amplicon sequence variants) or ESVs (exact sequence variants) follows advances in the ability to analyse marker gene sequencing data without the requirement to cluster at an arbitrary threshold, frequently 97 %. It is argued, therefore, that the reproducibility and ability to compare datasets outweighs any benefits which may be associated with OTUs which are invalid outside of the dataset in which they were defined (Callahan *et al.*, 2017). However, this is not a straightforward process as it is difficult to distinguish between ASVs that are truly different opposed to those that are a product of amplification or sequencing error. For this reason, the use of ASVs, which differ in a single nucleotide, may run the risk of over estimating diversity. Furthermore, many microbes hold multiple rRNA genes which may differ in nucleotide sequence. This is particularly pertinent for fungi which hold multiple copies of the ITS gene which may differ by as much as 20 % (Schoch *et al.*, 2012), again, this carries the risk of over estimating diversity because multiple different nucleotide sequences may originate from a single

microbe. While tools are improving to differentiate ‘true’ diversity from sequencing error, the more stringent ASV pipeline frequently means rare, but perhaps significant, sequences may be lost. Therefore, the argument could be made that analysis is not a comprehensive representation of the full microbial community. To conclude, while the debate surrounding ASVs or OTUs remains ongoing, and is perhaps specific to the ecological questions that are being asked, the inability to re-cluster and assign taxonomy in this chapter would offer a valid argument in favour of using ASVs.

Chapter 5

A shift towards sustainable agri-ecosytems: unveiling plant-microbe associations and the potential for their exploitation within and between cropping cycles.

5.1 | Abstract

Meeting the needs of a growing population demand while also protecting ecosystems from the deleterious impacts associated with conventional farming practices is gaining increased interest in sustainability research. Practices such as zero-till farming and biological control have been historically adopted in sustainable agriculture. However, manipulation of the soil microbiome to increase positive feedbacks while decreasing negative feedbacks has rarely been visited. Through two agricultural field trials, next-generation sequencing approaches were used to characterise the microbiome both within and between cropping cycles. Here I show that within plant species differences drove distinct soil-borne bacterial communities, implying informed cultivar selection could have implications for ecosystem functioning. Moreover, mixing of wheat cultivars saw a 97.4% and 60.87% reduction in unique fungal OTUs in three and nine variety plots respectively with significance met for richness loss in three variety mixes. This result suggests a significant loss of OTUs with increasing aboveground diversity and as such could mean the negative effects of NDD could be reduced by mixed cultivar cropping. Concerning potential sustainability approaches between cropping cycles, I show that cover-crop choice selection can have both positive and negative implications for soil microbial health. Cover cropping with Brassica species significantly supressed fungal OTU richness, while forage-rape and oilseed radish were associated with a greater abundance of beneficial saprotrophic and endophytic associations. Moreover, forage-rape and brown-mustard plots were associated with a

greater abundance of beneficial bacteria, specifically, Actinobacteria. Encompassed within these two studies, I have enhanced our understanding of plant-microbe interactions and how carefully managed cash versus cover crop rotations could see a replacement of conventional agriculture with low input sustainable cropping systems. While this is only the beginning, the data from this chapter suggests the incorporation of a microbial perspective may increase the speed at which arable farmers increase the self-sufficiency of agricultural ecosystems.

Abbreviations: NDD (negative density dependence), OM (organic matter).

5.2 | Introduction

One of the greatest constraints on agricultural production worldwide is soil-borne disease. However, in contrast to their aboveground conspicuous counterparts, soil-borne diseases are difficult to control and typically demonstrate disease symptoms that are difficult to distinguish from those caused by abiotic stress (ref). The key question I ask stemming from the developing results in this thesis would be; does this information, i.e. plant-microbe host specificity and NDD, provide scope for the development of more sustainable agri-ecosystems? Throughout Chapters 3 and 4, I have developed an understanding of plant-microbe interactions, the relationship this holds with plant diversity and have confirmed host-specificity of various fungal groups across differing plant taxonomic levels. These findings suggest the exploitation of plant-microbe interactions and knowledge of NDD may provide scope to increase the sustainability of traditional agricultural practices whilst reducing harmful and deleterious effects of heavy chemical application.

In part, the management of soil-borne disease relies on the planting of crop species and the rotation of these crops with alternative covers to try to restore soil health. Agriculturalists have recognised the importance of soil health as early as the 1960's. For example, crop rotation schemes have been employed frequently since the nineteenth century to overcome some of the difficulties caused by such negative feedbacks (Bullock, 1992). This is thought to work by interrupting the host-pathogen lifecycle (Krupinsky *et al.*, 2002). Alternatively, multiple agrochemicals, including fertilisers and pesticides, have been used to offset the effect of pathogenic fungi on plant health and subsequently crop yield. However, intense chemical inputs can have deleterious impacts on ecosystem health (Geiger *et al.*, 2010; Beketov *et al.*, 2013) and for this reason their use is also restricted under EU law (Hillocks, 2012). This highlights the need to develop more sustainable strategies to protect crops from soil-borne disease without the requirement of pesticides and fertilisers.

An increasing body of evidence shows the empirical biodiversity-productivity relationship is underpinned by the build-up of belowground host-specific soil pathogens under reduced diversity (M*aron et al*., 2011; Schnitzer *et al*., 2011; Mommer *et al*., 2018a). This mechanistic basis of diversity and soil-borne disease argues that 'biodiversity is essential' for more sustainable cropping systems. Therefore, satisfying and understanding the assumptions underlying the 'pathogen-hypothesis' within agricultural production systems to inform and develop more sustainable agri-ecosystems is the next logical step in this present theme of ecology.

Increasing diversity in cropping systems closely aligns with intercropping and mixed-cropping ecosystems which typically yield 20 % more biomass than the most productive monoculture (Yu *et al.*, 2015). However, this has practical considerations, for instance, growing distantly related crops involves a number of considerations such as the length of the growing cycle, nutrient requirements and growth form which may provide difficulty for harvesting and post-harvest sorting. However, one way to increase diversity aboveground could involve growing a cultivar mix as opposed to conventional single cultivar monocultures. While data from Chapters 3 and 4 suggest it would be unlikely different cultivars hold distinct pathogenic fungal communities, microbial profiling from distinct wheat cultivars has shown genotypes within a species are large enough to cause distinct changes in rhizosphere microbial communities (Aira *et al*., 2010). In addition, the previous Chapters have only focussed on fungi, and therefore we cannot make any assumptions regarding potential species-specific effects of bacterial communities which typically respond differently. Moreover, there may be an aspect of plant domestication by which microbial communities are modified and therefore plant responses differ between the two ecosystems, therefore this relationship requires further investigation. To enhance our understanding and potentially use this information to develop more sustainable cropping systems it is vital we explore species-specific interactions and the effects of increasing phylogenetic diversity in agricultural systems.

It is likely there will be differences in the response of bacterial and fungal communities to plant diversity. As stressed by Mommer *et al.*, (2018), a plant diversity gradient could have opposing effects on fungal diversity; response 1) if host-specificity for all taxonomic groups of microbes is strong (this could be likely as Chapter 3 indicated that all functional groups of fungi are host-specific), microbial diversity will increase with plant diversity because overall microbial diversity will be an outcome of all plant-specific microbes combined (Dassen *et al*., 2017). Conversely, and in align with the 'pathogen-hypothesis', response 2) if negative PSF and NDD type effects are strong, increased plant diversity may have a dilution effect on pathogenic microbial diversity because plots with high plant diversity hold a decreased density of any specific plant species population. However, I predict there will be minimal differences in bacterial communities between cultivars, or in association with an increase in aboveground diversity as bacteria are typically driven by soil abiotic properties as opposed to plant identity (Fierer, 2017; Navrátilová *et al.*, 2018).

Furthermore, not only are cropping systems constrained by soil-borne disease, but soil erosion, nutrient losses, soil quality and weed susceptibility also play a role in predicting crop losses. One way these variables can be minimised is through cover-cropping strategies implemented through two main crop cycles (Dabney *et al.*, 2001; Hartwig & Ammon, 2002; Dorn *et al.*, 2015). However, there is still a knowledge gap concerning how cover-crops effect belowground microbial communities and whether mixed cover-cropping has scope to suppress soil-borne disease remains untested. In align with the theme of plant host-microbial specificity explored throughout this thesis, informed selections of cover-crop varieties could be used to reduce the abundance of negative plant-microbial associations whilst simultaneously increasing beneficial plant-microbial relationships which may achieve improved ecosystem functioning. This idea builds upon the theme of microbiome-assisted agriculture. Cover cropping, and choice of cover crops, can directly influence microbial composition through root exudation properties regulated by plant identity effects (Badri & Vivanco, 2009), or

indirectly through subsequent modifications to key soil properties such as organic matter and total nitrogen (Nunes *et al.*, 2018). Coupled with advances in molecular tools and increased ease of usage, recent work has shown cover-cropping can have beneficial effects on soil microbial communities (Vukicevich *et al.*, 2016). In addition, in light of the 'pathogen hypothesis', cover-crop mixes (i.e. growing multiple species as opposed to single species cover) may provide a mechanism to reduce pathogen load within cropping cycles. Adopting such approaches that exploit plant-microbial interactions both within and between crop cycles could mean a change in modern day cultivation for more sustainable ecosystems will rely less heavily on external and manufactured chemical inputs. Not only will this benefit agriculture economically, but a reduction in harmful chemicals will lessen environmental pollution caused by typical modern day intensive farming practices.

To summarise, this Chapter covers two distinct proof of principle agricultural studies covering management strategies to be applied both within and between crop cycles. In this study, I utilised next-generation sequencing techniques firstly to test for host-specificity of plants grown in monoculture (i.e. are they cultivar or cover crop specific?). I then test for signalling of PSF and NDD when cultivars or covercrops are mixed. In addition, to explore beneficial plant-microbe interactions I determine the taxonomic diversity and infer a functional response of soil-borne and root-associated biota (bacteria and fungi) across an aboveground richness gradient (or in the case of cover crops, between single and three species mixes). The information learned from this study incorporates ecological understanding of plant-microbial interactions,PSF and NDD to help inform more sustainable agricultural management systems.

The first study is a biodiversity experiment in which nine wheat cultivars are grown in single-species mixes (monoculture), and are subsequently mixed in three-species and nine-species mixes. Here, the following hypotheses were tested:

H1: Root-associated and soil-borne microbial communities will be distinct.

H2: Root associated fungal communities will be distinct between cultivars.

H3: There will be no effect of aboveground diversity on bacterial diversity.

H4: Fungal diversity will decrease (or remain the same) as aboveground diversity increases due to theloss of pathogenic OTUs.

The second is a field trial consisting of single species and species-mixes of cover crops. This is very much a simple proof of concept study in which four species of commonly used cover crops were grown in monoculture, and a species mix of cover crops were sewn representing a ‘mixed’ species plot. Cover-crops were chosen for known beneficial effects such as nematode control or the ability to improve soil structure. The following hypotheses were tested:

H1: Root-associated microbial communities will be distinct between cover-crop varieties – this implies host-specificity.

H2: Fungal diversity will be lower in cover-crop varieties containing Brassica species.

H3: If host-specificity is confirmed, functional diversity will differ between cover-crop varieties.

H4: Pathogenic fungal load will be lower in mixed cover crop varieties.

5.3 | Methods

5.3.1 | Experimental setup

This chapter covers two distinct agricultural experiments at RAGT Seeds, UK (52.058667”N, 0.140688”E). All plots were treated with 40-60 kg/ha of nitrogen fertiliser prior to sewing of seeds.

5.3.2 | Wheat cultivars – biodiversity experiment

First, soil and root cores were obtained from a wheat cultivar biodiversity experiment (Cambridge). Briefly, setup in September 2018, this experiment was a randomised block design (each block 1.5 m x 4.5 m), with wheat variety richness (single, triplet, and nine variety plots) as the main factor (Figure 5.1). Wheat varieties comprised of nine different cultivars (specific details cannot be given as cultivars are patent protected by RAG-T seeds). In April 2019, three soil cores (3 cm diameter, 10 cm depth) were taken at random from each plot (n = 81) and pooled (n = 27). Corers were sterilised with 10 % NaOCl, followed by 70 % ethanol and washed with distilled water between samples. Soil cores were stored in sterile airtight plastic bags and transported on dry ice to the University of Essex. Samples were frozen at - 20 ^{o}C until analysis. For analysis, soil cores were defrosted, homogenised and sieved to remove roots for separate analysis. All roots were oven dried at 60 ^{o}C then homogenised in 6 ml reinforced tubes with screw caps and o-rings (Fisher, UK) including six stainless steel beads (QIAGEN, UK) using Precellys homogeniser. For species-specific soil and root characterisation of microbial communities, 0.05 g of fresh weight soil and 0.03 g of homogenised root sample were weighed for separate DNA extraction. DNA extraction followed the CTAB Griffiths protocol. For methods regarding DNA extraction from root and soil cores and soil chemistry analyses please see general methods.

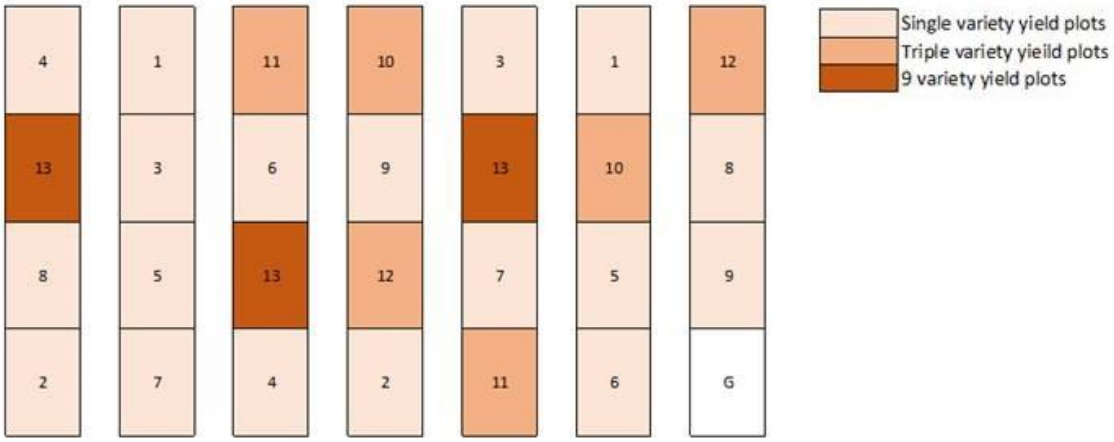


Figure 5.1. Schematic detailing the random block design of nine (1-9) wheat cultivars grown in single (monoculture), triple or nine variety yield plots (see coloured key), n = 27. ‘G’ represents an unused plot with bare ground.

5.3.3 | Cover-crop trial

Second, root samples were obtained from an agricultural cover crop trial (RAGT Seeds, UK. Cambridge). This trial consisted of four adjacent plots (6 m x 24 m) of branded monoculture cover crops and a fifth plot (3 m x 24 m) of a mixed cover crop variety, see Table 5.1 for details. Five replicates of single plants from each plot (n = 25) were carefully extracted from the soil (roots intact), roots were brushed to remove loose debris and stored in sterile airtight plastic bags on dry ice for transportation to the University of Essex. Roots were oven dried at 80 ^{o}C, then homogenised in 6 ml reinforced tubes with screw caps and o-rings (Fisher, UK) including six stainless steel beads (QIAGEN, UK) using Precellys homogeniser. For species-specific microbial root characterisation, 0.03 g of homogenised root sample were weighed for DNA extraction. DNA extraction followed the CTAB Griffiths protocol. For methods regarding DNA extraction from root and soil cores please see general methods.

Table 5.1. Details of cover crops used in trial at RAGT Seeds, UK, including sample ID, plant species and brand name. Samples are referred to by their associated ID throughout the Chapter.

ID	Cover crop (% mix)	Brand	Properties
Terra	Oil seed radish (100%)	Terranova	Resistance to nematodes beet cyst and root knot, frost resistant, late flowering, deep root system.
Exito	Japanese oats (100%)	Exito	Fast growth, vigorous deep root system, frost susceptible.
Ringo	Forage rape (100%)	Ringo	Fast growth, medium frost-resistance, high protein content (ideal for grazing cattle between cash crops).
Brons	Brown mustard (100%)	Brons	Early flowering (good for short harvest windows), trap-crop (host for beet cyst and root knot).
Mixed	Ethiopian mustard (50%): Oil seed radish (40%): Forage rape (10%)	Biofum	Reduction of soil-borne fungi through production of glucosinolates. Frost resistant.

5.3.4 | DNA Extraction

DNA was extracted from the roots/and soil of each sample following the Cetyl Trimethylammonium Bromide protocol (CTAB; Griffiths *et al.*, 2000). Details for DNA extraction are outlined in the general methods. Each sample was standardised to 1 ng/µL in PCR water to allow for high-throughput automated sample preparation.

5.3.5 | Molecular analysis

Root/soil associated bacterial and fungal communities were characterised using 2 x 300 bp Miseq amplicon sequencing of ~ 350 bp fragment targeting the 16S rRNA and ITS2 region of the Small Subunit (SSU) respectively. Library preparation methods are outlined in the general methods (Chapter 2). Sequencing was conducted at The University of Essex on an Illumina Miseq (providing 2 x 300 bp sequences).

Each sequencing run contained multiplexed samples of each target gene which were pooled in equimolar concentrations with a per library ratio of; 16S bacteria – 60%; fungal ITS - 40%. Next generation amplicon sequencing was conducted on an Illumina MiSeq platform (Illumina, 2013) at the University of Essex (UK).

5.3.6 | Soil chemistry

All edaphic properties from each soil core were measured as outlined in the general methods (Chapter 2). Edaphic variables were only measured for the wheat variety biodiversity study.

5.3.7 | Statistical analysis

Bioinformatic analyses were conducted using the methods outlined in the general methods (Chapter 2). The OTU table was filtered to remove negative controls, after confirmation that contamination was negligible, non-target amplicons (i.e. plant material belonging to the phylum Streptophyta and Chlorophyta) and low abundance OTUs (< 3) as these are more likely to be non-biological (Flynn *et al*., 2015). Further

community OTU subsets were created for functional groups of fungi (i.e. potential plant pathogens, saprotrophs and endophytes).

5.3.7.1 | Wheat variety – biodiversity experiment

Linear-models (LMs) were used to determine any differences in edaphic factors between sampling sites and the effect of edaphic factors on microbial community composition were explored using the Mantel test.

Differences in microbial OTU richness between soil-borne and root-associated communities were analysed using a linear-model (LM) or a negative binomial generalised linear model (GLM) if data was not normally distributed. Unique and core OTUs within soil and root samples were calculated and visualised within the package 'VennDiagram' (Chen & Boutros, 2011). To investigate how the abundance of OTUs changed between soil-borne and root-associated fungal communities multivariate generalized linear models (MV-GLMs) were used (Wang *et al*., 2012). A negative binomial mean-variance error family was used to account for over dispersion, a common property of count data. Heterogeneity in library size was accounted for by incorporating an offset term as the first fixed term in the model. Multivariate and unadjusted univariate P values were obtained by Wald tests, both using 10,000 Monte Carlo permutations.

To test for host-specificity differences in microbial alpha-diversity measures were compared between wheat varieties growing in monoculture. To determine if wheat variety growing in monoculture had an effect on microbial OTU richness a linear-model (LM) or negative binomial generalised model (GLM) within the package 'MASS' (Ripley, 2011) was implemented. A negative binomial GLM was used to account for over dispersion of OTU richness, a common feature of count data. Differences in library size were accounted for by including log(number of sequences) as the first (fixed) term in the model. This approach to dealing with differences in sequencing

depth is encouraged by Warton *et al.*, (2015) as it avoids many of the unfavourable aspects introduced by alternatives such as rarefraction (McMurdie & Holmes, 2014). This is particularly important within the context of this study as rarefraction frequently removed biologically important pathogenic taxa. The effects of wheat variety on community diversity indices (diversity and evenness) were explored using linear models with a Gaussian distribution. Community diversity and evenness were quantified using Shannon's diversity and Simpson's evenness index respectively (see Morris et al., 2014). All community evenness values were logit-transformed to meet linear modelling assumptions (Warton & Hui, 2011). For the purpose of visualisation, small sample sizes were removed and data were rarefied to the smallest library size prior to producing figures.

Differences in overall microbial community composition were performed on rarefied data and were visualized using nonmetric multidimensional scaling (NMDS) on Bray–Curtis distances. PERMANOVA was performed to determine how much variance in microbial community clustering can be explained by soil fraction (root versus soil) and wheat genotype (permutations = 10,000).

To explore the effects of aboveground diversity, the same statistical analysis as above was implemented with 'aboveground genetic richness' as a fixed term each model (i.e. one, three or nine variety richness). Unique and core OTUs within each biodiversity plot (one, three or nine variety mix) were identified and visualised using the package 'VennDiagram' (Chen & Boutros, 2011).

5.3.7.2 | Cover-crop trial

Overall taxonomic distribution of the top 10 most abundant genera (> 1 % relative abundance) were calculated and visualised within the package phyloseq (McMurdie & Holmes, 2013) and visualised using ggplot.

To test for host-specificity and species-specific microbial communities, differences in microbial alpha-diversity measures across the total bacterial and fungal community and the ten most abundant bacterial and fungal taxa were compared between cover crop varieties using a one-way ANOVA followed by a Tukey post-hoc test. An interaction between treatment plot and log(lib.size) was initially incorporated to explore the effects of unequal sampling depth on the response variable. If this wasn't significant (log)lib.size was included as a separate explanatory term. This same approach was used to explore further subsets of fungal guilds (pathogens, saprotrophs and endophytes).

Differences in overall microbial community composition was performed on rarefied data and were visualized using nonmetric multidimensional scaling (NMDS) on Bray–Curtis distances. PERMANOVA was performed to determine how much variance in microbial community clustering can be explained by cover crop variety (permutations = 10,000).

5.4 | Results

5.4.1 | Wheat cultivars - Biodiversity experiment

5.4.1.1 | Soil variables

Formate was the only soil variable to significantly differ between sampling plots (LM; $F_{12,14}$ = 2.95, P < 0.05). Overall, only soil moisture correlated with root-associated fungal community composition, there were no other significant correlations between microbial community composition from either root or soil sources with the edaphic properties measured in this study (Appendix 5.1).

Table 5.2. Linear modelled results of soil variables across treatment plots.

Anions		**Cations**		
Acetate	$F_{12,14}$ = 1.05, P = 0.46	Calcium	$F_{12,14}$ = 0.47, P = 0.9	
Butyrate	$F_{12,14}$ = 1.31, P = 0.31	Magnesium	$F_{12,14}$ = 0.54, P = 0.86	
Chloride	$F_{12,14}$ = 0.86, P = 0.59	Potassium	$F_{12,14}$ = 0.98, P = 0.51	
Chlorite	$F_{12,14}$ = 1.31, P = 0.31	Sodium	$F_{12,14}$ = 0.83, P = 0.62	
Fluoride	$F_{12,14}$ = 0.93, P = 0.54	**Abiotic**		
Formate	**$F_{12,14}$ = 2.95, P < 0.05**	pH	$F_{12,14}$ = 2.02, P = 0.11	
Lactate	$F_{12,14}$ = 1.00, P = 0.49	Total carbon	$F_{12,14}$ = 2.07, P = 0.09	
Nitrate	$F_{12,14}$ = 1.37, P = 0.28	Moisture (%)	$F_{12,14}$ = 1.01, P = 0.49	
Nitrite	$F_{12,14}$ = 4.48, P = 0.06			
Phosphate	$F_{12,14}$ = 0.58, P = 0.82			
Phosphite	$F_{12,14}$ = 1.08, P = 0.44			
Sulphate	$F_{12,14}$ = 1.03, P = 0.47			

5.4.1.2 | Effects of soil fraction: soil-borne versus root-associated microbial communities

Overall, there was no significant difference in bacterial richness (OTUs) between root-associated and soil-borne bacterial communities (GLM; $LR_{1,49}$ = 1.0, P = 0.31). Regarding fungal communities, fungal richness (OTUs) was significantly higher in root-associated communities in comparison to soil-borne communities (GLM; $LR_{1,52}$ = 37.96, P < 0.001).

To investigate persistent members of microbial communities that inhabit the root-associated and soil-borne microbiome, common and unique microbial taxa were explored. Unique bacterial OTUs to root and soil sources were only a subset of the shared OTUs (4047 OTUs), and roots held a comparatively lower number of unique OTUs (124 OTUs) compared to soil (281 OTUs). For the fungi, the ratio of unique:shared OTUs was much more even; root (63 OTUs) and soil samples (61 OTUs.

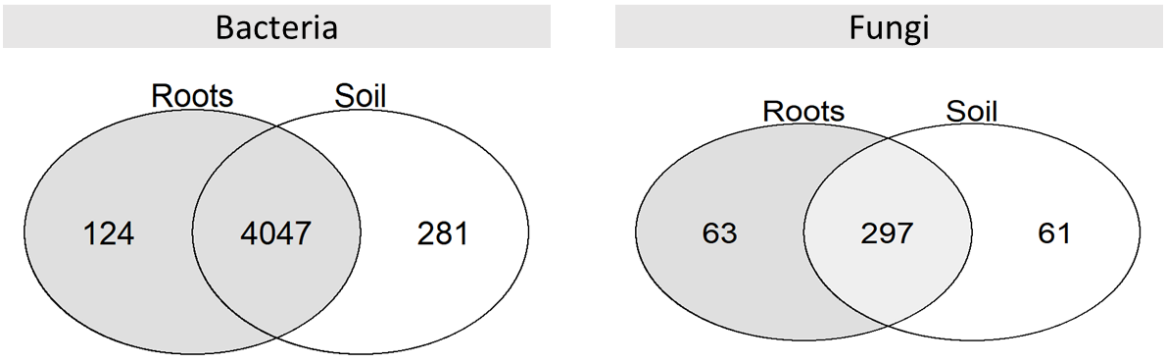


Figure 5.2. Venn diagram showing the overlap in operational taxonomic units (OTUs) between root-associated and soil-borne bacterial and fungal communities. Numbers represent total OTU counts.

Multivariate analysis were performed to determine what fungal taxa significantly increased/decreased in abundance between root-associated and soil-borne fungal communities. Because root-associated fungal communities demonstrated significantly higher OTU richness, soil-borne fungal communities were set as the intercept to determine what OTUs were driving this result. A total of 288 OTUs occurred > 1 % in the overall dataset. Multivariate modelling revealed that soil fraction (rhizosphere or bulk soil) significantly affected fungal OTU abundances (156 OTUs, Wald = 35.8, $P < 0.001$), after controlling for unequal sequencing depths. Of all the OTUs affected by soil fraction, 100 OTUs were significantly more abundant (4 Ascomycota; 4 Basidiomycota; 1 Chytridiomycota; 2 Glomeromycota; 2 Zygomycota; 89 unclassified) and 56 OTUs were significantly less abundant in roots in comparison to soil (3 Basidiomycota; 1 Chytridiomycota; 2 Glomeromycota; 2 Zygomycota; 48 unclassified). See Figure 5.3.

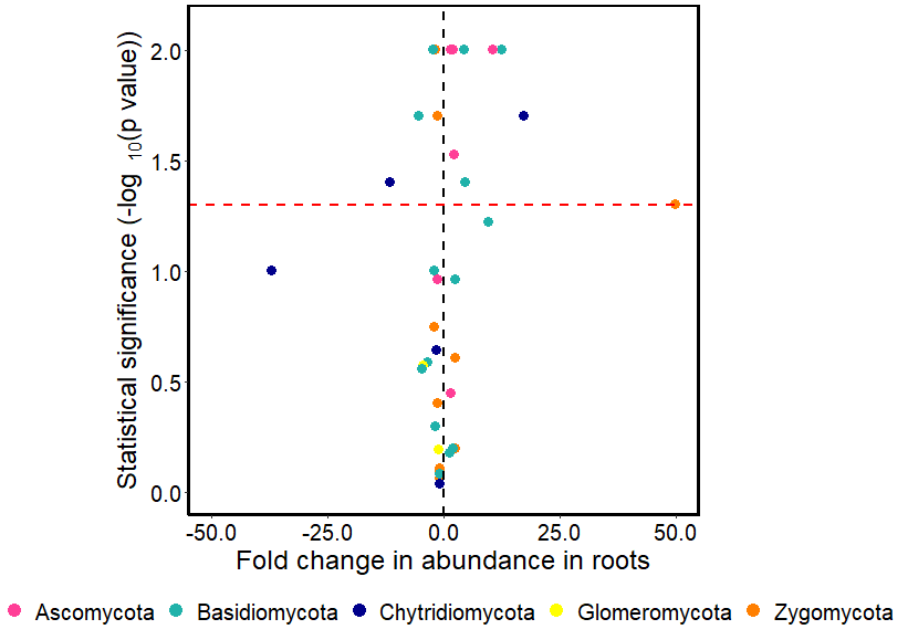


Figure 5.3. Volcano plot of MV-GLM modelled shifts in total fungal phyla abundances in root-associated microbes when compared to soil-borne microbes. Coloured points above the horizontal dotted line represent phyla that showed statistically significant shifts in abundance ($P < 0.01$), whereas points below the line represent phyla that did not show statistically significant shifts ($P > 0.05$). Phyla further to the left were statistically less abundant while phyla to the right were statistically more abundant when compared to soil-borne microbes.

5.4.1.3 | Alpha-diversity. Host-specificity in root-associated and soil-borne microbial communities

OTU bacterial richness of the root-associated (Figure 5.4. A-C; grey) communities were significantly different across wheat varieties in monoculture (GLM; $LR_{8,16,} = 20.85$, $P = < 0.01$), but diversity (LM; $F_{8,16} = 0.69$, $P = 0.69$) and evenness (LM; $F_{8,16} = 0.76$, $P = 0.65$) were not. Similarly, soil-borne (Figure 5.4. D-F; white) OTU richness of bacterial communities were significantly different across wheat varieties in monoculture (GLM; $LR_{8,16,} = 34.08$, $P = < 0.001$), but diversity (LM; $F_{8,16} = 1.24$, $P = 0.38$) and evenness (LM; $F_{8,16} = 2.14$, $P = 0.14$) were not.

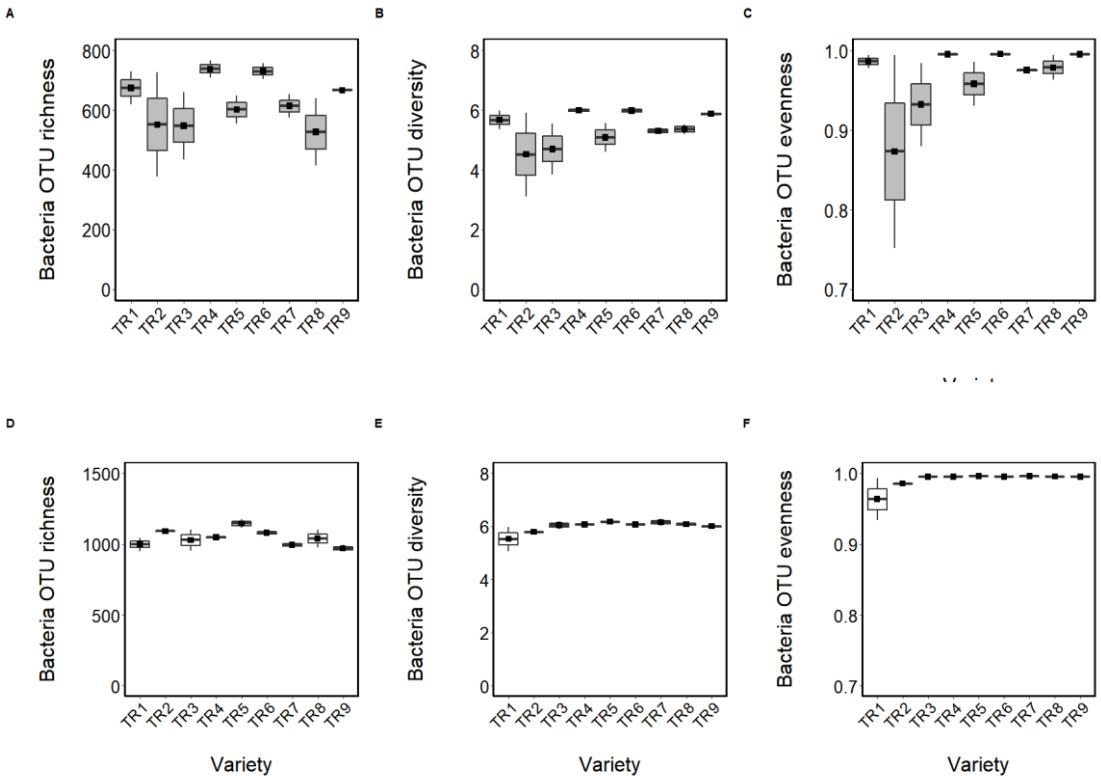


Figure 5.4. Host specific effects of plant wheat variety on alpha-diversity measure of root-associated (A-C; grey) and soil-borne (D-F; white) bacterial communities of plants in monoculture. Each box plot represents the median (horizontal line), mean (solid black square) and 25th to 75th percentiles, bars.

Measures of alpha-diversity for the root-associated fungal community (Figure 5.5. A-C; grey) were not significantly different across wheat varieties in monoculture; richness (GLM; $LR_{8,16}$ = 4.96, P = 0.76), diversity (LM; $F_{8,16}$ = 0.64, P = 0.73), and evenness (LM; $F_{8,16}$ = 0.49, P = 0.84). Similarly, fungal alpha-diversity measures of the soil-borne fungal community (Figure 5.5. D-F; white) were not significantly different across wheat varieties in monoculture; (GLM richness; $LR_{4,11}$ = 1.55, P = 0.98. LM diversity; $F_{4,11}$ = 0.17, P = 0.47. LM evenness; $F_{4,11}$ = 1.19, P = 0.46).

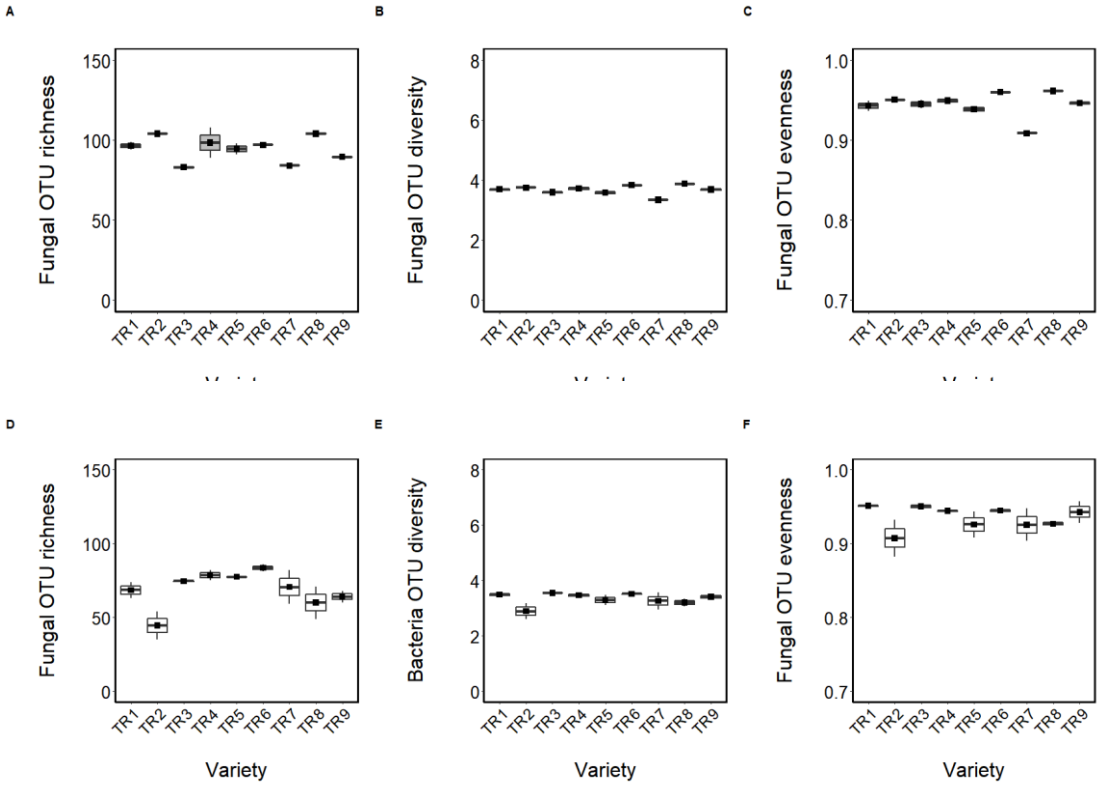


Figure 5.5. Host specific effects of plant wheat variety on alpha-diversity measure of root-associated (A-C; grey) and soil-borne (D-F; white) fungal communities. Each box plot represents the median (horizontal line), mean (solid black square) and 25th to 75th percentiles, bars.

5.4.1.4 | Beta diversity. Host-specificity and the effect of soil fraction

Exploration of host-specific effects between root and soil associated bacterial communities revealed overall significant differences in the soil-borne and root-associated bacterial composition of wheat varieties grown in monoculture (Figure 5.6. PERMANOVA; $F_{1,32} = 5.84$, $P < 0.001$, stress = 0.1191). However, when soil-borne and root-associated differences within a single wheat variety were compared the difference in bacterial composition was not significant (PERMANOVA; $F_{1,8} = 0.99$, $P = 1.07$, $P = 0.33$).

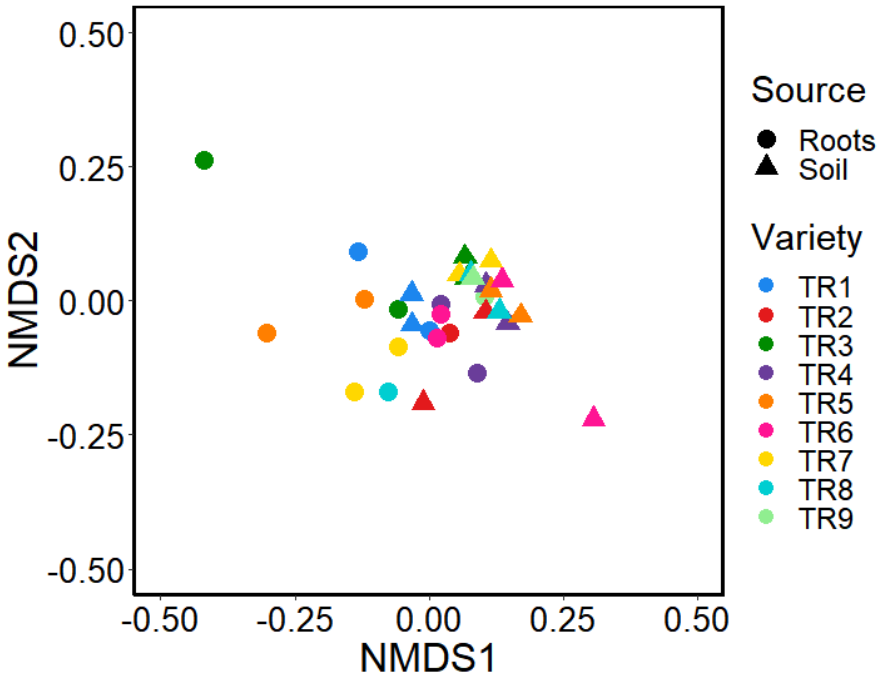


Figure 5.6. Total root-associated (circles) and soil-borne (triangles) bacterial communities from nine wheat varieties grown in monoculture. Nonmetric multidimensional scaling (NMDS) ordinations, based on Bray's distance matrix of the total fungal community. Wheat variety explained significantly different composition in soil-borne bacterial communities.

Exploration of host-specific effects between root and soil associated fungal communities revealed overall significant differences in the soil-borne and root-associated fungal composition of wheat varieties grown in monoculture (Figure 5.7. PERMANOVA; $F_{1,30}$ = 7.33, P < 0.001, stress = 0.1674). However, when soil-borne and root-associated differences within single wheat varieties were compared the difference in fungal composition was insignificant (PERMANOVA; $F_{1,8}$ = 1.03, P = 0.41).

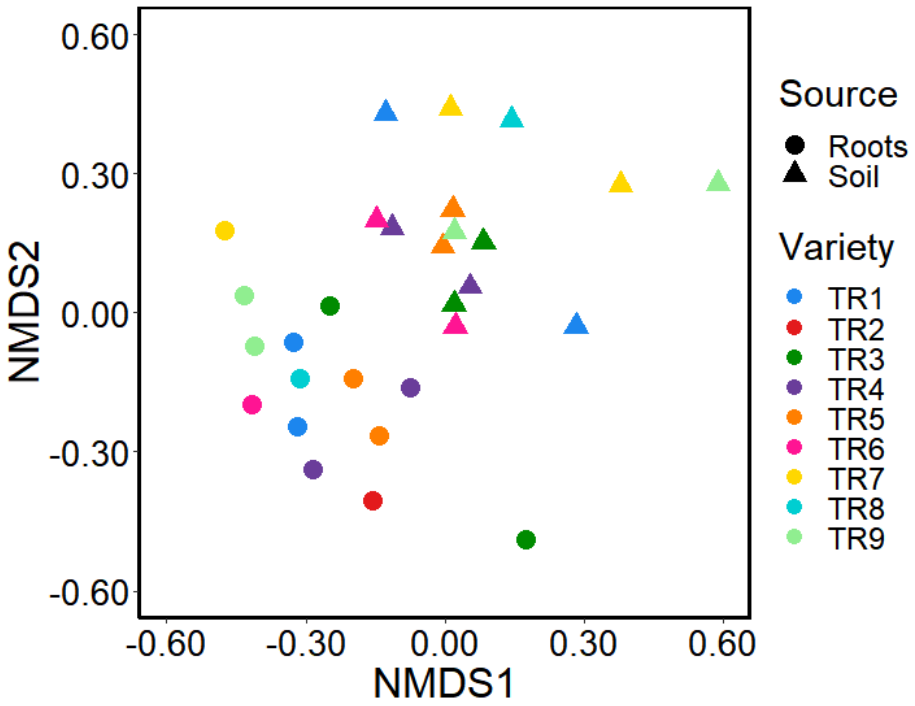


Figure 5.7. Total root-associated (circles) and soil-borne (triangles) fungal communities from nine wheat varieties grown in monoculture. Nonmetric multidimensional scaling (NMDS) ordinations, based on Bray's distance matrix of the total fungal community. Wheat variety explained significantly different composition in soil-borne bacterial communities.

5.4.1.5 | Composition – host specificity of microbial communities in monoculture

In this study, the total-root associated bacterial communities (i.e. all bacterial OTUs observed) in monocultures were not significantly different between wheat varieties grown in monoculture (PERMANOVA; $F_{8,16}$ = 0.99, P = 0.49, stress = 0.071). Conversely, the total soil-borne bacterial communities in monocultures were significantly different between wheat varieties in monoculture (Figure 5.6. PERMANOVA; $F_{8,17}$ = 1.93, P < 0.001, stress 0.0952).

Root-associated or soil-borne fungal communities did not show any significant differences in fungal composition between different wheat varieties grown in monoculture.

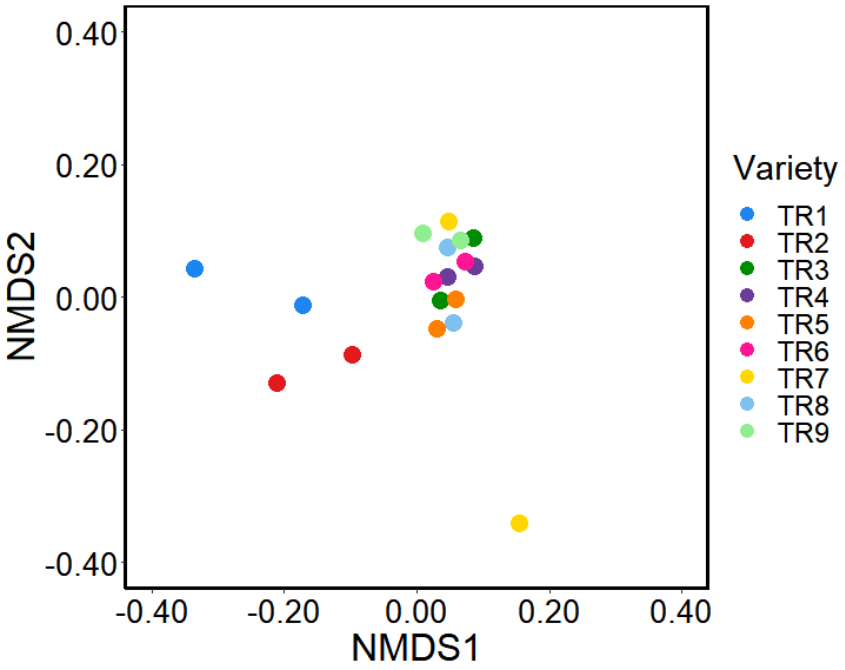


Figure 5.8. Total soil-borne bacterial communities from nine wheat varieties grown in monoculture. Nonmetric multidimensional scaling (NMDS) ordinations, based on Bray's distance matrix of the total fungal community. Wheat variety explained significantly different composition in soil-borne bacterial communities.

5.4.1.6 | Microbial diversity across a plant variety richness gradient

Measures for root-associated bacterial alpha-diversity did not significantly increase when wheat varieties were mixed but soil-borne bacterial communities did differ in OTU richness when wheat varieties were mixed, although this result was not linear (Table 5.3, Figure 5.9).

Root associated fungal OTU richness significantly differed when wheat varieties were mixed, although, this relationship was non-linear. Soil-borne fungal communities did not differ when wheat varieties were mixed.

Subsets of fungal functional guilds revealed no significant differences when wheat varieties were mixed (GLM root pathogens; $LR_{2,16} = 1.18$, $P = 0.55$, soil pathogens; $LR_{2,18} = 0.29$, $P = 0.86$, root endophytes; $LR_{2,23} = 0.71$, $P = 0.71$, soil endophytes; $LR_{2,23} = 0.47$, $P = 0.79$, root saprotrophs; $LR_{2,23} = 1.11$, $P = 0.57$, soil saprotrophs; $LR_{2,23} = 0.73$, $P = 0.60$).

Table 5.3. Linear-modelled analysis of bacterial and fungal alpha-diversity across a wheat variety richness gradient for both root-associated and soil-borne microbial communities. Significant results are in bold.

Bacteria		
	Roots	**Soil**
Richness	$LR_{2,22}$ = 2.26, P = 0.32	**$LR_{2,23}$ = 7.36, P < 0.05**
Diversity	$F_{2,22}$ = 0.11, P = 0.89	$F_{2,23}$ = 7.86, P = 0.36
Evenness	$F_{2,22}$ = 0.07, P = 0.93	$F_{2,23}$ = 1.77, P = 0.19
Fungi		
Richness	**$LR_{2,23}$ = 10.27, P < 0.01**	$LR_{2,23}$ = 2.23, P = 0.32
Diversity	$F_{2,23}$ = 2.31, P = 0.12	$F_{2,23}$ = 1.18, P = 0.32
Evenness	$F_{2,23}$ = 0.13, P = 0.88	$F_{2,23}$ = 0.98, P = 0.39

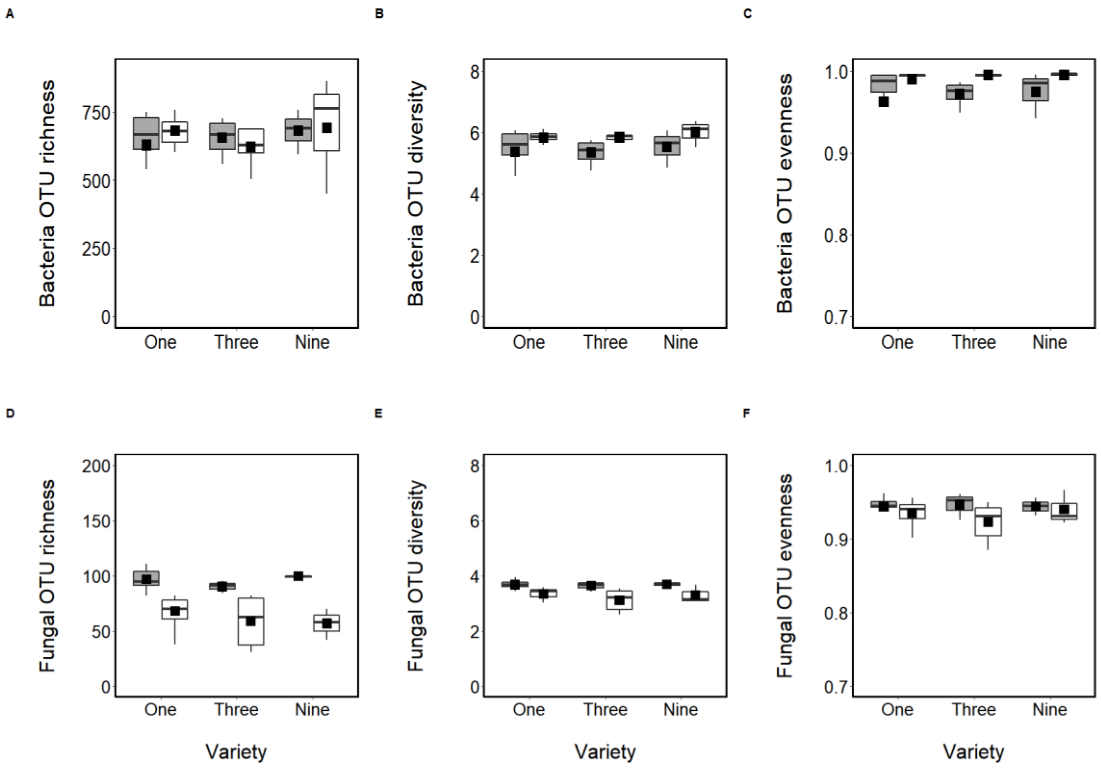


Figure 5.9. Total bacterial (A-C) and fungal (D-F) alpha-diversity (calculated from number of OTUs) across a wheat variety diversity gradient (monoculture, triplicate and nine species mix). Grey boxes represent the root-associated microbial community and white boxes represent soil-borne microbial communities. Each box plot represents the median (horizontal line), mean (solid black square) and 25th to 75th percentiles, bars.

5.4.1.7 | OTU similarity

Unique bacterial and fungal OTUs in mixtures of three or nine wheat varieties were only a subset of those found in monoculture plots (one-variety plots). Root-associated bacteria – 2233 OTUs were common to all wheat variety levels, 3570 OTUs in monocultures, 2743 in three-variety plots and 715 in nine-variety plots. Root-associated fungi – 140 OTUs were common to all wheat variety levels, 314 in monocultures, 201 in three-variety plots, and 188 in nine-variety plots (Figure 5.10). Soil-borne bacteria – 1889 OTUs were common to all wheat variety levels, 3795 in monocultures, 2678 in three-variety plots and 2456 in nine-variety plots. Soil-borne fungi – 106 OTUs were found across all wheat variety levels, 282 in monocultures, 196 in three-variety plots and 149 in nine-variety plots.

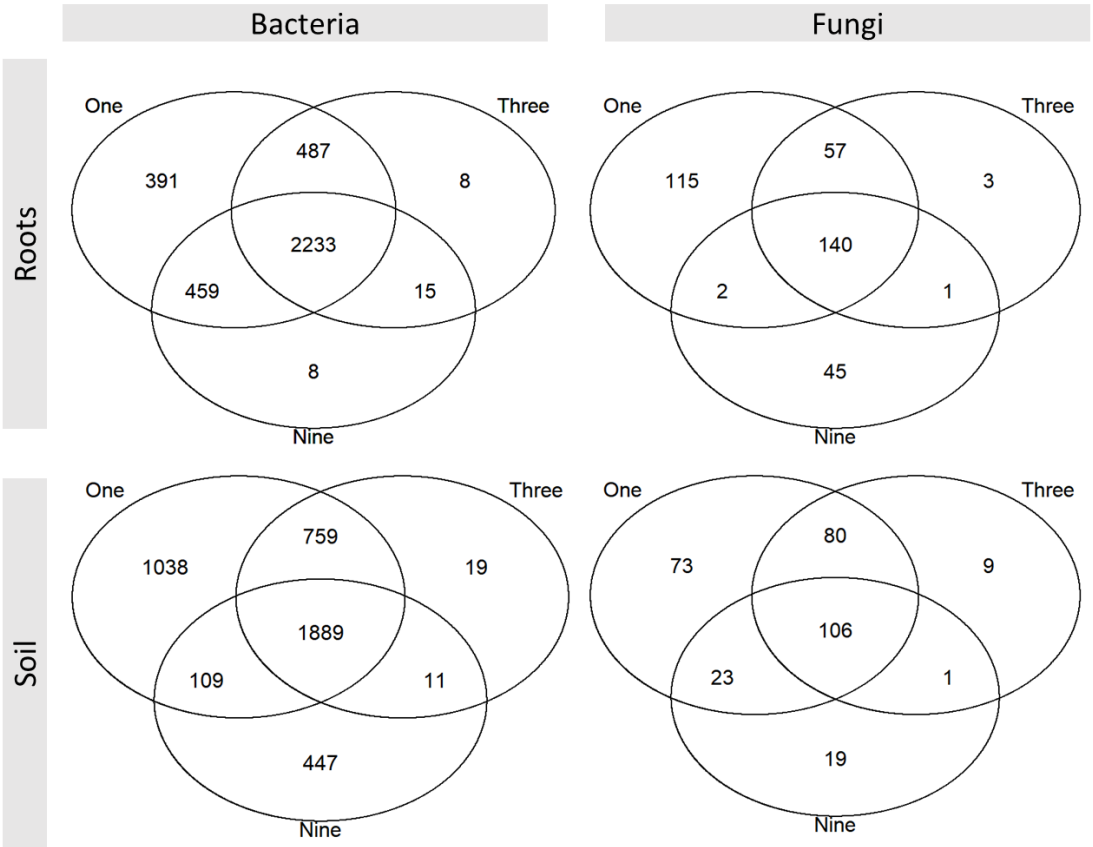


Figure 5.10. Venn diagram showing the overlap in operational taxonomic units (OTUs) among wheat variety levels for bacterial and fungal root associated and soil-borne microbial communities. Numbers represent total OTU counts.

5.4.2 | Cover crop trial

5.4.2.1 | Microbial taxonomic distribution between plots

A total of 467 and 29 unique bacterial and fungal genera (> 1%) were detected across all treatment plots. Taxonomic distributions within each cover-crop treatment plot with average relative abundances for the top ten bacterial genera (Figure 5.11). Taxa belonging to the top ten most abundant bacterial and fungal genera were explored for differences in OTU richness between treatment plots, Bacillus, Micromonospora, Nocardioides, Skermanella, Solirubacter and Streptomyces significantly differed between treatment plots independent of library size (ANOVA: $F_{4,19}$=15.47, P < 0.001, $F_{4,15}$=14.09, P < 0.001, $F_{4,15}$=18.85, P < 0.001, $F_{4,14}$=38.48, P < 0.001, $F_{4,14}$= 6.18, P < 0.01, $F_{4,15}$= 7.96, P < 0.01, $F_{4,14}$= 9.4, P < 0.001). Fungal taxa belonging to the genus Mortierella differed between treatment plots independent of library size (ANOVA; $F_{4,14}$= 6.48, P < 0.01).

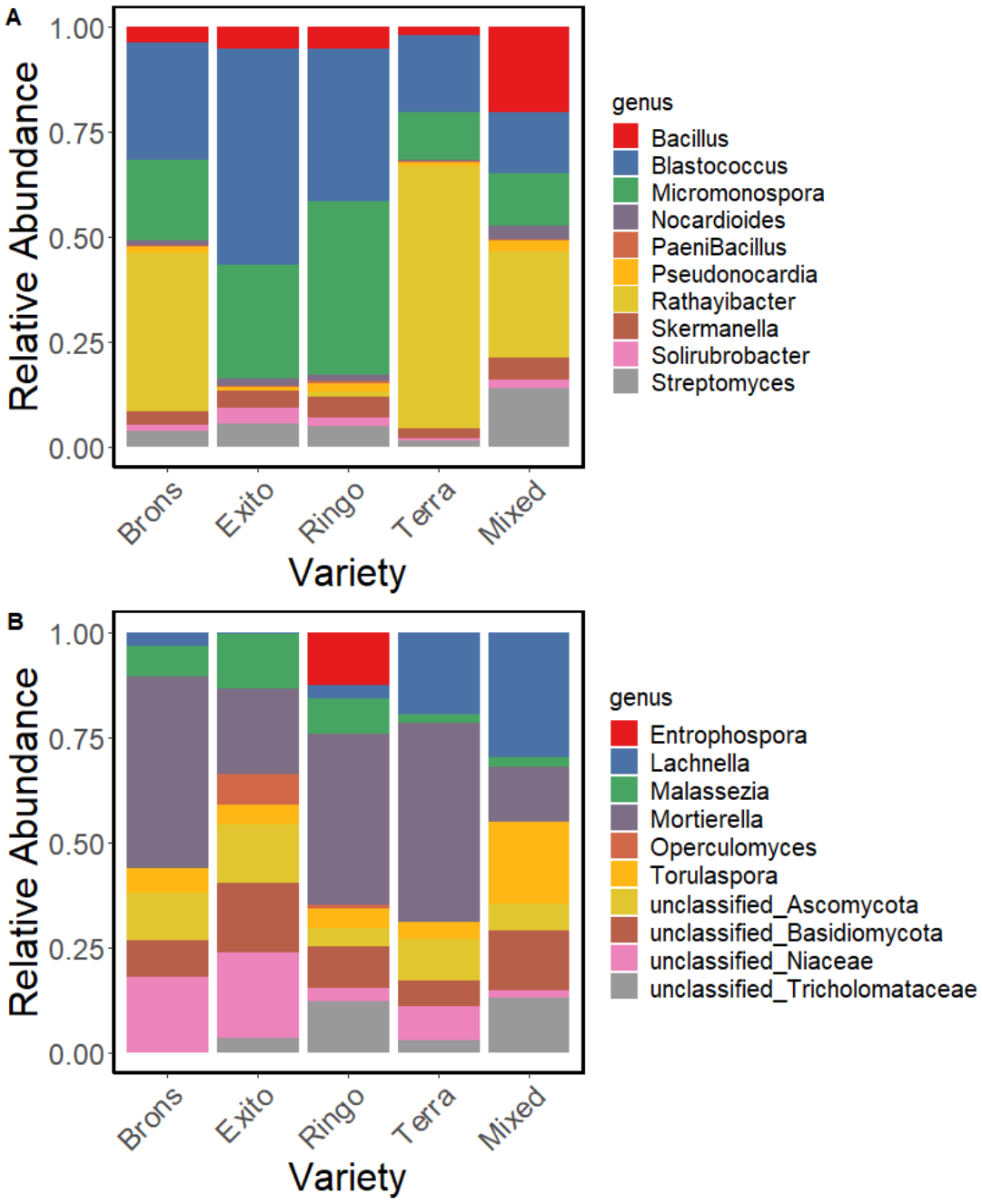


Figure 5.11. Comparison of bacterial (top) and fungal (bottom) taxonomic distributions between cover-crop treatment plots. OTUs were filtered to include those that only occur > 1% relative abundance in the total dataset. Bars are coloured according to bacterial phyla.

5.4.2.2 | Alpha-diversity of root associated microbial communities

All bacterial measures of alpha diversity were lower in monocultures when compared to mixed species plots, Figure 5.12. Treatment plots had a significant effect on bacterial OTU richness (ANOVA; $F_{4,19} = 11.48$, $P < 0.001$). Tukey-post hoc analysis revealed three distinct groups. Library size had a significant effect on OTU richness (ANOVA; $F_{4,19} = 14.84$, $P < 0.01$) but the interaction between treatment plot and library size was insignificant, therefore log(lib.size) was included as a separate factor to explore effects of unequal sequencing depth (Appendix 5.2). Diversity (ANOVA; $F_{4,19} = 2.71$, $P = 0.06$) and evenness (ANOVA; $F_{4,19} = 1.22$, $P = 0.33$) were not significantly different between treatment plots and library size had no effect.

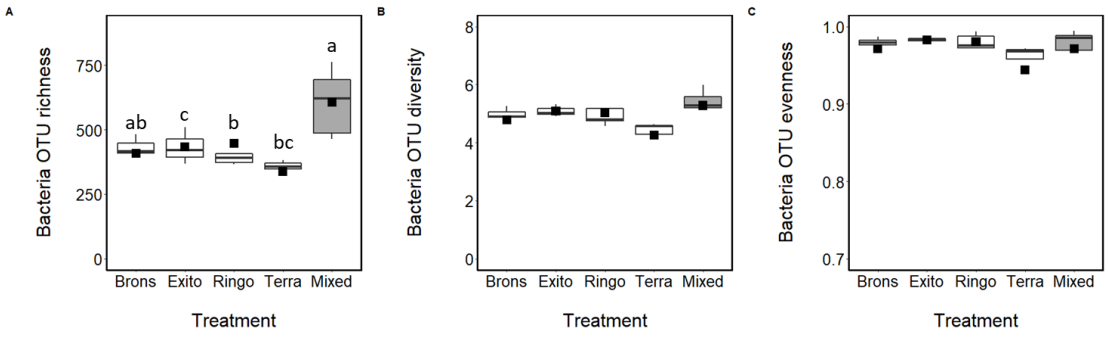


Figure 5.12. Root associated bacterial (A) richness, (B) diversity and (C) evenness effects from cover crop varieties. White boxplots represent monocultures, and grey boxplots represent mixtures. Each box plot represents the median (horizontal line), mean (solid black square) and 25th to 75th percentiles, bars.

Conversely, the fungi followed a less distinct trend (Figure 5.13). Treatment plots had a significant effect on fungal OTU richness (ANOVA; $F_{4,19} = 25.30$, $P < 0.001$). Tukey-post hoc analysis revealed two distinct groups. Library size had a significant effect on OTU richness (ANOVA; $F_{4,19} = 11.28$, $P < 0.01$) but the interaction between treatment plot and library size was insignificant, therefore log(lib.size) was included as a separate factor to explore effects of unequal sequencing depth (Appendix 5.3). Diversity (ANOVA; $F_{4,19} = 2.29$, $P = 0.09$) and evenness (ANOVA; $F_{4,19} = 1.36$, $P = 0.28$) were not significantly different between treatment plots and library size had no effect.

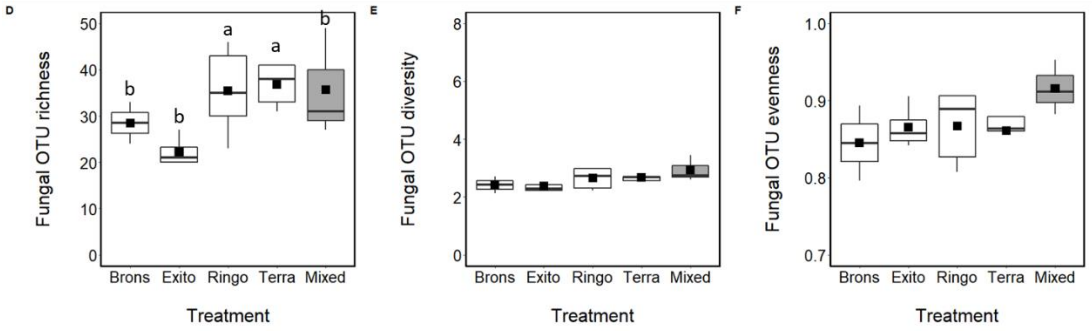


Figure 5.13. Root associated fungal (A) richness, (B) diversity and (C) evenness effects from cover crop varieties. White boxplots represent monocultures, and grey boxplots represent mixtures. Each box plot represents the median (horizontal line), mean (solid black square) and 25th to 75th percentiles, bars. White boxplots represent monocultures, and grey boxplots represent mixtures. Each box plot represents the median (horizontal line), mean (solid black square) and 25th to 75th percentiles, bars.

5.4.2.3 | Fungal guilds

Further exploration of functional guilds revealed there were no significant differences in OTU richness of pathogens between cover crop variety (ANOVA; $F_{3,6} = 0.62$, $P = 0.63$). Saprotrophs differed in OTU richness (ANOVA; $F_{4,19} = 11.49$, $P < 0.001$) and library size (ANOVA; $F_{1,19} = 10.52$, $P < 0.01$) across treatment plots, but there was no interaction between the two (ANOVA; $F_{4,15} = 0.59$, $P = 0.67$). Post-hoc analysis revealed two distinct groups (Figure 5.14.A). Endophytes differed in OTU richness (ANOVA; $F_{4,18} = 11.19$, $P < 0.001$) but not library size (ANOVA; $F_{1,18} = 1.26$, $P = 0.28$). Post-hoc analysis revealed two distinct groups (Figure 5.14.B).

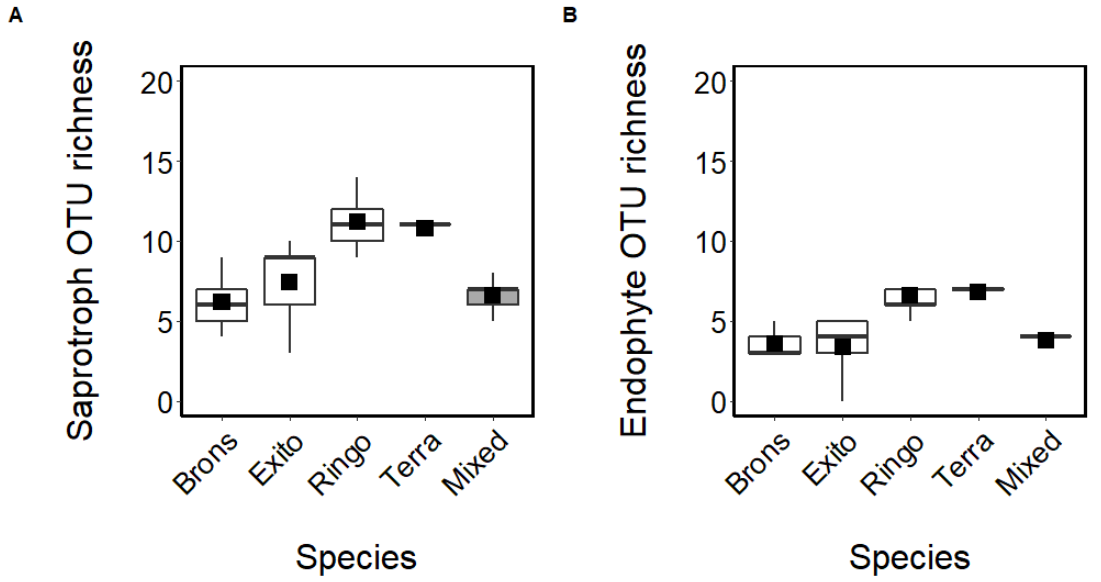


Figure 5.14. Effects of cover crop variety on OTU richness of (A) saprotrophs and (B) endophytes of root-associated fungal communities. White boxplots represent monocultures, and grey boxplots represent mixtures. Each box plot represents the median (horizontal line), mean (solid black square) and 25th to 75th percentiles, bars.

5.4.2.4 | Host-specificity. Beta-diversity of root associated microbial communities

In this study, the total-root associated bacterial community (i.e. all bacterial OTUs observed) in treatment plots were compositionally distinct (PERMANOVA; $F_{4,23} = 2.47$, $P < 0.001$, stress = 0.0624). Conversely, the total-root associated fungal community (i.e. all fungal OTUs observed) in treatment plots were not significantly different (PERMANOVA; $F_{4,18} = 2.05$, $P = 0.32$, stress = 0.1767).

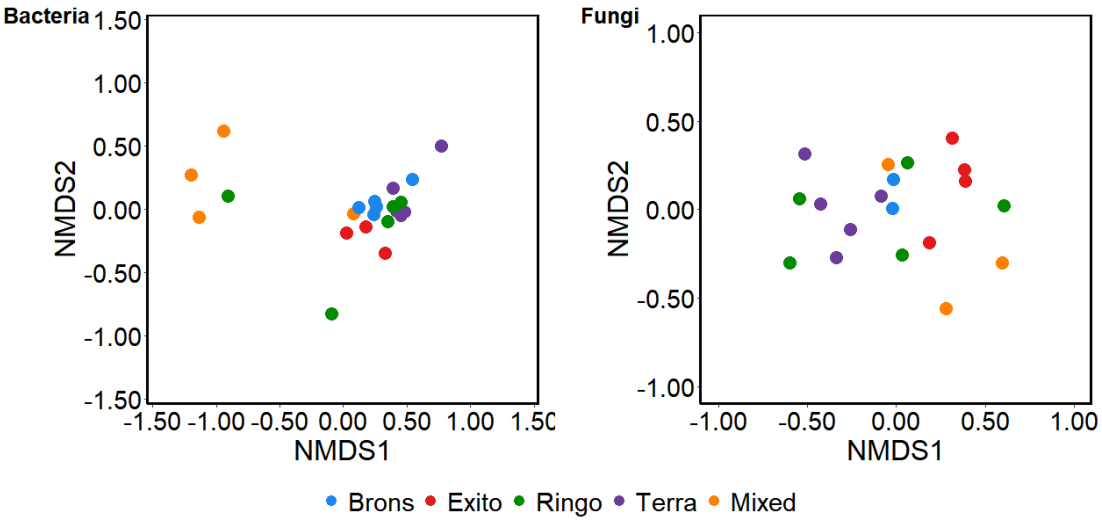


Figure 5.15. Specific root-associated bacterial communities (left) and fungal communities (right) from four cover varieties. Nonmetric multidimensional scaling (NMDS) ordinations, based on Bray's distance matrix of the total fungal community.

5.5 | Discussion

The overarching theme of this Chapter concerns enhancing our knowledge of host-specific root-associated and soil-borne microbiomes with the aim to inform more sustainable agricultural practices. Through both of the aforementioned studies, enhanced ecological understanding of the microbial effects associated with above-ground biodiversity, and host-specific effects of plants within and between crop cycles will help to inform management strategies that could potentially exploit plant-microbe interactions for their benefits in terms of ecosystem functioning and also provide scope for the suppression of soil-borne disease. In essence, this could lead towards a future of more 'self-sufficient' agri-ecosystems, and thus through lessened chemical inputs, not only has vast economic benefits, but undoubtedly will minimise environmental risks associated with traditional farming practices.

5.5.1 | Wheat cultivars - Biodiversity experiment

The findings from this study constitute some of the first field trials to utilise next-generation sequencing approaches to open the 'black-box' of root-associated and soil-borne microbial communities within an agricultural production system. By characterising the microbial communities of wheat cultivars grown in monoculture, and in three and nine species plots, I provide empirical tests of the two underlying assumptions of the 'pathogen hypothesis' as discussed in 5.1.The results from this study did not meet the prerequisite of the pathogen hypothesis, fungal host-specificity. Nonetheless, root-associated fungal richness significantly decreased in three variety mixed plots and mixing of wheat varieties in three cultivar mixes consistently saw a reduction in unique fungal OTUs, this could suggest effects of NDD through loss of pathogenic OTUs. Although interestingly, all microbial groups held this pattern. Moreover, soil-associated bacteria significantly differed in richness and composition between wheat varieties grown in monoculture. The findings from this study provide

encouraging evidence to imply the negative effects off NDD in agriculture could be lessened by increasing aboveground diversity by growing mixed cultivar stands and the choice in these cultivars can also have significant effects on bacterial diversity and composition. However, both of these theories require validation by making an assessment of soil function and assessing the impact of informed cultivar choice on yield.

5.5.1.1 | Effects of soil fraction: Root-associated vs soil-borne microbial communities.

H1: Root-associated and soil-borne microbial communities will be distinct within cultivars growing in monoculture

Average OTU richness of root-associated fungi were significantly richer than soil-borne fungi. This was further confirmed by multivariate analysis in which 100 OTUs were more abundant in roots while 56 OTUs were less abundant in roots compared to soil. Regarding composition, across wheat varieties bacterial and fungal communities differed between the roots and soil. However, this was not significant went looked at within a single wheat variety. This trend held true for both bacterial and fungal community composition. In literature, there is clear evidence to suggest plants shape the structure of their associated microbial communities (Badri & Vivanco, 2009; Philippot et al., 2013; Praeg et al., 2019). The enrichment of rhizosphere-specific taxa, i.e. the rhizosphere effect', such as symbiotic AMF and saprotrophs (Ranelli et al., 2015), is driven by root derived products termed rhizodeposits and plant-specific root exudates, both products of photosynthesis rich in organic carbon (Nguyen, 2003).

Interestingly, the number of unique fungal OTUs associated with the soil and root fraction were almost identical, however,multi-variate analysis of fungal diversity between root-associated and soil-borne fungi revealed a significantly greater number of taxa that occurred in significantly higher abundance in roots, compared to soil.

Members of the class Dothideomycetes, (order Pleosporales) were one group to dominate this trend, this is in align with Wang et al., (2017) in which Pleosporales were more abundant in rhizosphere soil compared to bulk soil. Fungi belonging this phylum, Ascomycota, quickly become dominant on plant roots as they are able to immediately utilise the carbon released by plant roots (Hannula et al., 2012). Saccharomycetales were another order of the Ascomycota that proliferated with the association roots of plants. Species of this order are known for their plant-growth promoting abilities of multiple crops (Karajeh, 2013) and thus has potential positive effects for the ecosystem functioning of these agricultural plots. These significant shifts in key fungal biota have implications for plant health as it could effect the response of plants to both abiotic and biotic stressors such as herbivory, or create a type of PSF response in which the proliferation of beneficial microbes drives a shift in the physicochemical properties of the soil to the benefit of conspecific plants (Bakker et al., 2018; Lladó et al., 2018).

Conversely, bacterial OTU richness did not significantly differ between root-associated and soil-borne communities. Nonethelessanalysis of OTU similarity suggests a greater abundance of unique bacterial OTUs are derived from the soil fraction, as opposed to the roots. This suggests bacterial communities may not be as dependent as fungi on root exudates and rhizodeposits for their growth and proliferation. The richer diversity in soils is likely because the soil serves as a microbial reservoir from which the roots recruit their associated microbes. However, to confirm any of these theories, characterisation of root exudates across the distinct wheat genotypes would be required.

5.5.1.2 | Host specificity of microbial communities from wheat cultivars in monoculture

H2: Fungal and bacterial communities will show host-specificity from root-associated samples.

The roots and soil of wheat varieties growing in monoculture harboured significantly different bacterial OTU richness, however, compositional analysis showed only soil-borne bacterial communities were distinct between monocultures of wheat varieties. This means differences in microbial richness of root associated bacterial communities were likely caused by rare OTUs that did not contribute significantly to the overall bacterial composition. Nonetheless, 53% of variation in soil-borne bacterial communities were explained by monoculture plots associated with different wheat genotypes. This finding shows within species differences at the cultivar level are capable of manipulating the soil bacterial microbiome. This could have beneficial applications in future agricultural practices if this shift in the soil microbiome is coupled with a modification of the functionality of the soil. To explore soil functionality, data on yield between monocultures plots of wheat varieties would be required.

Interestingly, root-associated and soil-borne fungal communities did not differ between wheat varieties growing in monoculture, as revealed by alpha and beta diversity analysis. This in contrast to multiple studies demonstrating host-specificity of fungal biomes (Mills & Bever, 1998; Liu et al., 2012; Bever et al., 2015; Mommer et al., 2018) and thus challenges the first underlying assumption of the pathogen-hypothesis regarding host-specificity. Nonetheless, this study does not undermine the assumptions of the pathogen-hypothesis but merely suggests the specific genotypic differences in this study might not be large enough to drive host-specific differences in fungal communities. This is not a surprising result as fungal host range is known to correlate with host phylogenetic distance (Gilbert & Webb, 2007) and data from

Chapters 3 and 4 show a clear phylogenetic host signal that becomes stronger at higher plant taxonomic ranks. It is likely this is because root exudates do not differ between wheat variants or fungi are not sensitive to potential differences in root exudates. This provides an avenue for future research. To validate this theory, techniques such as NMR or gas chromatography with mass spectroscopy would be required to characterise the metabolic profile of root exudates of each genetic variant (da Silva Lima et al., 2014).

5.5.1.3 | Aboveground diversity gradient effects on soil-borne and root-associated microbial communities

H3: Bacterial diversity will increase with cultivar species richness.

H4: Fungal diversity will likely decrease or remain the same with aboveground diversity, this is a result of a loss of pathogenic OTUs.

In align with the above results regarding host-specificity, only OTU richness of soil-borne bacterial communities differed when wheat varieties were mixed, although this relationship was not linearly correlated. It is sensible to suggest a significant loss of bacterial diversity in three cultivar mixes could be met with impaired ecosystem functioning, however, measurements on yield and biomass would be required to validate this theory. The lack of a relationship between root-associated bacterial diversity and aboveground richness is not surprising as host specificity of root-associated bacterial communities was not met. If host-specificity held true, it would be predicted that when multiple cultivars are grown together, their unique species-specific microbial consortia would also exist together and thus plant species richness would be correlated with microbial richness.

Despite lack of host-specificty of fungal communities grown in monoculture, root-associated fungal communities followed a similar trend in which there was a non-linear

difference in fungal richness as wheat varieties were mixed. This result aligns with aligns with Mommer et al., (2018) who revealed a loss of fungal diversity when plant species were mixed. This could be signalling of a loss of pathogenic fungal OTUs at increased genetic richness aboveground. To consolidate this theory it was important to investigate diversity effects at the level of functional guild (i.e. plant pathogen, saprotrophs, endophytes), however, no fungal guilds differed when wheat varieties were mixed. However, this could be an artefact of the extremely low taxonomic resolution of this dataset. Nonetheless, analysis of core and unique OTUs between aboveground diversity did show some supportive evidence in align with the dilution effect as seen by Mommer et al., (2018). 115 unique root-associated fungal OTUs were found in monoculture plots, whereas only 3 and 45 unique OTUs were found in three and nine variety plots respectively. This loss of fungal diversity could be fungal pathogens, and as such could have vast implications for more sustainable agriculture by reducing the negative effects associated with monoculture cropping. Nonetheless, this is only an assumption and to make more reliable predictions ecologists require enhanced coverage of taxonomic databases for cultivated crops, this would involve isolation and purification of fungal strains for use in subsequent plant bioassays. Whilst possible, this was beyond the scope of this project but certainly provides a basis for further research.

5.5.1.4 | Conclusion

The data learned from the first experimental series in this Chapter provides encouraging evidence to imply within species differences could be large enough to drive informed exploitation of beneficial plant-microbial interactions and reduce the negative effects associated with monoculture cropping. However, these interactions cannot be confirmed without a replicated study in which crop yield and simultaneously measured. Furthermore, characterisation of the relative importance of root-derived

products in structuring microbial communities could be the one of the first stepping stones for targeted microbiome engineering. However, the control we have over creating genetic variants and manipulating the identity of each variant is beyond the scope of an ecologist and therefore relies on the expertise of geneticises and plant physiologists

5.5.2 | Cover crop trial

The second half of this Chapter explores the impact cover-crop choice selection has on the diversity and composition of root-associated microbial communities and the subsequent consequences this may have for ecosystem functioning. Small-scale mesocosm experiments (Maherali & Klironomos, 2007; Wagg et al., 2014) and global scale studies (Delgado-Baquerizo et al., 2016) reveal microbial diversity is linked to ecosystem functioning. To be more specific, it is ubiquitously accepted that an ecosystem with greater microbial diversity perform better. This means an enhanced understanding of how we can manipulate belowground microbial diversity through host specific plant-microbial interactions could lead to agri-ecosystems that are more self-sufficient. In this study, each cover crop variety (single species or a species mix) were chosen for potential abiotic (i.e. rigorous root system to infiltrate soil) or biotic (i.e. disease suppression) beneficial effects. In line with the theme throughout this thesis, I test for the first assumption of the pathogen-hypothesis, host-specificity and then test for potential signalling of NDD when cover-crops are grown in mixes. Moreover, I explore differences in abundant bacteria and fungi between plots and infer predictions regarding ecosystem functioning. The characterisation microbial profiles between cover-crop varieties will help to inform more efficient crop-rotations systems; exploiting beneficial plant-microbe interactions and increasing microbial diversity could mean the reliance of external inputs (i.e. herbicides and fertilisers) is reduced, this not only has economic benefits, but reduced vast environmental risks.

5.5.2.1 | Host specificity of root-associated bacterial and fungal communities

H1: Root-associated microbial communities will be distinct between cover-crop varieties – this implies host-specificity.

H2: Fungal diversity will be lower in cover-crop varieties containing Brassica species.

Measures of bacterial OTU richness were significantly different between monoculture plots and comparatively, OTU richness was consistently higher in the mixed cover-crop plot. This hints towards host-specificity of bacterial communities and indicates there is an accumulation of host-specific relationships in mixed-species plots. This result alone would advocate the use of mixed cover-crop varieties as increased bacterial diversity is known to improve ecosystem functioning (Delgado-Baquerizo et al., 2016). Furthermore, the roots of cover-crop species growing in monocultures harboured compositionally distinct bacterial communities, providing strong support for host specificity. These results are in align with Fitzpatrick et al., (2018) in which 30 phylogenetically distant plant species harboured compositionally distinct bacterial communities. It is hypothesised that plant species, age and developmental stage drive differences in microbial community composition (Bardgett & Van Der Putten, 2014), most likely through the release of photosynthates (root-exudates) between plant species that can be used as a carbon source by bacteria (Philippot et al., 2013).

Similarly, fungal OTU richness differed between both monoculture and mixed plots. However, in contrast to bacteria, mixed plots did not harbour the greatest OTU richness, nor did they see a suppression of fungal pathogens. This result implies the choice of cover-crop plays a greater role in driving fungal diversity than growing mixed cover-crop varieties. This is not a surprising result when the properties of each cover-crop variety are considered. The Biofum species mix contains 50% Ethiopian mustard.

Ethiopian mustard belongs to the Brassica family known for enhanced production of glucosinolates, these secondary metabolites have previously been shown to supress total fungal and fungal pathogen microbes (Sotelo et al., 2015). Similarly, Brons plots, a monoculture of Brown mustard (Brassica juncea), share the same low level of total fungal richness. This provides support of the fungal suppression properties of cover-crop varieties belonging to the Brassica family. However, the difference in total fungal richness did not differ between Brons (100% Brassica species) or mixed plots (50% Brassica species). This implies there could be a difference in the suppressive ability between different Brassica species. However, without complete metabolic profiling of root-exudates in mixed plots, we can only speculate, but not confirm that this result is driven by an increased production of glucosinolates by the inclusion of Brassica species.

Moreover, root-associated fungal communities were not compositionally distinct between plots and therefore fails to meet the first assumption of the pathogen-hypothesis, host-specificity. This result goes against empirical evidence of fungal host-specificity in literature (i.e. Mills & Bever, 1998; Schnitzer et al., 2011; Ranelli et al., 2015; Mommer et al., 2018). This result is likely an artefact of experimental design, this study did not provide the scope for true biological replicates nor were abiotic variables measured. Further field trials including a randomised block design including true biological replicates and thorough sampling of both biotic and abiotic factors would enable more reliable ecological predictions to be made.

5.5.2.2 | Functional response – fungi

H3: If host-specificity is confirmed, functional diversity will differ between cover-crop varieties.

Within the context of this study, it was important to investigate cover-crop choice effects at the functional level of guilds (i.e. plant pathogens, endophytes and

saprotrophs). For fungal taxa, this comes with relative ease because phylogenetic data sets for fungal communities benefit from the use of a widely used open source annotation tool called FunGuild to parse OTUs by functional guild (Nguyen et al., 2016). In light of the pathogen dilution effect of 'pathogen hypothesis' (van der Putten et al., 2013) and due to the pathogen-suppressive properties of the Biofum mix, in a truly replicated experimental design, I would have predicted a decrease in pathogen richness in mixed cover crop plots. However, pathogens did not differ between cover-crop variety, or between single species cover crops and three species cover crop mixes. However, this relationship may have been masked by poor guild coverage of the relevant OTUs within the FUNguild database or the small-scale approach of the implemented experimental design.

Interestingly, saprotrophs and endophytes differed in OTU richness between plots, both occurring in greater richness in Ringo (forage rape) and Terra (oil seed radish) plots. Analysis of the top ten most abundant fungal genera revealed taxa belonging to the genus Mortierella were predominantly driving this relationship. Taxa belonging to the genus Mortierella contain a large number of species (~100) and commonly occur in soils as saprotrophs (Kendrick, 2003). Saprotrophic fungi are key regulators of nutrient cycling in terrestrial ecosystems (Baldrian & Valášková, 2008) and endophytic fungi are regarded as beneficial plant symbionts for their plant growth promoting capabilities such as phosphate solubilisation (Chhabra & Dowling, 2017). As such, the proliferation of both of these groups could have beneficial implications for plant growth and ecosystem functioning. Further experiments to monitor soil legacy effects of beneficial interactions for subsequent cash crops would inform more successful crop rotation schemes. Furthermore, while total-fungal suppression is advocated because it is coupled with pathogen suppression, the aforementioned results show all fungal guilds were reduced in Biofum mixed plots. In addition, Brons plots (Brown mustard) harboured the lowest saprotrophic and endophytic diversity, again, this is likely due to

the fungal suppressive properties of glucosinolates produced by Brassicas. The next step would be to determine if suppressing all fungal guilds (in the hope to supress fungal pathogens) outweighs the benefits of symbiotic saprotrophic and endophytic interactions. This would require repeated trials in which the microbial community is characterised simultaneously with biomass production for quantitative assessment of cover-crop yield, but also of subsequent cash crop rotations.

General analysis of the top ten most abundant genera revealed a number of abundant fungal taxa that were not present in all treatment plots. Only two fungal taxa (OTUs) contributed to the genus Operculomyces. Interestingly, these taxa were only present in high relative abundances (top ten) in Exito and Ringo plots. Unfortunately, the ecological function of this taxa is unknown, but other genera in the order Rhizophydiales are known for their parasitic and saprotrophic potential. This could therefore have significant consequences, either positive or negative, for ecosystem functioning (Letcher et al., 2006). Furthermore, taxa that belong to the genus Entrophospora, arbuscular mycorrhizal Glomeromycete, were found in exclusive association with Ringo plots (forage rape). AMF have positive implications for nutrient acquisition and research has shown plant associations with Entrophospora have been known to increase yields in fruit (Vázquez-Hernández et al., 2011). Entrophospora symbiosis might have the potential to leave positive soil legacies for the cultivation of subsequent cash crops and as such could contribute to the success of sustainable agriculture. While these results only provide a glimpse of plant-microbe interactions in a few selected cover crop varieties, this data shows strong proof of principle evidence to advocate the use of carefully informed cover cropping regimes to enhance ecosystem functioning. Future experiments investigating potential plant hosts of beneficial microbes and subsequent positive PSF (i.e. increased yield) would help to inform more efficient crop rotation systems.

5.5.2.3 | Functional response - bacteria

H4: If host-specificity is confirmed, functional diversity will differ between cover-crop varieties.

Within the scope of this dataset, bacterial functional assessment between treatment plots was only possible by identifying differences in taxonomic (OTU) relative abundance between treatment plots. To do this, I compared alpha-diversity measures for the top ten most abundant bacterial genera. Brons and Terra plots consistently held the greatest OTU richness for subsets of bacterial genera, while Exito plots held the lowest. Collectively, taxa belonging to the phyla Firmicutes and Actinobacteria (i.e. Bacillus, Micromonospora, Streptomyces, Nocardioides and Solirubrobacter) were consistently and significantly higher in Brons and Terra plots. Many of these Gram-positive bacteria have excellent biocontrol, plant-growth promoting, and bioremediation properties (Trujillo et al., 2015). Of these groups, species of Streptomyces are well studied and research has shown the ability of certain species to produce auxin indol acetic acid (IAA) (Goudjal et al., 2013), a compound involved in seed germination and root elongation, and antifungal molecules that have shown the potential to inhibit crop disease (Cao et al., 2005; Goudjal et al., 2014). In contrast, research is still in its infancy regarding other plant-actinobacteria associations. Fuelled by recent molecular advances, Micromonospora are increasingly isolated from root-nodule tissues and host plants inoculated with some of these strains showed better rates of growth (Trujillo et al., 2010; Deng et al., 2011). Micromonospora were previously overlooked in the microbial nitrogen-fixating niche due to its slow growth on culture dependent analyses. However, work by Carro et al., (2013) strongly suggests this microbe is a common inhabitant of actinorhizal plants. However, the ecological role of the interaction between rhizobia and Micromonospora is unknown. While Micromonospora has no known nitrogen-fixating abilities, plant co-inoculation indicates Micromonospora has a positive effect on plant growth in which an increase in number of nodules and height of plants was seen in co-inoculation as opposed to

plants inoculated with rhizobia and Micromonospora separately (Martínez-Hidalgo et al., 2014). While still in early stages, the aforementioned evidence, and the high abundance of Micromonospora in two out of five treatment plots, suggests agricultural farming practices could be modified to exploit this interaction which may see an increase in crop yields. While data available is currently very limited, reduced reliance on culturing techniques and increased implementation of 16S rRNA bacterial sequencing implies the presence of Micromonospora among nitrogen fixating plants (legumes and actinorhizal) may be more widespread than previously thought. This implies there is a potential to use this bacterium as a promising plant growth promotor in combination with rhizobia. The knowledge of this interaction requires a lot more work to fully understand the ecological process, but nonetheless, this information has the potential help to inform more successful crop rotation schemes between cover crops and cash crops. This is particularly relevant to the cultivation of leguminous crops.

Rathayibacter were another notable genera when exploring differences in relative abundance between treatment plots, but interestingly, the high abundance of this genera was driven by a single OTU (OTU.83). This species was highly abundant in three of five treatment plots, but the overall contribution to the taxonomic distribution in Exito and Ringo plots was near negligible. There are six recognised species that belong to the genus Rathayibacter. The negative impacts associated with the presence of Rathayibacter are threefold and should not be underestimated. Firstly, all Rathayibacter species are recognised as phytopathogenic bacteria and as such can have significant consequences for global food production (Martins et al., 2018). Secondly, and perhaps more importantly, all phytopathogenic Rathayibacter species identified to date are unique in that they are vectored by plant parasitic nematodes (Murray et al., 2017). Seed gall nematode, Anguina tritici, causes ‘ear cockle’ in which grain is converted into cockles and as such has vast consequences for seed losses (Ozberk et al., 2011). Whilst most prominent in wheat, they are known to parasitise a

wide range of Poaceae plant species. From an agricultural perspective, this result could have massive ecological implications. Control of seed gall nematode including rotations and seed cleaning has effectively eradicated wheat gall nematode and as of a decade ago there had been no evidence of wheat gall nematode in previously infected regions of the world (Nicol & Rivoal, 2008). However, more recent evidence has shown global re-emergence of this nematode in agriculture and this could have devastating consequences for arable production systems (Mukhtar et al., 2018). Finally, A. tritici is related to the transmission of the bacterial pathogen Rathayibacter toxicus that produces toxins in annual ryegrass often resulting in the fatal toxicity in grazing animals. The re-emergence of Rathayibacter should not be taken lightly due to the risk of multiple and wide spread negative effects across the entire agricultural industry. While the plots in this study were not part of cultivatable land for food supply, I would recommend a period of fallow, seed sterilisation, and careful planning for subsequent studies not to include plant species belonging to the family Poacae to prevent any risk there might be associated with the transmission of disease, this includes common cereal crops such as maize, wheat, rice and barley. In addition, it might be worthy to sample local arable land for the presence of Rathayibacter and thus its presence can be eradicated before it has long lasting effects on crop production. Although 16s rRNA amplicon sequencing is widely used for screening purposes, it is not the most reliable way to predict functional profiles of microbial communities. Future studies would benefit from analysis of functional genes, for example, amoA and denitrification genes can be used as molecular markers to quantify the bacterial and thaumarchaeal ammonia-oxidizing and the denitrifying communities (Leininger et al., 2006).

5.5.2.4 | Conclusion

This study investigated the impact of a selected few cover-crop varieties on the taxonomic diversity of microbial communities found in the rhizosphere soil while

making informed predictions for functional diversity. The findings from this study have several implications. This study confirms choice of cover crop variety/species and carefully designed cover-cropping strategies could be used to exploit plant-microbial interactions for the benefits of ecosystem functioning. This includes beneficial plant-saprotrophic/endophytic interactions, reduced negative plant-fungal pathogen interactions and increased plant-actinobacteria associations. This improved understanding of plant-microbe interactions specific to cover crop species/varieties will contribute to the success of potential microbiome engineering and increased plant production in the future. Nonetheless, a future for sustainable agriculture does not have a static or single correct answer and this data alone hints towards, but does not provide the answer to sustainable agriculture. Looking forward, to confirm positive effects of potentially beneficial plant-microbe interactions, further work that encapsulates quantification of plant biomass and yield are required. It is also important to investigate legacy effects to inform productive cash crop versus cover crop rotations. Doing so would validate the cause and effect relationship between host specific plant-microbial interactions and enhanced ecosystem functioning. While cover crops are traditionally selected for their biotic and physical influences on the surrounding soil environment (i.e. deep root system or weed suppressive ability), the incorporation of a microbial perspective into cover crop rotations might increase the speed at which we develop more sustainable agri-eco practices.

5.6 | Limitations

Concerning Chapter 5, it could be argued that strong inferences were made based on the detection of abundant microbial taxa and their potential ecological function. However, this approach must be viewed with caution because phylogenetic identification through rRNA gene profiling does not necessarily correlate with the presence of metabolically active functional genes at the time of sampling. Nonetheless, the ability to make predictions based on the presence of abundant

microbes has potential to provide a quick overview of broad shifts in the total microbial community. This serves as a valuable tool by providing a starting point to select targeted analysis of functional genes such as NOB (nitrogen oxidising bacteria) and AOA (ammonia oxidising archaea) as examples. Alternatively, the relationship between phylogenetic data and ecosystem functioning could have been further explored if biomass data for each plot were available. For example, this would indicate whether there was a correlation between shifts in abundant microbial groups, i.e. saprotrophic bacteria such as Mortierella, which may beneficial for nutrient cycling, and plant productivity.

To further this, the ability to draw reliable and strong conclusions in trials 5.3.2 and 5.3.3 was confounded by experimental design in which there were only two and one true biological replicates per treatment respectively. To mitigate the concerns associated with inadequate biological replication it was ensured within treatment differences were minimised by repeating sufficient technical replicates (i.e. 3-5 cores were taken per plot). Nonetheless, the product of lack of true replication raises issues concerning the uncertainty of data and additionally, microbial diversity may be underestimated as it is uncertain whether species-sampling saturation was achieved. However, it must be stressed the purpose of this chapter was proof of principle, thereby, aimed to provide an initial glimpse into the effect of cultivar choice on the root associated microbiome. Both studies provide promising results to advocate that a similar experiment should be replicated on a larger scale and provides scope to inform the selection of cultivars with non-overlapping microbiomes.

Chapter 6

Managing oil palm plantations more sustainably – Effects of understory vegetation management on soil-borne microbial communities

6.1 | Abstract

In recent decades, rapid expansion and intensification of oil palm cultivation has been met with huge controversy due to its inherent impact on native biodiversity and associated loss of ecosystem functions. As such, the requirement for sustainable oil palm cultivation is increasing. One avenue that has not yet been explored is the role of soil-borne microbes in the promotion of more sustainable cultivation of palm oil. In this study, I utilised next-generation sequencing techniques to investigate the effects of understory vegetation diversity on soil-borne microbial communities (bacteria, archaea, micro-eukaryotes and fungi). Understory vegetation within oil palm plantations was manipulated in three treatments: 'enhanced' understory complexity (increased native biodiversity), 'normal', and 'reduced.' Results showed 'enhanced' treatment plots had the strongest effect on the diversity and composition of microbial communities. Bacterial taxa were richer in 'enhanced' treatment plots, with increased diversity of beneficial taxa including Actinobateria and Burkholderiales which could have positive implications for ecosystem functioning including biocontrol and N fixation. While eukaryotes did not reveal any overall significant variability between treatment plots, univariate analysis revealed heterotrophs (i.e. Cercozoa), were positively enriched within 'enhanced' treatment plots and as such could have further beneficial implications for nutrient turnover and ecosystem functioning. Conversely, fungal diversity appeared to be significantly impaired by management strategies implemented within 'enhanced' treatment plots, it is plausible to suggest fungal communities were supressed through the enrichment of mycophagous bacteria such

as Enterobacteriales. The data from this study demonstrates adopting more ‘wildlife’ friendly approaches could result in more efficient cycling of nutrients between key microbial functional groups. This means a future of more sustainable oil palm production might depend less heavily on external inputs and as such not only has vast environmental benefits, but could also increase yield while reducing costs of herbicides and manual labour.

6.2 | Introduction

Global agriculture has rapidly expanded to meet the consumption needs of our growing human population *(Tilman et al*., 2001; Godfray *et al*., 2010). Unfortunately, the tropics, centred on some the most biologically diverse terrestrial ecosystems on earth (Myers *et al.*, 2000), also serves as the location for the fastest rate of agricultural expansion in the world. More than 80 % of agricultural expansion has come at the expense of tropical forests (Gibbs *et al.*, 2010). However, the loss of 13.5 million ha of tropical, high rainfall, low-lying areas (Corley & Tinker, 2003) to plantations for oil palm cultivation, is one of the most controversial land uses associated with high ecological, economic and social impacts. As such, agricultural development, one of the largest drivers of land use change, is receiving ever-increasing demand to develop more ecologically friendly practices to maximise sustainability and mitigate potential threats imposed by conventional management practices.

Palm oil (*Elaeis guineensis*), native to Africa, with high output, easy establishment and relatively low costs, is one of the most widespread commercial crops and holds the highest yield per unit area (Zimmer, 2010), producing on average ~ 5 tonnes of oil per hectare per year (May *et al.*, 1992). Malaysia and Indonesia, the two largest contributors, account for 85 % of global palm oil output (Koh & Wilcove, 2007), with a combined total of 46 million tonnes annually. Amongst the largest drivers of agriculturally driven biodiversity loss in oil palm plantations includes reductions in shading, changes to the microclimate (young plantations with an open canopy have soils that are up to 6.5 hotter than old growth forest, Hardwick *et al.*, 2015), pest susceptibility induced by single-aged monoculture plantations and changes to soil chemical and physical properties - including initial clearing, considered the time point when environmental impacts are at their highest resulting in exposure which can make soils more vulnerable to erosion, compaction and leaching (Matson *et al.*, 1997; Benton *et al.*, 2003; Wilson *et al.*, 2005; Clarke & Walsh, 2006; Luskin & Potts, 2011;

Drescher *et al.*, 2016; Meijide *et al.*, 2018). In addition, extensive application of chemical fertilisers and pesticides are applied during the cultivation process (Corley & Tinker, 2015). Whilst management practices within oil palm plantations varies widely, it is not rare for 90 % of the pesticide budget to be used for management of understory plants that are typically thought of as 'weeds' that compete with oil palms for nutrients. Pesticides are known to reduce biodiversity worldwide (Geiger *et al.*, 2010; Beketov *et al.*, 2013). Correspondingly, chemical fertilisers and pesticides can pollute water sources and pose threats for aquatic ecosystems and human health (Schiesari & Grillitsch, 2011; Comte *et al.*, 2012). Consequently, dramatic losses in species diversity are predicted to be reflected in disproportionate losses in ecosystem functions (Barnes *et al.*, 2014; Ashton-Butt *et al.*, 2018), including changes in functional diversity (Tscharntke et al. 2008; Bihn et al. 2010; Sekercioglu 2012), and loss of functional services with potential impacts for climate change such as carbon storage (Foley et al., 2007) and pollination (Kremen *et al.*, 2002).

The growing environmental concern associated with oil palm cultivation has increased demand for production of sustainable palm oil. Reducing unnecessary expansion by ensuring current plantations are managed in a sustainable and optimum way is crucial to reduce negative impacts on ecosystem functioning whilst simultaneously maximising yield and productivity for farmers. Oil palm is a perennial crop with long rotation cycles (mature at 25 years), an output-to-input ratio exceeding all other oil seed crops (Basiron, 2007), and provides areas differing in habitat complexity because of its tree-like structure (e.g. harvesting path, frond heaps, weeded circles and windrows). This offers substantial potential for developing wildlife-friendly management strategies within these exceptionally important and widespread landscapes. The Roundtable on Sustainable Palm Oil (RSPO), a not-for-profit organisation, has developed a set of global standards for the production of Certified Sustainable Palm Oil (CSPO). For example, governmental policy prohibits land

clearing by means of fire in Indonesia, kernel shells are burned to generate energy for milling processes, and empty fruit bunches, mill effluent and decanter solids are used as an organic mulch (Basiron, 2007; Khatu*n et al*., 2017; Tao *et al*., 2018) to reduce the risk of nutrient fluxes and address waste management. One of the key core management criteria for CSPO is to improve soil sustainability through an encompassed approach of increasing fertility and reducing degradation of soils (Roundtable on Sustainable Palm Oil, 2018). Soil health can be enhanced by land use decisions and is impaired by decisions that only focus on a single aspect, such as yield.

A new and emerging avenue is the role microbes could play in the development of more sustainable palm oil production. Evidence suggests largescale conversion to monoculture, subsequent reductions in plant diversity via removal of the understory, and inherent landscape simplification causes a marked difference in the abundance and composition of soil microbes from those in natural systems (McGuire *et al.*, 2015; Tripathi *et al.*, 2016). Yet knowledge of soil fauna in both natural and agricultural systems and its effects on ecosystem functioning remains in its infancy (Bent & Forney, 2008). The dynamic interface between soil, its microbial constituents, and agriculture includes a number of multi-trophic interactions that can either have deleterious or beneficial effects for plant growth (Figure 6.1). The ratio between pathogenic and beneficial microbes is determined by the quality and quantity of rhizodeposits secreted by plants (Somers *et al.*, 2004), these root exudates are largely determined by plant species. It is plausible to predict differences in aboveground plant communities can alter microbial diversity and community composition through variations in plant identity, plant characteristics including rooting depth and canopy cover which can result in altered metabolic niches by inducing subsequent changes to the soil environment such as moisture, temperature and pH. Data already shows enhancing understory vegetation in plantations increases rates of litter decomposition

(Ashton-Butt *et al.*, 2018) and there is an already rich body of evidence to show understory complexity (plant diversity) is linked to higher levels of biodiversity (Chung *et al.*, 2000; Nájera & Simonetti, 2010). Gaining a better understanding of soil microbes, and subsequent ecosystem functioning, as a function of aboveground diversity will vastly improve efforts for the sustainable provision of ecosystem services.

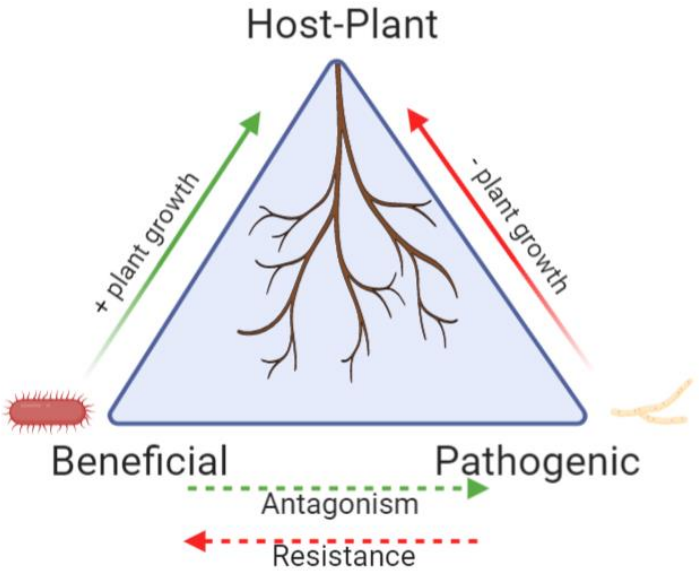


Figure 6.1. Tripartite interaction between plant host, beneficial and pathogenic microbiota within the soil profile and rhizosphere. Colour of arrow represents the overall outcome for plant growth and fitness (green; beneficial, red; negative). Based on a review by Raaijmakers *et al.*, (2009).

To examine these ideas I exploited a long-term biodiversity experiment that directly manipulates the understory within oil palm plantations (see Luke *et al.*, 2020). This experiment was originally designed to test whether increasing landscape structural complexity can enhance oil palm sustainability at little or no cost to yields and profitability. For this study, I survey the microbial communities present within mature oil palm plantations operated under different management practices with the

effort to support biodiversity and associated microbial driven ecosystem processes with implied relevance to soil sustainability. This integrative approach considering all microbial constituents in the soil profile will undoubtedly improve our understanding of ecosystem processes mediated via microbes and therefore offer ecological insight into more sustainable management regarding a shift to greater reliance on microbes and less reliance on external inputs in agricultural systems. Understanding the constituents of the soil microbial profile enhances our knowledge on key ecosystem processes that they mediate and potentially could inform more sustainable practices regarding biocontrol and microbial driven nutrient recycling. Specifically we look at three levels of understory vegetation management ("Reduced", "Normal", "Enhanced") which span levels of management currently used across the oil palm industry. Based on evidence regarding species identity and host specific plant-microbe associations (Chapters 3,4 and 5, Fitzpatric*k et al*., 2018; Mommer *et al*., 2018) I predict increasing plant diversity of the understory within oil palm plantations will be positively correlated with microbial diversity, i.e. when multiple plant species coexist their unique root associated microbiomes would also exist together. Moreover, heavy herbicide and intermediate use in Reduced and Normal treatment plots respectively will not only reduce the opportunity for plant-microbe associated interactions, but could supress non-target organisms including soil-borne microbes that are important for a number of ecosystem processes important for crop production (Nur *et al.*, 2013). As such, in this chapter the following hypothesis are tested:

H1: Microbial rRNA abundance will be greatest in Enhanced plots and suppressed in Reduced and Normal treatment plots.

H2: Microbial communities will show decreased taxonomic alpha-diversity in Reduced and Normal treatment plots.

H3: Microbial communities will differ in composition between treatment plots.

The BEFTA programme is a fully-integrated collaboration between universities, research institutes, and the oil palm industry, ensuring that management practices are realistic and that the outcomes of the project are readily implemented by land managers. The overarching aim of the BEFTA Programme is to demonstrate whether it is practicable to enhance the sustainability of oil palm through various management strategies that increase vegetation complexity within the crop ecosystem with the minimum possible impact on yield and profits. This study was designed on the basis that the view of agriculture and biodiversity as mutually exclusive entities should be discontinued, careful design and planning of agricultural practices can benefit both agriculture (i.e. industry and yield) and wildlife (biodiversity).

6.3 | Methods

6.3.1 | Study site

Fieldwork took place in the Riau Province in central Sumatra, (0° 56′0″ N, 101°18′0″ E). This region is amongst the highest percentage oil palm coverage in the country (Uryu *et al.*, 2008). The climate of this region is described as tropical humid, with a high average rainfall, 27 °C, > 2300 mm/year (from SMARTRI data, 1998 - 2018), with lowland rainforest as the natural vegetation type. The soil type is ferralitic with gibbsite and kaolinite (Ferric 114 Acrisol according to the FAO classification). The study area was selectively logged in the 1970 s prior to clearing and transformation to oil palm plantations between 1985 and 1995: little natural forest remains and the region is now heavily dominated by oil palm agriculture. The plantations in this study were on average 25 years old (23 – 29 years old). These sites therefore represent a tropical ecosystem that has been fundamentally altered from a pre-disturbance system. The sites form part of an established research project in collaboration with Sinar Mas Agro Resources and Technology Research Institute (SMARTRI), the Research and Development entity for upstream activities of Golden Agri Resources (GAR), and the University of Cambridge: The Biodiversity and Ecosystem Function in Tropical Agriculture (BEFTA) Programme (Foster et al., 2014, Luke et al., 2020, ww.oilpalmbiodiversity.com, BEFTA).

As part of this collaboration the BEFTA Understory Vegetation Project has been established to investigate the impact of understory management on plantation biodiversity, ecosystem functioning and oil palm yield, and involves a large-scale, long-term Before After Control Impact (BACI) experiment (note, microbial data is not available as part of BACI). The BEFTA Understory Vegetation Project, established in October 2012, comprises 18 study plots, distributed across two oil palm estates (Ujung Tanjung and Kandista). Plots are located in flat areas of the plantation distant to

habitation, between 10 and 30 metres above sea level and in areas of mature palm (planted between 1988 and 1993). Plots were established at the ends of three neighbouring 300 x 1000 m plantation planting blocks, adjacent to plantation access roads and therefore include both core plantation areas and more open roadside edge vegetation, including a drainage ditch. Each block is 150 m by 150 m (2.25 ha) in area, with a 50 x 50 m inner area where the majority of data are collected. The 150 m x 150 m area contains a mixture of harvesting paths, weeded circles around palms, and windrows (including fronds piles and vegetation). All blocks were managed in the same way, following 'Normal' as outlined below, prior to the experimental set up in 2014. In total there are six replicates of each understory management treatment. The blocks are arranged adjacently in randomly assigned triplets at least one kilometre away from any other replicate, with one block in each triplet assigned one of the three understory management treatments. Within each triplet three different understory management treatments were allocated at random:

1. **Reduced complexity understory vegetation**: representing the most intense level of management used by growers. This treatment involves spraying of all understory vegetation with herbicides. Herbicides used included Glyphosate (Rollup 480 SL), metsulfuron- methyl (Erkafuron 20 WG), Fluroxypyr (Starane 290 EC), Paraquat Dichloride (Rolixone 276 SL – until 2016), and ammonium glufosinate (Rolifos 150 SL – after 2016, following Golden Agro Resources (GAR) policy change on the use of Paraquat Dichloride). Herbicides were re-applied as necessary, three to five times per year, to maintain a clear understory throughout the plots. Hereafter referred to as Reduced.

2. **Normal complexity understory vegetation**: representing an intermediate level of management used by growers and standard practice within GAR estates. This treatment includes an intermediate level of herbicide spraying (using the herbicides listed above) along harvesting paths and within 1.5 m circles around

palms, manual removal of woody vegetation (using a machete), but other vegetation are allowed to regrow. Herbicides were re-applied as necessary, three to five times per year, to maintain clear paths and circles. All plots were managed in this way pre-treatment and therefore act as a control in this experiment. Hereafter referred to as Normal.

3. **Enhanced complexity understory**: representing the lowest intensity level of management commonly used by growers, represents conservation management. This treatment involves no herbicide spraying and only limited hand-cutting of vegetation (using a machete) to create harvesting paths which allow access to the palms, and in the 1.5 m circle around palms. It also includes removal of large woody vegetation (also using a machete). Manual cutting in Enhanced plots began one year after treatments started, but then was carried out at the same frequency as herbicide application in the Reduced and Normal plots. Hereafter referred to as Enhanced.

Standard fertilizer treatment were applied to all treatment plots of oil palm in the study site (Ashton-Bu*tt et al*., 2018): 1.75 kg tree^{-1} yr^{-1} urea (46% N); 0.5 kg tree^{-1} yr^{-1} triple super phosphate (45% P_2O_5, 15% Ca); 2.5 kg tree^{-1} yr^{-1} muriate of potash (61% K_2O, 46% Cl); and 0.5 kg tree^{-1} yr^{-1} Kieserite (16% Mg, S: 22%).

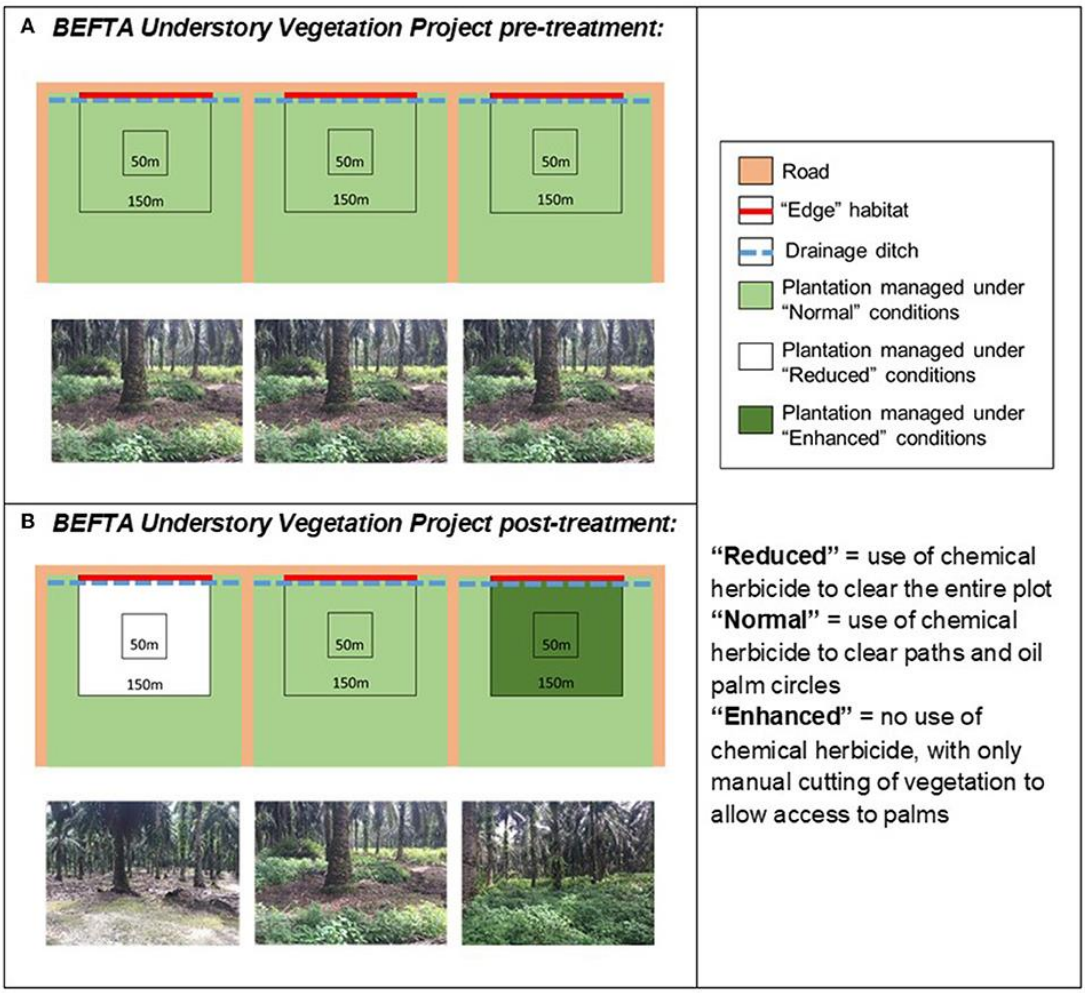


Figure 6.2. Schematic to show the design of the BEFTA Understory Vegetation Project. Each diagram represents a set of three experimental plots ('Reduced', 'Normal' and 'Enhanced'), each of which is 150 x 150 m within which is a 50 x 50 m area (core sampling area) where samples were taken. (A) depicts the plots pre-treatment, whilst (B) shows how management was altered 'post-treatment'. The triplet design is replicated six times (n = 18). In each triplet, the order of in which Reduced, Normal and Enhanced treatments were allocated was randomized. This figure is taken from (Luke *et al.*, 2020).

6.3.2 | Soil Microbial Sampling

Sampling took place in plantations within Riau Province in central Sumatra (21st - 25th January 2019), Indonesia (0° 56′0″ N, 1010°18′0″ E). Within each of the 18 study plots, three random points were chosen (n = 18 * 3) within the inner 50 m x 50 m core sampling area with close proximity to the harvesting path (1 – 2 m). An equilateral triangle (sides of 10 cm) was placed at each random point and a core (4 cm diameter) was taken from each corner (n = 18 * 3 * 3) to a depth of 10 cm. The soil corer was sterilized with 96 % ethanol between each sample. The cores were stored separately at – 20 °C in an airtight sterile bag until DNA extraction. A total of 162 cores were collected across the 18 study plots spanning across the two oil palm estates.

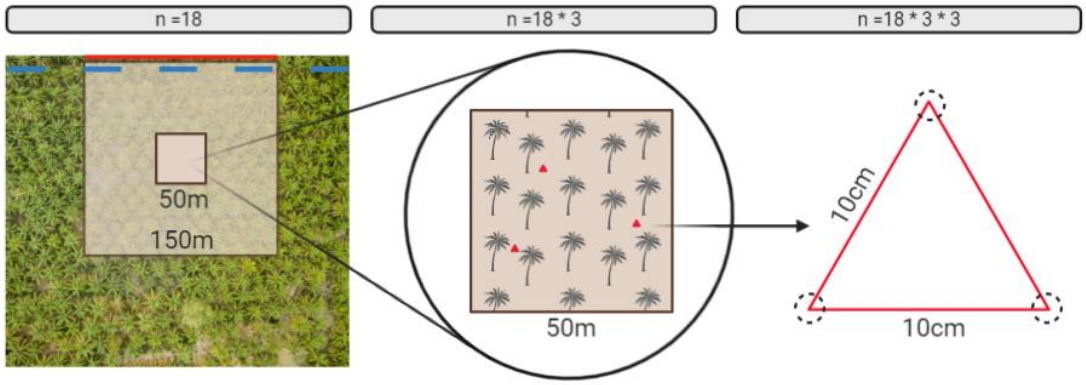


Figure 6.3. Schematic to illustrate sampling protocol for phylogenetic soil microbial profiling. For each study plot (n = 18), three random points were chosen within the 50 m * 50 m core sampling area (n = 18 * 3), at each random sampling point an equilateral triangle (sides 10 cm) was placed and a soil core (depth 10 cm) was taken (n = 18 * 3 * 3). Total number of samples = 162.

6.3.3 | Soil Abiotic Sampling

All edaphic properties were predetermined by the field team at BEFTA in 2016. Soil samples for abiotic analysis were taken from the same sample locations as the soil microbial sampling, within the 50 x 50 m inner core sampling area with close proximity to the harvesting path (1 – 2 m). The following soil properties were measured; pH, soil organic carbon, total soil nitrogen, total phosphate and total potassium content, available phosphate, and cations including calcium, magnesium, potassium and sodium. Total soil P was analysed using the hydrogen chloride extraction method. Cation exchange efficiencies were calculated. Soil moisture was recorded at each point of soil microbial sampling using a wet sensor (DELTA-T). Soil analysis was performed at SMARTRI.

6.3.4 | DNA extraction

DNA extraction from 0.25 g of fresh weight soil was performed using the DNAeasy Power Soil kit (Qiagen) according to the user manual and DNA was eluted into 100 µl of elution buffer. Extracted DNA samples were transported on dry ice to The University of Essex, UK, for molecular processing.

6.3.5 | Molecular analysis

Soil borne microbial communities were characterised using 2 x 300 bp Miseq amplicon sequencing of the bacterial and archaeal 16S rRNA genes, eukaryote 18S rRNA gene, and the fungal internal transcribed spacer (ITS) region. Libraries were prepared separately using Illumina's Nextera™ XT Library Prep Kit (Illumina, Cambridge, UK) following the associated protocol 16S Metagenomic Sequencing Library Preparation Protocol (Illumina, 2013). Sequencing was conducted at The University of Essex on an Illumina Miseq (providing 2 x 300 bp sequences). Each sequencing run contained multiplexed samples of each target gene which were pooled within gene libraries in

equimolar concentrations with a per library ratio of; 16S bacteria – 35%; 16S archaea – 15%; 18S eukaryote - 15%; fungal ITS - 35%. Next generation amplicon sequencing was conducted on an Illumina MiSeq platform (Illumina, 2013) at the University of Essex (UK). Library preparation methods are outlined in the general methods (Chapter 2).

6.3.6 | Quantitative PCR

Abundance of bacteria, archaea, fungi, and eukaryotes were determined by qPCR using the taxon specific primer sets in Table 2.1 without Illumina adaptors (general methods). DNA standards for qPCR were prepared by end point PCR of an equal mixture of three environmental samples. PCR mixtures for preparing DNA standards followed the identical reaction volumes, primers, and thermocycling conditions as above for the first stage PCR NGS protocol. The resulting end point PCR amplicons were purified using a GenElute PCR purification kit (Sigma) and quantified with a Quant-iT dsDNA assay kit (Thermo Fisher Scientific Inc. USA) with a NanoDrop 3300 fluorospectrometer (Thermo Fisher Scientific) according to the manufacturer's instructions. Copy numbers of each target gene within each qPCR standard were calculated using the Avogadro constant as described by McKew and Smith (2017). For each qPCR assay a serial dilution of each qPCR standard ranging from 10^2 to 10^8 target copies μl^{-1} and were run in triplicate, with at least three no template controls (NTCs) included on each plate. Each biological sample was ran in triplicate (technical replicates) with all samples randomized across two 384 well plates plate using a Bio-Rad CFX384 Touch Real-Time PCR Detection System (Bio- Rad Laboratories) to prevent between plate variation. Each 10 µl reaction contained 2 µl of DNA template, 5 µl 1X SensiFASTTM SYBR No-ROX dye (Bioline Reagents), 0.2 µl of 10 µmol of each primer and 3.6 µl of PCR water, prepared in a CFX384 quantification plate (Bio-Rad). Due to low amplification of the 18S target region 0.4 µl $MgCl_2$ (25 mM) was added to the reaction mix, and PCR water was adjusted accordingly. For 16S rRNA

bacteria, 16S rRNA archaea and ITS target regions thermocycling consisted of 95 °C for 3 minutes, followed by 40 cycles of 95 °C for 10 seconds, 60 °C for 0.5 minutes. qPCR targeting the 18S region followed the same above protocol with an annealing temperature of 53 °C. To check for product specificity and potential primer dimer formation, runs were completed with a melting analysis starting from 65 °C to 95 °C with temperature increments of 0.5 °C and a transition rate of 5 seconds. Determination of target gene copy numbers for each sample were quantified against the standard curves (R^2 > 0.99 with efficiencies between 60.8 and 78.5%) using cfx manager software (Bio-Rad Laboratories) with automatic settings for Cq values and the baseline. Melt peaks were studied to confirm no non-specific amplification or contamination had occurred.

6.3.6 | Statistical analysis

Bioinformatic analyses were conducted using the methods outlined in the general methods (Chapter 2).

Sampling cores taken from the three points of each equilateral triangle were pooled for all downstream analysis, n = 54. After confirmation that contamination was negligible, the resultant OTU table was filtered to remove negative controls, non-target amplicons and low abundance OTUs (< 3) as these are more likely to be non-biological (Flynn *et al*., 2015).

Prior to subsequent analysis ITS fungal and 18S eukaryote OTU communities were further subdivided for downstream analysis. ITS fungi were analysed as a total community, followed by separation by taxonomic assignment to the phyla levels *Ascomycota*, *Basidiomycota, Glomeromycota* (only 11 OTUs found) and *Zygomycota*. Trophic guild was also used to extract pathogenic and saprotrophic OTUs. For analysis of 18S eukaryote communities, based on existing literature (Simpson *et al*., 2017), all fungi were removed from the original 18S dataset (18S targeted fungi were

subset as a separate OTU table for taxonomic analysis). To ensure all further analysis were conducted on the microbial 18S eukaryote communities, larger eukaryotes from the phyla Metazoa and Streptophyta were removed. The following classes Embryophyceae, Ulvophyceae, Bangiophyceae, Florideophyceae and Compsopogonophyceae were also removed. While Bangiophyceae and Florideophyceae contain microbial eukaryotes, only macrophytes were present in the OTU tables generated in this study. To characterise microbial autotrophs the following phyla were included in subsequent analysis; Ochrophyta, Dinoflagellata, Chlorophyta, Haptophyta, Discoba and Rhodophyta. Phylum Discoba have classes of both autotrophs and heterotrophs, to retain Eugelnozoa only, Orders Kinetoplastida and Heterolobosea were also removed from the autotrophic dataset. To characterise microbial heterotrophs; the following phyla were included in analysis; Stramenopiles_X, Ciliophora, Conosa,Lobosa, Apicomplexa, Apusomonadidae, Katablepharidophyta, Hilomonadea, Mesomycetozoa, Metamonada, Centroheliozoa, Protalveolata_X, Telonemia, Perkinsea, Choanoflagellida, Foraminifera, Cercozoa and Discoba. The class Euglenozoa, belonging to the Phyla Discoba, were removed as those present in the dataset were from photosynthetic strains. Assignment to phototrophs or heterotrophs for members of the Discoba phylum was based on the presence/absence of chloroplasts.

I investigated the effects of the understory treatment management (hereafter referred to as ‘treatment’) on bacterial, archaeal, eukaryote and fungal richness using negative binomial general linear mixed - effect models (GLMM). To determine the effects of treatment on community (OTU) diversity and evenness I used linear mixed-effects models. Community diversity was calculated using Shannon’s diversity index and evenness was quantified using Simpson’s evenness index (Morris et al., 2014). Measures of evenness were logit transformed to meet linear modelling assumptions (Warton & Hui, 2011). In all mixed effects models, treatment was included as a fixed

effect, while spatial differences between plots were accounted for by including plots as a random effect, hereafter referred to as 'Blocks'. These models quantify the effects of treatment while controlling for differences between sampling blocks. Differences in amplicon library sizes were controlled for by including log (number of sequences) as the first (fixed) term in the model. This method for dealing with differences in sequencing depth is supported by Warton et al., (2015) as it avoids poor detection for differentially abundant species as often introduced to data by alternative methods such as rarefaction (McMurdie & Holmes, 2014). Differences in soil abiotic variables across both depths (0-5 cm, 5-10 cm) were explored using linear mixed effect models.

Differences in total bacterial, archaeal, eukaryote and fungal composition were visualised using nonmetric multidimensional scaling (NMDS) on Bray distances. To investigate how the abundance of OTUs changed between treatment (reduced, normal and enhanced) and blocks, multivariate generalized linear models (MV-GLMs) were used (Wang *et al*., 2012). A negative binomial mean-variance error family was used to account for over dispersion, a common property of count data. As described above heterogeneity in library sizes was accounted for by incorporating an offset term as the first fixed term in the model and the interaction between block and treatment allowed us to determine whether the influence of treatment varied between spatial blocks. Multispecies GLMs with the 'mvabund' package were used to investigate the effects of the understory treatment on microbial community composition. The multispecies GLM cannot handle random effects thus 'Block' was accounted for including it as an interaction term. Multivariate and unadjusted univariate P values were obtained by Wald tests, both using 10,000 Monte Carlo permutations.

All statistical, diversity and community analyses were conducted using the R statistical language version 3.3.1 with standard R libraries (Team, 2016), community analysis specific package vegan (Oksanen *et al*., 2018) and statistical packages mvabund (Wang *et al.*, 2012) and lme4 (Bates *et al.*, 2015).

6.4 | Results

6.4.1 | Effects of understory management treatment on soil abiotic variables

Means + SE for 13 soil variables were quantified at two sampling depths (Table 6.1). Soil variables frequently differed across the two sampling depths and between treatment (Table 6.2). pH, C:N, total phosphate, total potassium, calcium, sodium and CEC-Eff were significantly different between treatment plots. Bacterial OTU richness linearly decreased with total potassium (LMM; 0-5 cm, coeff = -17.18, t = -3.01, $P < 0.001$). Fungal OTU richness linearly increased with pH at both soil depths (LMM; 0-5 cm, coeff = 195.74, t = 2.94, $P < 0.01$; 5-15 cm, coeff = 190.25, t = 2.72, $P < 0.05$).

Table 6.1. Mean values (+ SEs) for 13 soil variables, 12 of the 13 soil variables were taken at two depths (0-5 cm and 5-15 cm).

Variable	Depth (cm)	Treatment					
		Reduced		**Normal**		**Enhanced**	
		Mean	**SE**	**Mean**	**SE**	**Mean**	**SE**
pH	0 - 5 cm	4.96	0.06	5.24	0.03	5.1	0.04
	5 - 15 cm	5.07	0.05	5.18	0.04	4.93	0.06
Organic C	0 - 5 cm	2.8	0.37	2.67	0.17	2.64	0.27
	5 - 15 cm	3	0.26	3.25	0.363	2.98	0.22
Total N	0 - 5 cm	0.173	0.02	0.18	0.02	0.15	0.01
	5 - 15 cm	0.171	0.01	0.19	0.02	0.15	0.02
C:N	0 - 5 cm	16.2	0.393	15.5	0.464	17.3	0.71
	5 - 15 cm	18.7	0.64	15.8	0.61	20.4	1.03
Total P	0 - 5 cm	118	11.9	190	26.5	113	14.5
	5 - 15 cm	57	5.71	107	21.9	33.9	3.97
Total K	0 - 5 cm	52.2	10.5	79.5	17.2	48.5	10.2
	5 - 15 cm	38.8	3.46	84.7	17.3	52.3	9.06
Available P	0 - 5 cm	20.8	2.9	21.7	3.42	18.9	2.84
	5 - 15 cm	9.29	2.4	13.4	3.22	6.39	1.08
Available K	0 - 5 cm	0.17	0.33	0.16	0.04	0.09	0.02
	5 - 15 cm	0.124	0.03	0.17	0.04	0.08	0.01
Calcium	0 - 5 cm	1.03	0.35	0.37	0.04	0.25	0.03
	5 - 15 cm	0.81	0.33	0.25	0.02	0.19	0.05
Magnesium	0 - 5 cm	0.37	0.09	0.32	0.05	0.19	0.02
	5 - 15 cm	0.27	0.06	0.22	0.03	0.16	0.02
Sodium	0 - 5 cm	0.09	0.01	0.07	0.003	0.08	0.01
	5 - 15 cm	0.09	0.01	0.08	0.01	0.09	0.01
CEC-Eff	0 - 5 cm	5.39	1.91	1.93	0.15	1.71	0.09
	5 - 15 cm	4.69	1.53	1.79	0.19	1.69	0.12
Moisture	0-10 cm	24.9	1.43	26	1.52	27.3	2.16

Table 6.2. Linear-modelled test statistics for 13 soil variables. 12 soil variables were taken at two depths (0-5 cm and 5-15 cm). Significant differences are highlighted in bold. “Normal” understory treatment was set as the intercept. Significant increases and decreases in comparison to normal treatment plots are shown in the table as ↓ and ↑ respectively.

Variable	Depth (cm)	Treatment			
		Reduced		Enhanced	
		t-statistic	P-value	t-statistic	P-value
pH	0 - 5	-5.33 ↓	**<0.001**	-2.71 ↓	**<0.01**
	5 - 15	-3.02 ↓	**<0.01**	-6.72 ↓	**<0.001**
Organic C	0 - 5	0.44	0.65	-0.08	0.93
	5 - 15	-0.71	0.47	-0.77	0.44
Total N	0 - 5	-0.29	0.77	-1.86	0.06
	5 - 15	-1.87	0.30	-1.87	0.06
C:N	0 - 5	2.84 ↑	**<0.01**	4.73 ↑	**<0.0001**
	5 - 15	3.64 ↑	**<0.001**	5.85 ↑	**<0.0001**
Total P	0 - 5	-2.71 ↓	**<0.01**	-2.91 ↓	**<0.01**
	5 - 15	-2.73 ↓	**<0.01**	-3.99 ↓	**<0.0001**
Total K	0 - 5	-4.07 ↓	**<0.0001**	-4.62 ↓	**<0.0001**
	5 - 15	-3.68 ↓	**<0.001**	-2.60 ↓	**<0.01**
Available P	0 - 5	-0.24	0.81	-0.77	0.44
	5 - 15	-1.33	0.18	-2.26	**<0.05**
Available K	0 - 5	0.55	0.58	-2.43	**<0.05**
	5 - 15	-1.59	0.11	-2.95	**<0.01**
Calcium	0 - 5	2.73 ↑	**<0.01**	-0.50	0.62
	5 - 15	2.55 ↑	**<0.01**	-0.26	0.79
Magnesium	0 - 5	0.84	0.40	-1.89	0.06

	5 - 15	0.98	0.33	-1.38	0.17
Sodium	0 - 5	2.09 ↑	**<0.05**	-0.50	0.62
	5 - 15	2.45 ↑	**<0.05**	1.69	0.09
CEC-Eff	0 - 5	2.68 ↑	**<0.01**	-0.16	0.87
	5 - 15	2.64 ↑	**<0.01**	-0.09	0.93
Moisture	0 - 5	-0.44	0.66	0.54	0.59

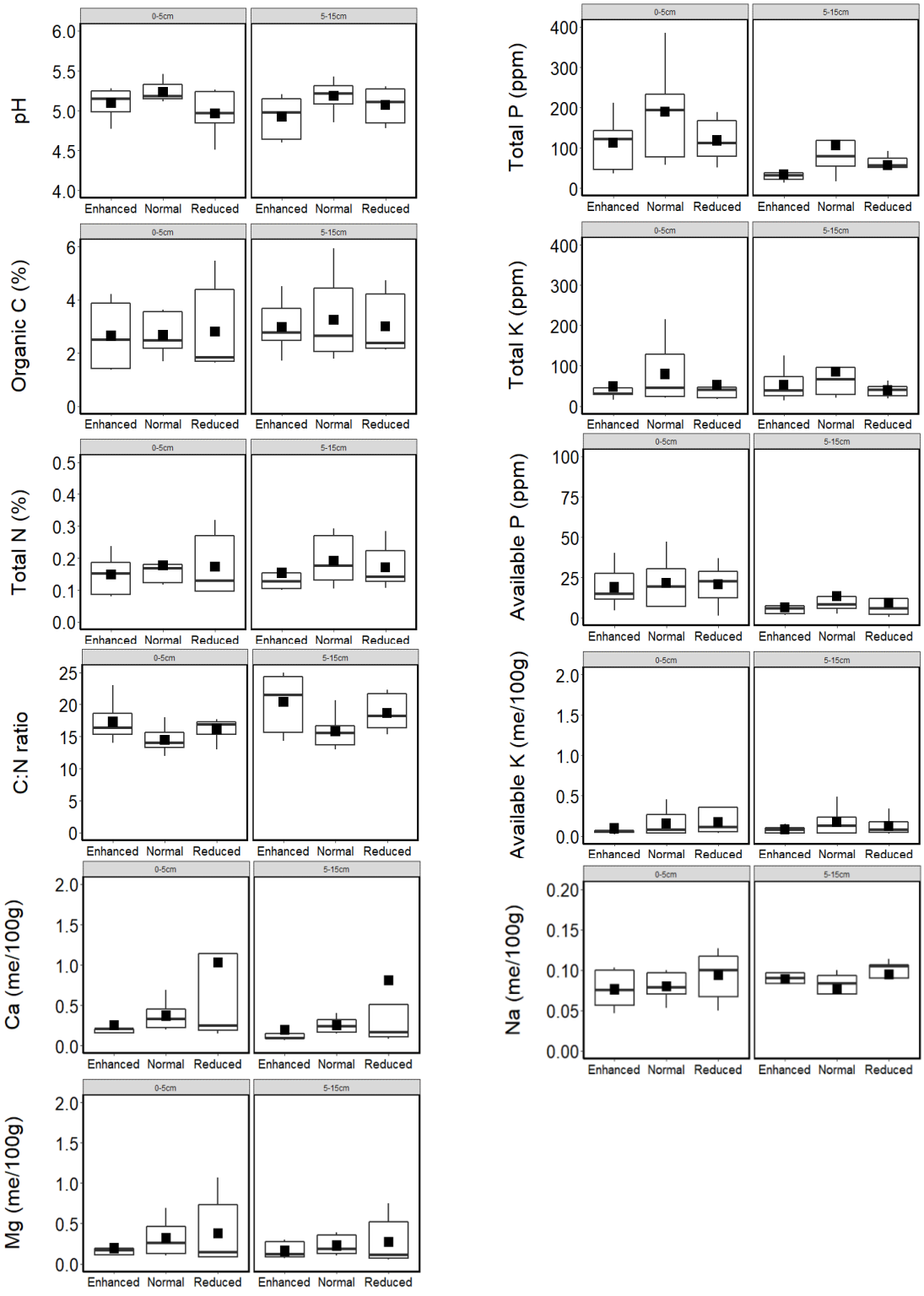


Figure 6.4. Plots of soil variables across treatments and sampling depth. Each box plot shows the median (horizontal line), mean (black-square), inter-quartiles and whiskers. Each box plot represents the median (horizontal line), mean (solid black square) and 25th to 75th percentiles, bars.

6.4.2 | qPCR analysis of 16S rRNA genes (bacteria and archaea), 18S rRNA and ITS.

A total of 162 soil microbial OTU communities for 16S bacteria, 16S archaea, 18S Eukaryotes and ITS fungi were sampled across the three understory treatments (Reduced, Normal, and Enhanced). All 162 samples successfully amplified for each target gene. Understory management treatment had no significant effect on DNA copy numbers for 16S rRNA bacteria (Figure 6.5. LMM; (a) enhanced t = -1.85, P = 1.19, reduced t = -0.995, P = 0.32), 16S rRNA archaea (Figure 6.5. LMM; (b) enhanced t = -0.72, P = 0.47, reduced t = -0.82, P = 0.41), 18S rRNA eukaryotes (Figure 6.5. LMM; (c) enhanced t = 1.14, P = 0.26, reduced t = -1.32, P = 0.19), and ITS fungi (Figure 6.5. LMM; (d) enhanced t = 0.43 P = 0.67, reduced t = -1.38, P = 0.17).

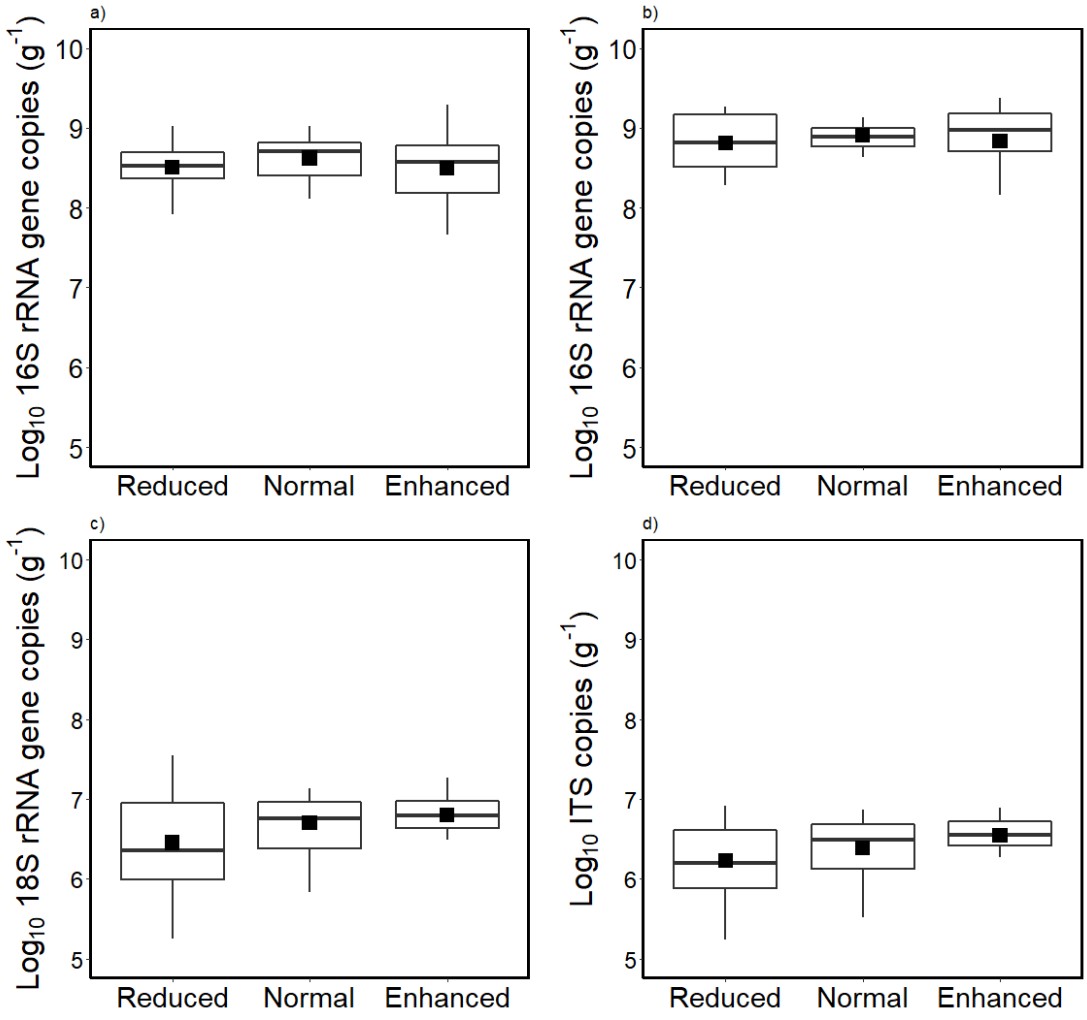


Figure 6.5. Copy number per 1 g of fresh weight soil based on qPCR quantification for 16S rRNA genes; bacteria (a) and archaea (b), 18S rRNA eukaryotes (c) and ITS fungi (d). Each box plot represents the median (horizontal line), mean (solid black square) and 25th to 75th percentiles, bars.

6.4.3 | Impacts of treatment on measures of microbial alpha-diversity

6.4.3.1 | Soil-borne microbes summary

After stringent quality control, a total of 12368 bacterial OTUs were produced on which our analysis were based. The most abundant ten bacterial OTUs represented a total of 15.8 % of all detected 16S bacterial sequences. Among these, OTU.25 and OTU.32 are the two most abundant bacterial OTUs across all sampling plots in the BEFTA experiment, belonging to the phyla Proteobacteria (class; Rhizobiales) and Acidobacteria (class *"*unclassified*"*) respectively. After quality control, 1231 16S archaeal OTUs were produced. The most abundant ten archaeal OTUs represented a total of 17.8% of all detected sequences. Among these, OTU.15 and OTU.370 are the two most abundance bacterial OTUs across all sampling plots in the BEFTA experiment, both belonging to the phyla Thaumarchaeota (species; *Nitrososphaera*). After stringent quality control and further sub-setting for multiple eukaryotic groups, a total of 2707 microbial eukaryote OTUs were produced (342 autotrophs and 2358 heterotrophs) on which our analysis were based. The most abundant ten OTUs from the total microbial eukaryote community represented a total of 51.3% of all detected sequences. Among these, OTU.4258 and OTU.261 are the two most abundance microbial eukaryote OTUs across all sampling plots in the BEFTA experiment, both belonging to the super-phyla Amoebozoa, phylum Conosa (species; *Kelleromyxa fimicola*). After stringent quality control and further sub-setting for multiple fungal groups, a total of 3441 fungal OTUs were produced on which our analysis were based. The most abundant ten OTUs represented a total of 30.6 % of all detected sequences. Among these, OTU.489 and OTU.3119 are the two most abundant fungal OTUs across all sampling plots in the BEFTA experiment, belonging to the phyla Zygomycota (genus; *Archaeorhizomyces*) and Ascomycota (genus; *Mortierella*) respectively.

6.4.3.2 | Bacteria

Treatment had no effect on bacterial OTU richness (LMM; $X^2_{2,54} = 3.45$, $P = 0.16$) , but diversity (LMM; $X^2_{2,54} = 7.24$, $P < 0.05$) and evenness (LMM; $X^2_{2,54} = 8.69$, $P < 0.05$) were significantly higher in enhanced plots in comparison to normal treatment plots (Figure 6.6).

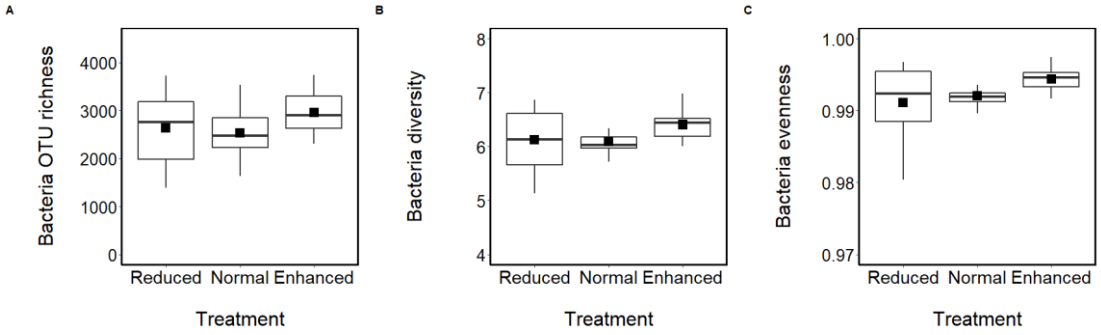


Figure 6.6. Differences in bacterial (A) OTU richness, (B) diversity – Shannnon's Diversity Index and (C) evenness - Simpson's Diversity Index) between understory treatments at BEFTA. Each box plot represents the median (horizontal line), mean (solid black square) and 25th to 75th percentiles, bars.

6.4.3.3 | Archaeal

Treatment had no effect on archaeal OTU richness (LMM; $X^2_{2,54} = 1.55$, P = 0.46), diversity (LMM; $X^2_{2,54} = 0.66$, P = 0.71), or evenness (LMM; $X^2_{2,54} = 0.78$, P = 0.67) between treatment plots (Figure 6.7).

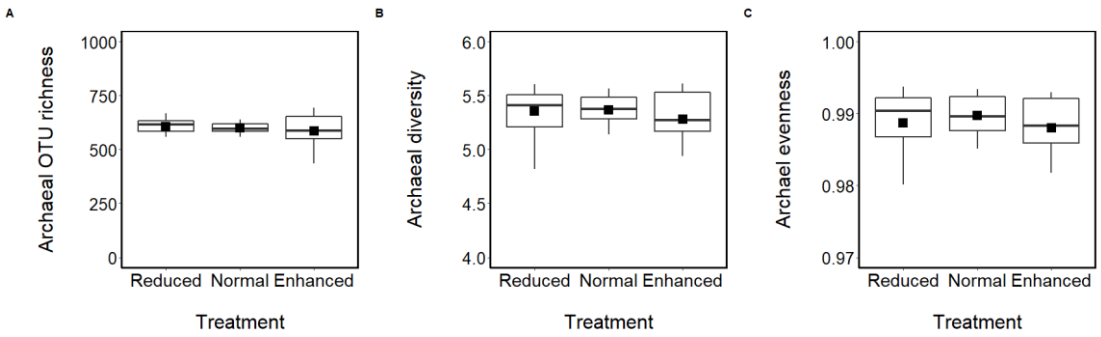


Figure 6.7. Differences in archaeal (A) OTU richness, (B) diversity – Shannnon's Diversity Index and (C) evenness - Simpson's Diversity Index) between understory treatments at BEFTA. Each box plot represents the median (horizontal line), mean (solid black square) and 25th to 75th percentiles, bars.

6.4.3.4 | Microbial – eukaryotes

Treatment had a marginal effect on microbial-eukaryote OTU richness (LMM; $X^2_{2,54}$ = 5.57, P = 0.06), this marginal significance was caused by greater OTU richness in enhanced treatment plots when compared to normal (LMM; coeff = 161.26, t = 1.92, P = 0.053), there was no effect of treatment on microbial eukaryote diversity or evenness (Figure 6.8.A-C). Further subsets for microbial heterotrophs followed a corresponding pattern, treatment had a marginal significant effect on OTU richness (LMM; $X^2_{2,54}$ = 5.92, P = 0.051), which was higher in enhanced treatment plots (LMM; coeff = 143.87, t = 1.91, P = 0.055). Diversity and evenness of heterotrophs were not significantly different between treatment plots (Figure 6.8.D-F). Microbial autotrophs did not differ in richness (LMM; $X^2_{2,54}$ = 4.53, P = 0.1), diversity (LMM; $X^2_{2,54}$ = 0.98, P = 0.61), or evenness (LMM; $X^2_{2,54}$ = 0.69, P = 0.71) between treatment plots. Taxonomic assignment for fungi from the 18S eukaryote gene revealed treatment had a significant effect on fungal OTU richness (LMM; $X^2_{2,54}$ = 9.38, P < 0.01), this was caused by enhanced treatment plots that were significantly lower than normal treatment plots (LMM; coeff = -0.18, z = -3.01, P < 0.01). Treatment had no effect on 18S fungal diversity or evenness (Figure 6.8.J-L).

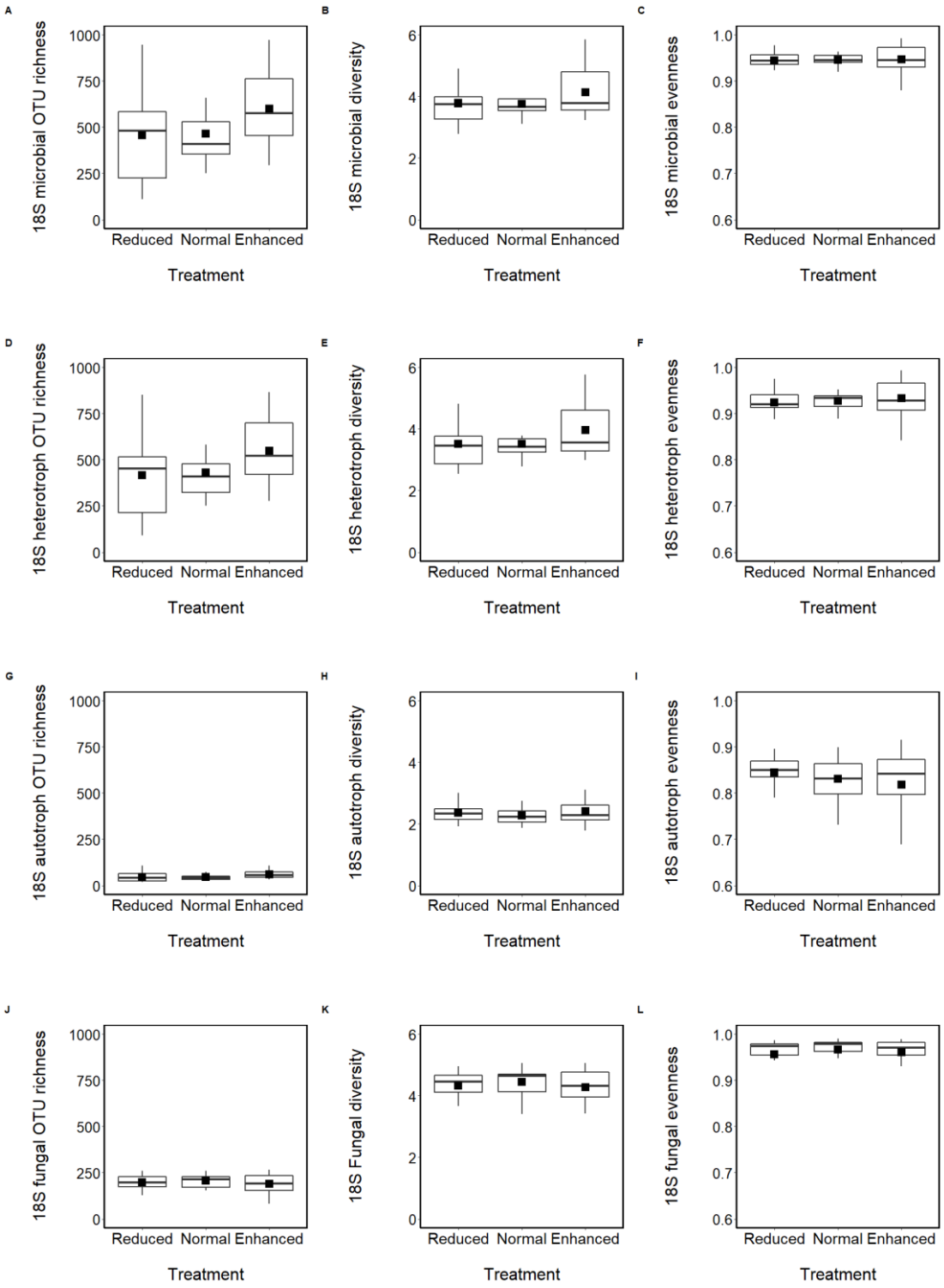


Figure 6.8. Differences in total microbial-eukaryote (A) OTU richness, (B) diversity – Shannon's Diversity Index and (C) evenness - Simpson's Diversity Index, and heterotrophic microbial eukaryotes (D-F), autotrophic eukaryotes (G-I) and fungi (J-L) between understory treatments at BEFTA. Each box plot represents the median (horizontal line), mean (solid black square) and 25th to 75th percentiles, bars.

6.4.3.5 | Fungi

Treatment had no effect on fungal OTU richness (LMM; $X^2_{2,54} = 4.24$, P = 0.11). But, diversity (LMM; $X^2_{2,54} = 10.36$, P < 0.01), and evenness (LMM; $X^2_{2,54} = 10.79$, P = 0.1) were significantly different between treatment plots. Both diversity in reduced and enhanced treatment plots were lower than in normal treatment plots, but this was only significant for enhanced plots (LMM; coeff = -0.62, t = -3.08, P < 0.05). Evenness was significantly lower in enhanced treatment plots when compared to normal (LMM; coeff = -0.71, t = -2.77, P < 0.05), and in reduced treatment plots evenness was greater when compared to normal plots but this result was insignificant (Figure 6.9.A-C). Ascomycota did not differ in OTU richness (LMM; $X^2_{2,54} = 2.81$, P = 0.24), diversity (LMM; $X^2_{2,54}$ 3.07, P = 0.21) or evenness (LMM; $X^2_{2,54} = 4.2$, P = 0.12) between treatment plots. Basidiomycota significantly differed in OTU richness (LMM; $X^2_{2,54} = 7.57$, P < 0.05) and evenness (LMM; $X^2_{2,54} = 6.03$, P < 0.05), but not diversity (LMM; $X^2_{2,54} = 4.93$, P = 0.08) between treatment plots. Glomeromycota did not differ in richness (LMM; $X^2_{2,54} = 1.02$, P = 0.6), diversity (LMM; $X^2_{2,54} = 2.41$, P = 0.29), or evenness (LMM; $X^2_{2,54} = 2.63$, P = 0.27) between treatment plots.

Fungal communities were further subset and explored for treatment effects on fungal functional guilds. Fungal pathogens did not differ in OTU richness (LMM; $X^2_{2,54} = 2.88$, P = 0.24) between treatments. Diversity (LMM; $X^2_{2,54} = 5.68$, P = 0.06) and evenness (LMM; $X^2_{2,54} = 5.29$, P = 0.07) met marginal significance for fungal pathogens between treatments. Diversity (LMM; coeff = -0.26, t = -2.36, P < 0.05) and evenness (LMM; coeff = -0.77, t = -2.31, P < 0.05) were significantly lower in reduced treatment plots only (Figure 6.8.D-F). Saprotrophs did not differ in richness (LMM; $X^2_{2,54} = 1.72$, P = 0.42), diversity (LMM; $X^2_{2,54} = 0.09$, P = 0.95) or evenness (LMM; $X^2_{2,54} = 0.17$, P = 0.92) between treatment plots (Figure 6.9.G-I).

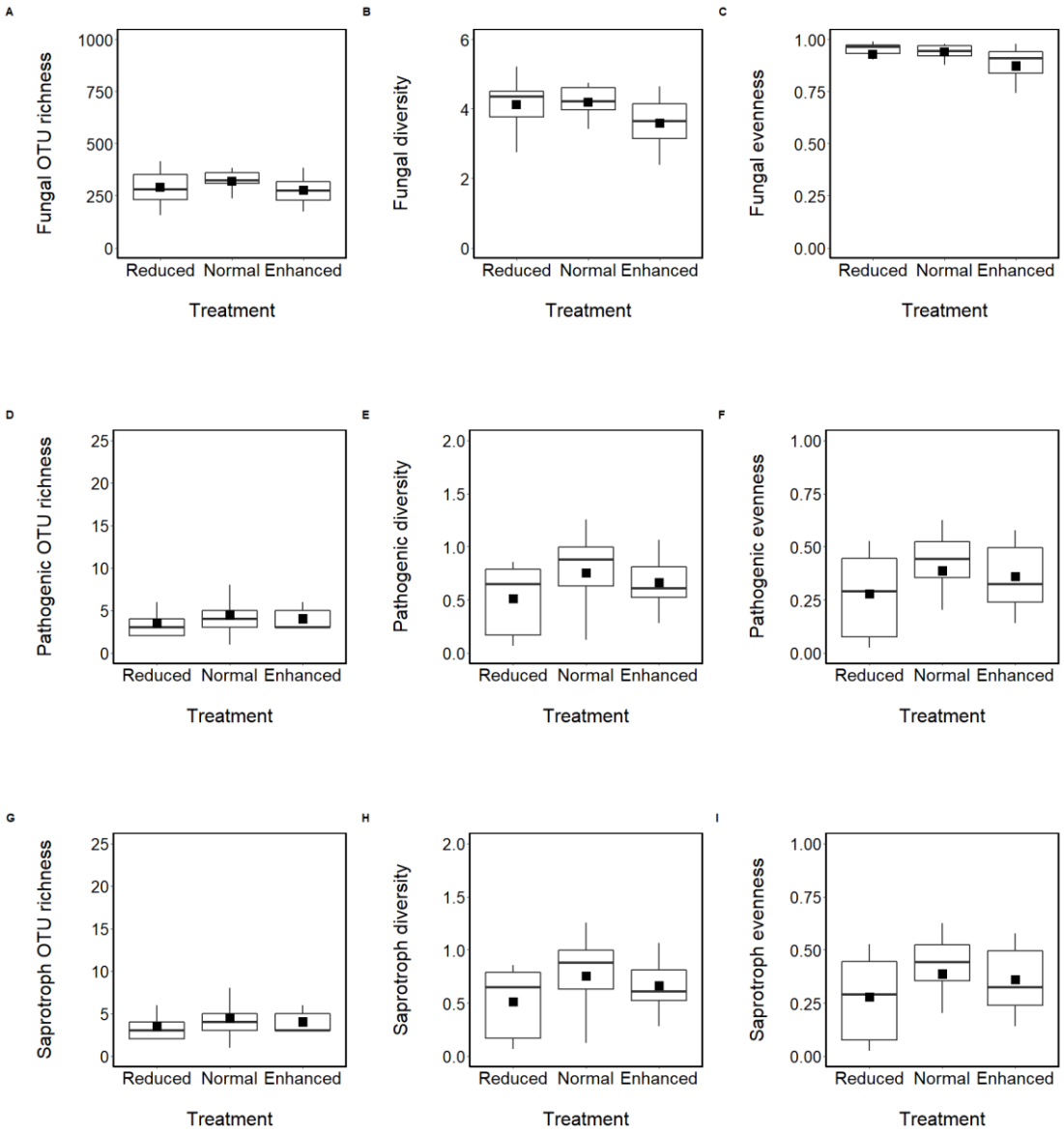


Figure 6.9. Differences in total fungal (A) OTU richness, (B) diversity – Shannnon's Diversity Index and (C) evenness - Simpson's Diversity Index, and pathogenic fungi (D-F), and saprotrophic fungi (G-I) between understory treatments at BEFTA. Each box plot represents the median (horizontal line), mean (solid black square) and 25th to 75th percentiles, bars.

6.4.3.6 | Alpha-diversity summary

Table 6.3. Summary of alpha-diversity measures indicating a significant increase(↑) /significant decrease(↓) or no significance (NS) in comparison to normal treatment plots. 'R' refers to reduced treatment plots and 'E' to enhanced treatment plots.

		Richness		**Diversity**		**Evenness**	
		R	**E**	**R**	**E**	**R**	**E**
16S	**Bacteria**	NS	NS	NS	↑	NS	↑
	Archaea	NS	NS	NS	NS	NS	NS
18S	**Eukaryotes**	NS	↑	NS	NS	NS	NS
	Heterotrophs	NS	↑	NS	NS	NS	NS
	Autotrophs	NS	NS	NS	NS	NS	NS
	Fungi	NS	↓	NS	NS	NS	NS
ITS	**Fungi**	NS	NS	NS	↓	NS	↓
	Pathogens	NS	NS	↓	NS	↓	NS
	Saprotrophs	NS	NS	NS	NS	NS	NS

6.4.4 | Effects of understory management on soil microbial composition

Treatment had a significant effect on bacterial composition (PERMANOVA; $F_{2,53}$ = 2.05, P < 0.01, stress = 0.12) composition, a marginally significant effect on fungal community composition (PERMANOVA; $F_{2,53}$ = 1.22, P = 0.07, stress = 0.23) and microbial-eukaryote community composition between treatment plots (PERMANOVA; $F_{2,53}$ = 1.35, P = 0.08, stress = 0.15). Fungal pathogens or saprotrophs were not significantly different in composition between treatment plots. Conversely, treatment had no effect on archaeal composition between treatment plots (Figure 6.10, A-D).

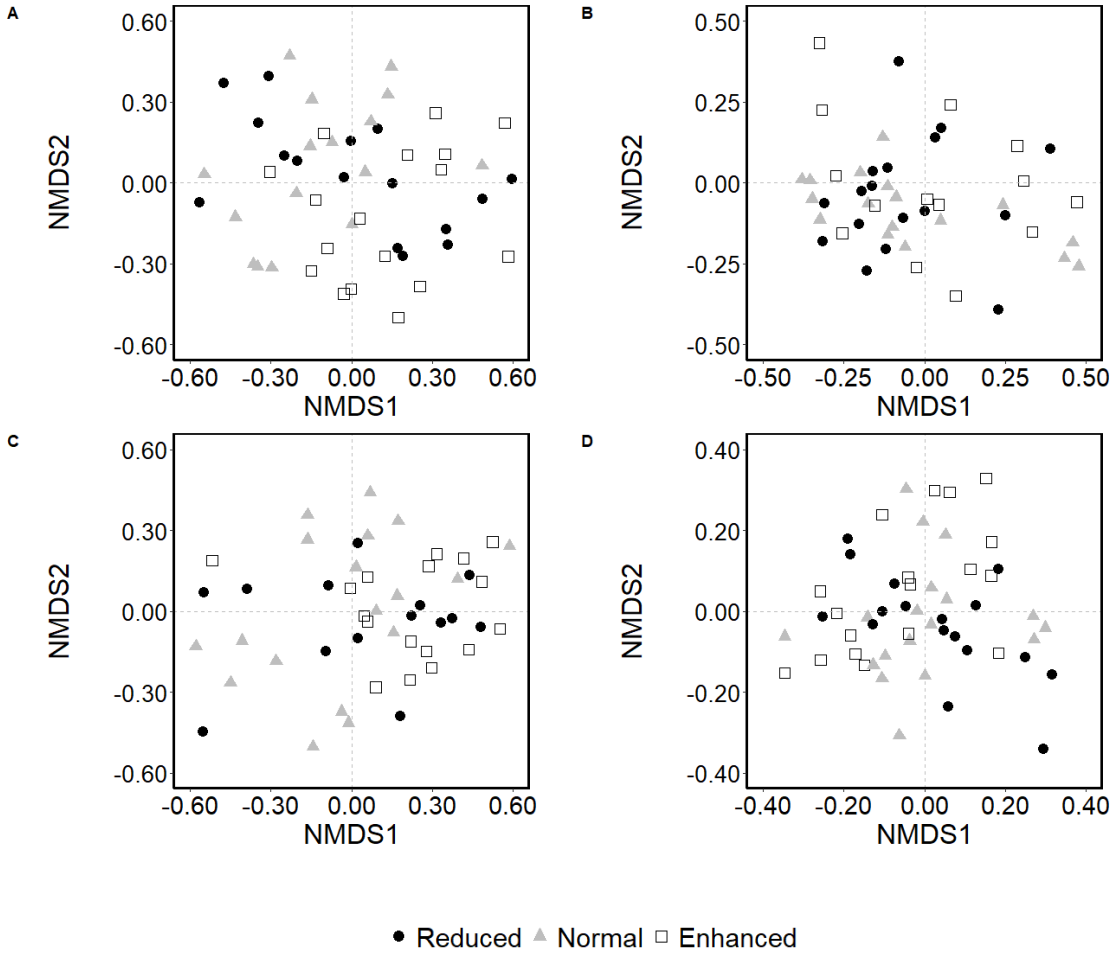


Figure 6.10. Differences in total fungal (A) bacterial, (B) archaeal, (C) micro-eukaryote and (D) fungal composition between treatment plots. Nonmetric multidimensional scaling (NMDS) ordinations based on Bray's distance matrix.

6.4.5 | MV-GLM results

6.4.5.1 | 16S bacteria

A total of 801 OTUs occurred > 1% in the overall dataset. Multivariate modelling revealed that enhanced treatment (247 OTUs; Wald = 55.65, $P < 0.001$) and reduced treatment (182 OTUs; Wald = 46.43, $P < 0.001$) significantly effects bacterial OTU abundances when compared to normal, after controlling for unequal sequencing depths. Of all the OTUs effects by understory treatment, 129 OTUs were significantly more abundant and 118 OTUs were significantly less abundant in enhanced treatment in comparison to normal. Whilst 99 OTUs were significantly more abundant and 83 OTUs were significantly less abundant in reduced treatments in comparison to normal (Figure 6.11).

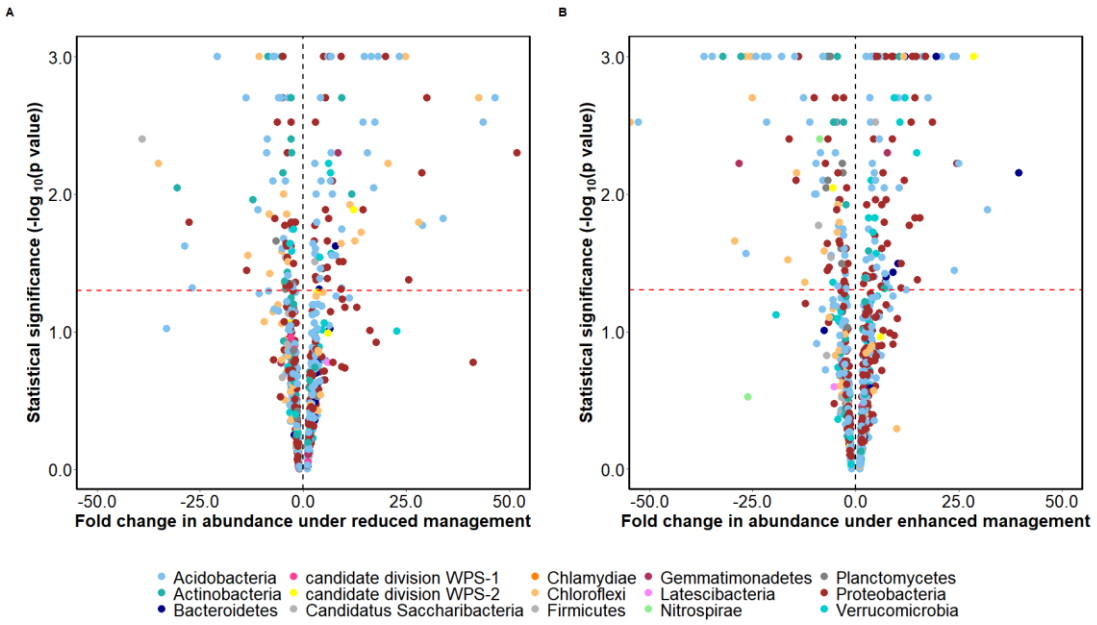


Figure 6.11. Volcano plot of MV-GLM modelled shifts in bacterial phyla abundances under A) reduced management, and B) enhanced management. Coloured points above the horizontal dotted line represent phyla that showed statistically significant shifts in abundance ($P < 0.01$), whereas points below the line represent phyla that did not show statistically significant shifts ($P > 0.05$). Phyla further to the left were statistically less abundant while phyla to the right were statistically more abundant when compared to normal.

6.4.5.2 | 16S archaea

A total of 658 OTUs occurred > 1% in the overall dataset. Multivariate modelling revealed that enhanced treatment (205 OTUs; Wald = 46.04, $P < 0.001$) and reduced treatment (148 OTUs; Wald = 40.64, $P < 0.001$) significantly effects archaeal OTU abundances when compared to normal, after controlling for unequal sequencing depths. Of all the OTUs effects by understory treatment, 89 OTUs were significantly more abundant and 116 OTUs were significantly less abundant in enhanced treatment in comparison to normal. Whilst 82 OTUs were significantly more abundant and 66 OTUs were significantly less abundant in reduced treatments in comparison to normal (Figure 6.12).

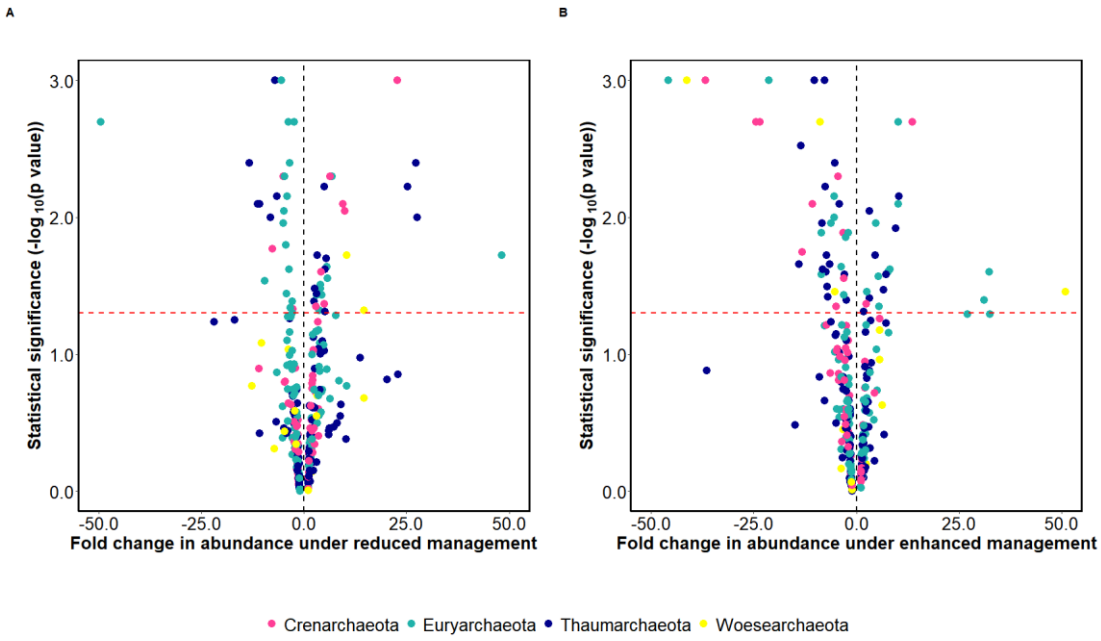


Figure 6.12. Volcano plot of MV-GLM modelled shifts in archael phyla abundances under A) reduced management, and B) enhanced management. Coloured points above the horizontal dotted line represent phyla that showed statistically significant shifts in abundance ($P < 0.01$), whereas points below the line represent phyla that did not show statistically significant shifts ($P > 0.05$). Phyla further to the left were statistically less abundant while phyla to the right were statistically more abundant when compared to normal.

6.4.5.3 | 18S eukaryotes

A total of 333 OTUs occurred > 1% in the overall dataset. Multivariate modelling revealed that enhanced treatment (115 OTUs; Wald = 3.56, P < 0.001) and reduced treatment (62 OTUs; 26.29, P < 0.001) significantly effects microbial eukaryote OTU abundances when compared to normal, after controlling for unequal sequencing depths. Of all the OTUs effects by understory treatment, 73 OTUs were significantly more abundant and 42 OTUs were significantly less abundant in enhanced treatment in comparison to normal. Whilst 29 OTUs were significantly more abundant and 32 OTUs were significantly less abundant in reduced treatments in comparison to normal (Figure 6.13).

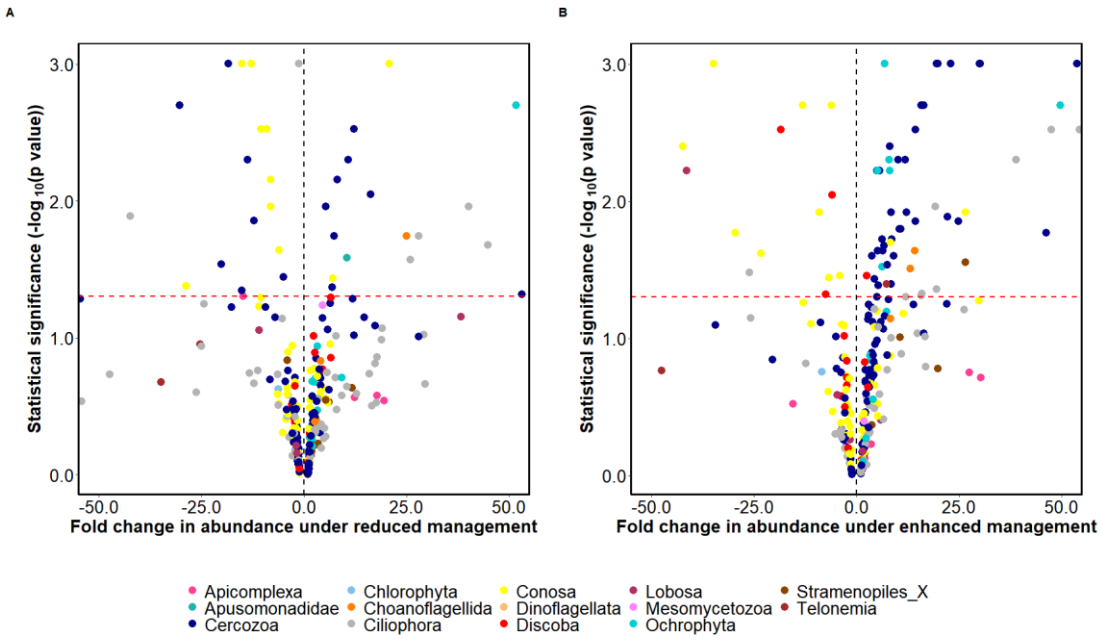


Figure 6.13. Volcano plot of MV-GLM modelled shifts in microbial eukaryote phyla abundances under A) reduced management, and B) enhanced management. Coloured points above the horizontal dotted line represent phyla that showed statistically significant shifts in abundance ($P < 0.01$), whereas points below the line represent phyla that did not show statistically significant shifts ($P > 0.05$). Phyla further to the left were statistically less abundant while phyla to the right were statistically more abundant when compared to normal.

18S eukaryotes; autotrophs)

A total of 151 OTUs occurred > 1% in the overall dataset. Multivariate modelling revealed that enhanced treatment (49 OTUs; Wald = 18.86, $P < 0.001$) and reduced treatment (31 OTUs; Wald = 14.20, $p < 0.001$) significantly effects autotrophic microbial eukaryote OTU abundances when compared to normal, after controlling for unequal sequencing depths. Of all the OTUs effects by understory treatment, 14 were significantly more abundant and 17 were significantly less abundant in enhanced treatment in comparison to normal. Whilst 29 were significantly more abundant and 20 were significantly less abundant in reduced treatments in comparison to normal (Figure 6.14).

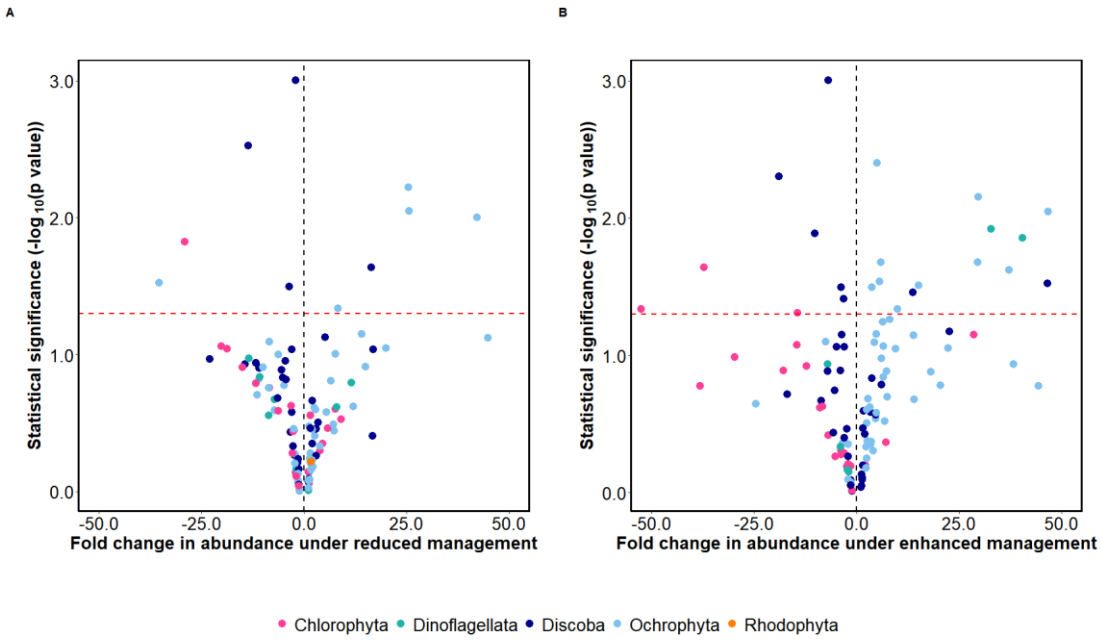


Figure 6.14. Volcano plot of MV-GLM modelled shifts in autotrophic phyla abundances under A) reduced management, and B) enhanced management. Coloured points above the horizontal dotted line represent phyla that showed statistically significant shifts in abundance ($P < 0.01$), whereas points below the line represent phyla that did not show statistically significant shifts ($P > 0.05$). Phyla further to the left were statistically less abundant while phyla to the right were statistically more abundant when compared to normal.

18S eukaryotes; heterotrophs)

A total of 345 OTUs occurred > 1% in the overall dataset. Multivariate modelling revealed that enhanced treatment (115 OTUs; Wald = 32.49, $P < 0.001$) and reduced treatment (69 OTUs; Wald = 26.63, $P < 0.001$) significantly effected heterotrophic microbial eukaryote OTU abundances when compared to normal, after controlling for unequal sequencing depths. Of all the OTUs effects by understory treatment, 75 OTUs were significantly more abundant and 40 OTUs were significantly less abundant in enhanced treatment in comparison to normal. Whilst 33 OTUs were significantly more abundant and 36 OTUs were significantly less abundant in reduced treatments in comparison to normal (Figure 6.15).

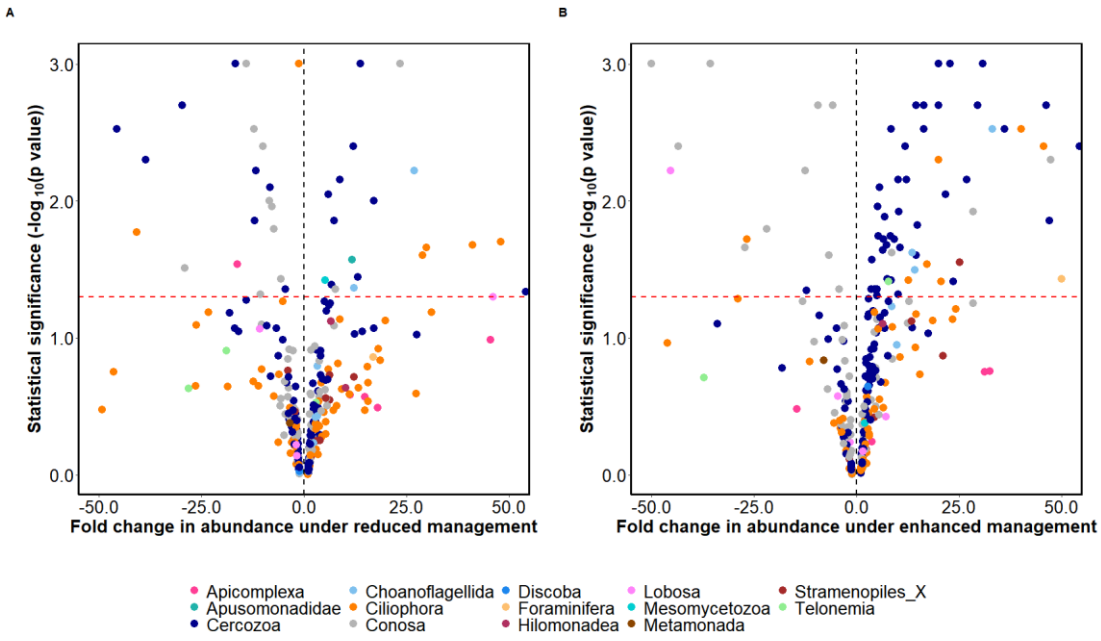


Figure 6.15. Volcano plot of MV-GLM modelled shifts in heterotrophic phyla abundances under A) reduced management, and B) enhanced management. Coloured points above the horizontal dotted line represent phyla that showed statistically significant shifts in abundance ($P < 0.01$), whereas points below the line represent phyla that did not show statistically significant shifts ($P > 0.05$). Phyla further to the left were statistically less abundant while phyla to the right were statistically more abundant when compared to normal.

6.4.5.4 | ITS fungi;

A total of 658 OTUs occurred >1% in the overall dataset. Multivariate modelling revealed that enhanced treatment (234 OTUs; Wald = 39.27, $P < 0.001$) and reduced treatment (223 OTUs; Wald = 38.02, $P < 0.001$) significantly effects fungal OTU abundances when compared to normal, after controlling for unequal sequencing depths. Of all the OTUs effects by understory treatment, 89 OTUs were significantly more abundant and 145 OTUs were significantly less abundant in enhanced treatment in comparison to normal. Whilst 74 OTUs were significantly more abundant and 149 OTUs were significantly less abundant in reduced treatments in comparison to normal (Figure 6.16).

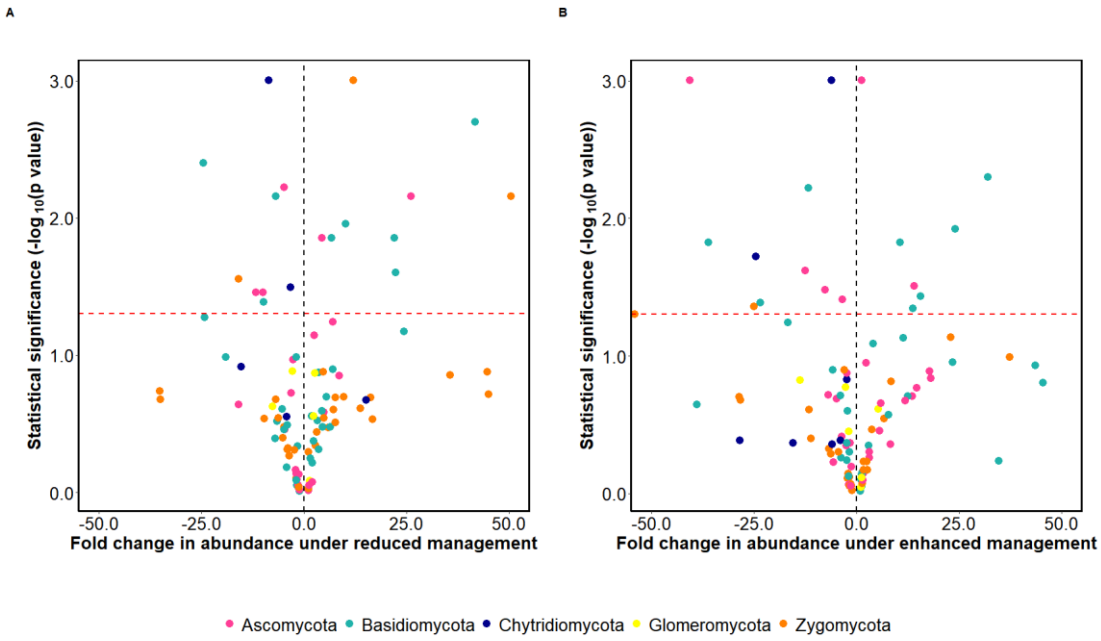


Figure 6.16. Volcano plot of MV-GLM modelled shifts in ITS fungal phyla abundances under A) reduced management, and B) enhanced management. Coloured points above the horizontal dotted line represent phyla that showed statistically significant shifts in abundance ($P < 0.01$), whereas points below the line represent phyla that did not show statistically significant shifts ($P > 0.05$). Phyla further to the left were statistically less abundant while phyla to the right were statistically more abundant when compared to normal.

6.5 | Discussion

In this study, I examined the changes in four community attributes (abundance, richness, diversity and composition) of four microbial communities in response to long-term biodiversity manipulation project within mature oil palm plantations (Sumatra). Treatments - Reduced (minimal understory), Normal (moderate understory) and Enhanced (rich understory), span a range of agricultural practices that are commonly operated within oil palm plantations today. There were no significant differences in rRNA gene abundance between treatment plots, and as such, differences at the diversity and compositional level become much more important to consider for effects on ecosystem functioning. Results demonstrate a number of findings that could have scope for the development of more sustainable management practices within oil palm plantations. I hope the data in this study encourages the incorporation of microbial perspective when it comes to designing models for a future of sustainable agriculture, specifically for palm oil. These results suggest that more sustainable palm oil cultivation practices could be produced by increasing the native diversity within oil palm plantations, this not only could result in increased yields and therefore minimise the risk of native tropical forest conversion, but it could also mean there will be reduced reliance on chemical inputs which not only has economical benefits, but will undoubtedly reduce the negative impacts associated with conventional oil palm cultivation.

6.5.1 | The effect of oil palm management on microbial diversity and composition

6.5.1.1 | Bacteria

In alignment with our expectations, measures of bacterial alpha-diversity were higher in enhanced plots when compared to normal and thus satisfies the hypothesis

that microbes will be enriched in enhanced treatment plots. The underpinning reasons for this could be two fold, it is probable inherent plant diversity will contain species associated with greater microbial diversity (i.e., “sampling effect”), and secondly, evidence in literature reasonably suggest increased plant diversity increases microbial diversity and the key ecosystem functions that they mediate (Heijden *et al*., 1998; Carney & Matson, 2006; Prober *et al*., 2015). Linear-modelling revealed bacteria belonging to the phylum Chloroflexi were driving this result. Chloroflexi are dominant thermophilic aerobic heterotrophs of tropical soils and provide a significant contribution to decomposition of organic matter (Delgado-Baquerizo *et al*., 2018). Interestingly, in terms of terrestrial environments, Chloroflexi are documented for their almost exclusive association with oil palm soils (Bouskill *et al.*, 2013).

Of all microbial communities explored, management strategies implemented within each treatment at BEFTA only had a significant effect on bacterial community composition. Therefore, in part, this satisfies one of the hypotheses underpinning this chapter by showing microbial community composition differs between treatment plots. At the species level, some bacterial OTUs increased, some decreased, and some stayed the same within both enhanced and reduced treatments when compared to normal. The most notable differences in bacterial composition as revealed by multivariate analysis were dominated by Actinobacteria, Bacteroidetes, Chloroflexi and Proteobacteria. Taxa (OTUs) belonging to the phylum Actinobacteria occurred in significantly greater abundance in enhanced treatment plots in comparison to normal, while their abundance appeared to be impaired by practices implemented within reduced treatment plots. Actinobacteria are documented for sharing similar characteristics to fungi for their role in decomposition of refractory biomaterials in dead organic matter and the subsequent production of secondary metabolites (Hopwood, 2007). Members of the order Actinobacteria, including Actinomycetales, known for their nitrifying capabilities (Bhatti *et al.*, 2017), and Acidimicrobiales, are amongst

those OTUs that occurred in significantly higher abundance in enhanced treatment plots. This suggests increased rates of carbon turnover and N cycling in enhanced treatment plots and could therefore imply more efficient plant growth in enhanced treatment plots. Whilst this is a step in the right direction, however, because soil N did not change between treatment plots, it is unlikely the shift in Actinobacteria in enhanced treatment plots is large enough to have positive implications N limited productivity. Moving on, taxa belonging to the phylum Proteobacteria and Bacteroidetes were also enriched in enhanced treatment plots. Further exploration of Proteobacteria revealed members of the class Betaproteobacteria, order Burkholderiales, were largely driving this difference. Firstly, these microbes are significant in the soil environment for their biocontrol ability and beneficial effects on plant growth and health through fungal disease suppression (Sessitsch *et al.*, 2005; Eberl & Vandamme, 2016). This is mediated through mycophagous feeding strategies of bacteria that can supress fungal populations and cause significant shifts in fungal community composition. As such, the suppressive nature of Burkholderiales could have positive implications for ecosystem functioning by reducing the risk of soil-borne diseases. This may be one reason why fungal richness appears to be impaired in enhanced treatment plots. Secondly, an increase in Bacteroidetes in enhanced treatment plots could have important ecological implications at higher trophic levels. This is based on the assumption that enhanced plots likely support a wider diversity of higher trophic levels because by association, Bacteroidetes increase in abundance in response to invertebrates. For instance, several Bacteroidetes families are recognised as decomposers of chitin (Wieczore*k et al*., 2019), an important polysaccharide that is the major component within exoskeletons of insects and cell walls of fungi (Merzendorfer & Zimoch, 2003). Whilst only assumption, this is a sensible prediction to make based on previous evidence published from the BEFTA plots showing increased faunal diversity in enhanced treatment plots (Ashton-Butt *et al.*, 2018). Ultimately, this suggests understory vegetation can increase food web

complexity in oil palm plantations by providing a greater variety of resources. Based on the aforementioned results, compositional differences in key bacterial groups indicates potential for greater biocontrol and enhanced nutrient recycling in enhanced treatment plots, when compared to normal. This has implications for the sustainability of oil palm cultivation as nutrient turnover is a valuable diagnostic feature of soil systems.

6.5.1.2 | Microbial-eukaryotes

The total eukaryotic and heterotrophic eukaryotic community aligned with alpha-diversity measures for bacterial communities by approaching significance for greater alpha-diversity in enhanced treatment plots. It is sensible to suggest the proliferation of bacterial communities in enhanced treatment plots acts as a carbon source to support the survival and proliferation of heterotrophic bacterial grazers. This interaction within the 'microbial loop' has important implications for ecosystem functioning because it results in the subsequent release of labile compounds into the soil profile making them available for assimilation by plant roots. Consequently, efficient cycling within the microbial loop is beneficial for plant growth (Bonkowski, 2004). Whilst there were no overall compositional differences in micro-eukaryote groups between treatments and as such cannot satisfy the hypothesis that micoribial composition will differ between treatment plots, univariate analysis did reveal specific (but shared) OTUs that significantly varied in response to understory management treatment. The most notable of these groups were species within the phyla Cercozoa in which more taxa (OTUs) occurred in significantly higher abundances within enhanced plots, when compared to normal. Cercozoa frequently occur as the most abundant eukaryote in soils (Howe et al., 2009) and obtain energy from feeding upon bacteria thus their presence is proposed to be the strongest biotic driver of bacterial communities (Fiore-Donno *et al.*, 2019), this could have positive implications for nutrient turnover. These results highlight the role of bacteria in shaping eukaryote

communities (Rønn *et al.*, 2002). But, perhaps more importantly and from a management perspective, the ability to promote heterotrophic microbial communities might hint towards a future relying less heavily on external nutrient inputs. Overall, this will have a positive effect for the sustainability of oil palm cultivation. The extensive exploration using advanced univariate analysis and use of next-generation techniques means this study begins to shed light on the ecological importance of the often overlooked protist diversity within terrestrial and soil ecosystems.

6.5.1.3 | Fungi

In contrast to bacteria, measures for fungal alpha-diversity were lower in both enhanced and reduced treatment plots when compared to normal, but again, this was only significant in enhanced treatment plots. This therefore opposes the hypothesis that microbes will be greater in enhanced treatment plots. Coupled with the above data regarding bacterial communities, this begins to imply management strategies applied within enhanced treatment plots have stronger implications for driving the diversity of microbial communities than those implemented in normal or reduced treatment plots. This is not surprising as enhanced treatment plots were the only treatment not exposed to the deleterious impacts associated with herbicide application. It would be sensible to predict a linear correlation between fungal diversity and aboveground understory diversity, not only will fungi likely be lower in reduced plots due to heavy herbicide use (Garciá-Orenes *et al.*, 2013), but fungi also monopolise the nutrient rich and dynamic rhizosphere niche and frequently form symbiotic relationships, therefore, the more plants, the more plant-fungal associations. While this sentiment supports reduced fungal diversity in reduced treatment plots and intermediate fungal diversity in normal treatment plots, it does not explain why fungal diversity appears to be impaired by management strategies implicated within enhanced treatment plots. Firstly, to explore if this was a signal of the dilution effect underlying the ‘pathogen-hypothesis’ as presented by Mommer et al., (2018), I explored differences in alpha-diversity

measures of pathogenic fungal communities between treatment plots. Pathogenic fungal taxa were significantly lower in both reduced and enhanced plots when compared to normal, although this result was insignificant. Nonetheless, tropical ecosystems are relatively understudied with respect to next-generation sequencing, so the insignificance of this relationship could be an artefact of an under curated taxonomic database. Interpreting this relationship with reliable significance will depend on future expansion of the coverage of taxonomic and functional databases. A second avenue to explore is the suppression of fungal populations in enhanced treatment plots via bacterial mycophagy. Fungus derived carbon is a major resource for bacterial secondary consumers and as such some bacteria develop mycophagous feeding strategies to cope with high competition for limited nutrients (Leveau & Preston, 2008; Leveau *et al.*, 2010). In enhanced treatment plots, bacteria consistently increase in all alpha-diversity measures whilst fungi decrease. This theory is supported by multivariate analysis of bacterial communities in which the presence of mycophagous bacteria, such as those belonging to the class *Burkholderiales* and order Enterobacteriales, occurred more frequently in significantly greater abundance in enhanced treatment plots in comparison to normal.

Coupled with the aforementioned evidence, I propose there are two further considerations that must be made with respect to possible drivers of fungal communities. Analysis revealed soil pH was positively correlated with fungal richness, this could be why normal treatment plots, with the least acidic conditions, were associated with the greatest fungal richness. This contrasts evidence in previous literature in which fungi exhibit wide pH ranges for optimal growth (La*uber et al*., 2008; Rousk *et al*., 2010). This suggests pH alone is unlikely to be driving significant differences in fungal richness between treatment plots. Moreover, cited work in literature provides a further plausible explanation for the enrichment of fungal diversity in normal treatment plots. Berkelmann et al., (2018) and Schneider et al., (2015) both

demonstrate enhanced microbial diversity associated with intensely managed land systems. Despite the fact a number of studies are in agreement that habitat heterogeneity is the largest driver of diversity, how this heterogeneity and the subsequent functional response is structured remains unresolved. As opposed to the native complexity and heterogeneity provided by the original tropical rainforest, it may be that native habitat complexity is compensated for by the implemented heterogeneity associated with the cultivation regime (e.g. soil disturbance from machinery and harvesting, patchiness in use of herbicides). As documented in Ashton-Butt et al., (2018) plantations have a typical zonation of soil and vegetation management. This produces three distinct zones; the weeded circle, harvesting path, and windrow. It is probable the perimeters of these zones are more distinguishable in normal treatment plots that use a combination of manual and chemical management strategies. While increasing habitat complexity through intense chemical application would be controversial as it would have obvious negative implications on the environment, this does however, serve as a baseline for potential further management strategies, i.e. the possibility to increase habitat complexity within oil palm plantations through manual management. This data begins to suggest habitat complexity could play a role of almost equal importance as plant diversity with regards to microbial diversity.

6.5.1.4 | Archaea

Overall, archaea were relatively insensitive to the treatment practices operated within plots at BEFTA. However, a general overview of archaeal communities aligned with results previously documented from literature. A total of 1231 Archaeal taxa (OTUs) from 14 families were recorded from the treatment plots at BEFTA. The estimated, and relatively rich, archaeal OTU richness and diversity is as expected. Whilst we have very limited research on tropical soil microbial communities, archaea in the environment are ubiquitous (Bintrim *et al*., 1997; Jurgens *et al*., 1997; Buckley *et al*., 1998; Robertson *et al*., 2005) and are frequently shown to demonstrate high

levels of phylogenetic diversity (Ochsenreiter *et al.*, 2003). Unsurprisingly, Thaumarchaeota*,* genus *Nitrososphaera* (Brochier-Armanet *et al.*, 2008), were the most abundant phyla within this dataset, this is also in align with findings by (Schneider *et al.*, 2015). Species from this phyla are described as the dominant archaea in most soil systems, accounting for up to 5% of all prokaryotes (Ochsenreiter *et al.*, 2003; Lehtovirta *et al.*, 2009). Members of this phyla are most commonly known for their role in aerobic ammonia oxidisation (Bagchi *et al*., 2014) and therefore are important constituents to consider within the soil microbial profile for their pivotal role within the global nitrogen cycle (Leininger *et al.*, 2006; Stieglmeier *et al.*, 2014). The high abundance and diversity of archaea in BEFTA treatment plots suggest efficient nitrogen cycling; this is a key parameter often indicative of improved soil health and therefore has vast implications for sustainability. Despite this, Tupinambá et al., (2016) compared archaeal communities of the Amazon forest with areas under oil palm cultivation. This study showed compromised archaeal diversity (1.7 times lower) associated with the oil palm cultivated area. Whilst sampling for BEFTA did not include native forest for comparison, it is plausible to suggest archaeal community diversity is degraded through agriculturally driven land use changes. Furthermore, whilst on average, the rich archaeal diversity across treatment plots at BEFTA supports enhanced ecosystem functioning, this was not reflected in compositional differences and therefore it can be concluded archaea have a minimal effect on ecosystem functioning between plots.

6.6 | Conclusion

Our results reveal evidence for human modification of soil microbial communities via the implementation of different understory management regimes within soils beneath oil palm cultivation and that such changes are mediated in part by plant diversity and possibly habitat complexity. Of the four microbial communities investigated, bacterial, fungal and heterotrophic eukaryotes were the most effected by

management strategies within treatment practices. Furthermore, management strategies implemented within enhanced treatment plots hold a stronger ability to structure microbial communities than those implemented in reduced plots. In contrast, the overall diversity and composition of archaea were relatively insensitive to the span of oil palm management frequently implemented across Southeast Asia. The data from this study predicts there is more efficient cycling within the 'microbial loop' within enhanced treatment plots and as such could have positive effects on ecosystem functioning. It is probable to predict shifts in bacterial abundance, diversity and composition are the largest driver of both diversity and composition in other microbial groups, i.e. suppression of fungi through mycophagous feeding and enrichment of microbial heterotrophs supported by increased bacterial diversity. The greater diversity of bacterial communities, compositional differences driven by beneficial taxa (i.e. Actinobacteria involved in nutrient cycling and Betaproteobacteria involved in biocontrol) and the effect this has on structuring microbial communities in enhanced treatment plots has obvious environmental and economical advantages that may lead to a future of more sustainable oil palm management. As our study is the first study of this size and duration on soil microbial communities across oil palm treatment regimes, the work encompassed within this chapter provides a basis for further investigation that has direct application to the future of economically viable and more sustainable oil palm plantations.

6.7. | Limitations

The key limitation concerning Chapter 6 is an artefact of differences in coverage of trophic mode in the FunGuild database between different ecosystems. For example, microbial diversity in European grasslands (Bever., 1994; Bev*er et al*., 2015; Prober *et al*., 2015; Mommer *et al*., 2018; Francioli *et al*., 2020) and agricultural systems (Agtmaal *et al.*, 2017; Romdhane *et al.*, 2019) have been well explored using both species-specific testing and rRNA gene profiling. This means it can be confirmed

with a relatively high certainty that identified microbes are either a pathogen, saprotroph or symbiotroph. However, tropical ecosystems, particularly oil palm plantations, remain in early infancy with respect to the number of studies exploring their microbial diversity. This means resolution to guild assignment remains relatively low and may limit analysis of the 'whole' pathogenic population in this study. This could lead to taxonomic bias towards certain ecosystems which is particularly important to consider if comparisons are being made. Nonetheless, the data in this study contributes to our understanding of microbial diversity and their potential ecological function in oil palm plantations differing across a gradient of management regimes.

Chapter 7

General discussion

7.1 | Summary of thesis findings

7.1.1 | Chapter 3 summary

In Chapter 3, I adopted a biologically replicated observational approach to explore root-fungal interactions in natural hyperdiverse grassland ecosystems. Due to their high intrinsic aboveground diversity and late successional stage, this made the chosen study sites an ideal location to explore plant vegetation dynamics as mediated by the root-associated fungal community. The research within this study was centred around Janzen-Connell type effects which encompasses theories of NDD and the 'pathogen hypothesis' in which host-specific fungal pathogens are proposed as the key regulators of plant community dynamics. Results showed both the total and pathogenic fungal community were host specific across all plant taxonomic levels and thus satisfies the first assumption of the 'pathogen-hypothesis'. Further exploration of the pathogenic community within plant functional group, i.e. grasses and forbs, revealed a phylogenetic host signal in which families within forbs held distinct pathogenic communities but grasses, that belong to a single family, held pathogenic communities that were compositionally similar between plant species. This has vital implications regarding vegetation dynamics because grasses are more likely to be effected by the negative impacts associated with negative PSF as a broad group, as opposed to forbs that contain multiple families. Secondly, results showed a significant linear relationship between pathogenic fungal diversity and aboveground spatial distribution of plants. This finding provides strong evidence to suggest plants at high conspecific density acquire enhanced pathogen loads and thus negative PSF could drive plant species coexistence by reducing the fitness of superiorly competitive

plants. This Chapter advocates the incorporation a microbial perspective in order to understand and predict plant species vegetation dynamics.

7.1.2 | Chapter 4 summary

Chapter 4 is analogous to chapter 3. Here, I tested the generality of fungal host-specific relationships across an aboveground diversity gradient. To do this I chose study sites across the UK and Norway that offered intermediate and low levels of plant diversity respectively. Again, these studies were biologically replicated within each geographical region. Interestingly, results from this Chapter revealed (1) a phylogenetic host signal driven by plant functional group drives host-specificity of fungal pathogens and (2) the strength of plant-fungal pathogenic host specificity is much weaker as aboveground diversity decreases. The data from Norway, the UK and Slovenia combined (Chapters 3 and 4), implies host specific plant-fungal interactions could be key regulators of aboveground vegetation dynamics and as such drive the overall diversity of an ecosystem. Simultaneously, this data supports negative PSF mediated by fungal pathogens as a key driver of high plant species diversity, but conversely, positive feedback mediated by host specific saprotrophs could be a key driver of low plant species diversity. The difference in significance of the outcome of PSF (negative or positive) within an ecosystem might be the driving force between the overall aboveground diversity grassland ecosystems.

7.1.3 | Chapter 5 summary

In chapter 5, I explore two experimental studies adopting approaches centred on the theme of plant-microbe interactions and their utilisation for a future of more sustainable agri-ecosystems. The success of both of these agricultural management practices rely on the overarching assumption throughout this thesis of plant-microbial host specificity. First, I comprehensively characterised the root-associated and soil-borne bacterial and fungal communities across nine wheat cultivars growing in

monoculture, three and nine variety mixes. I first test for host-specificity, soil-borne bacterial communities were host-specific to wheat cultivar, but neither bacterial nor fungal root-associated communities demonstrated host specificity between cultivars. Distinct soil-borne bacterial communities in association with monocultures of different wheat cultivars implies there is potential to exploit host specific cultivar-microbe interactions. Regarding the 'pathogen-hypothesis' and the subsequent pathogen dilution effect, increasing aboveground genetic diversity (i.e. mixing cultivars in three or nine species plot) did not suppress OTU richness of pathogenic fungal communities. Although, this could be an artefact or low taxonomic coverage. Nonetheless, total fungal populations were significantly supressed in three variety mixes and exploration of core and unique OTUs between monocultures, three variety and nine variety saw a 97.4 % and 60.87 % reduction in unique root-associated fungal OTUs respectively. While these results provide mixed evidence, the loss of unique OTUs when wheat cultivars are mixed provides encouraging support to imply this approach could be adopted to mitigate potential yield losses due to negative PSF. However, because taxonomic coverage was low, and for the simple reasoning that the presence pathogens does not infer pathogenicity, further trials exploring the effect of fungal pathogens on crop yields is required. Moreover, similarities in fungal communities indicate differences within the same species, but distinct genetic varieties of wheat species, are not large enough to drive cultivar-specific fungal communities. It is likely this is driven by non-distinct exudation profiles or similar rhizodeposition between genetic varieties. Nonetheless, future work could rely on targeted genome editing to successfully manipulate properties of root exudation profiles with the aim to modify the soil microbial community. Second, I explored the effect of cover-crop varieties on root-associated microbial communities. Interestingly, only bacteria demonstrated host-specificity between cover-crop varieties. Nonetheless, this study did highlight the potential to manipulate plant-microbial interactions with scope to modify specific functional processes between treatments. For example, two of the five plots were

shown to form more plant-Actinobacteria relationships which are considered beneficial for ecosystem functioning, and treatment plots containing Brassica species supressed fungal communities. Enhanced knowledge of belowground plant-microbial interactions will help to inform more efficient crop-rotation schemes with the potential to exploit specific microbial interactions with the aim to increase yield of subsequent cash crops. Both of these studies provide only a glimpse of plant-microbial interactions in agriculture, nonetheless, the data from this chapter provides a baseline for future work and suggests the incorporation of a microbial perspective and assumptions underlying the 'pathogen hypothesis' has the potential to increase the speed at which agriculturalists develop more sustainable farming practices.

7.1.4 | Chapter 6 summary

In Chapter 6, I explored the relationship between aboveground diversity and soil-borne microbial communities in the world's largest and most productive agricultural system, oil palm. To do this I utilised a long-term biodiversity experiment that directly manipulates the understory within oil palm plantations. This is a vast collaborate study designed to test the effect of increasing landscape structural complexity with the aim enhance oil palm sustainability at little or no cost to yields and profitability. For this study, I comprehensively characterised the soil microbial communities (Bacteria, Archaea, Eukaryote and Fungi) from three experimental treatment plots. In this study, I showed adopting more 'wildlife' friendly management strategies by increasing habitat complexity within industrially viable oil palm plantations could have beneficial implications for soil health and ecosystem functioning. In align with Chapter 5, this complements the growing body of evidence to advocate the exploitation of plant-microbial interactions, i.e. bacteria, fungal and heterotrophic-eukaryotes, for a future of more sustainable agri-ecosystems. The diverse microbial interactions between different taxonomic groups, for example bacterial mycophagy and heterotrophic bacterial grazers and their relative importance

within the ‘microbial-loop’, highlights the importance to explore all taxonomic levels when it comes to understanding soil health and ecosystem functioning.

7.2 | Key insights

7.2.1 | NGS as a means to explore ecosystem functioning as driven by fungal microbes

Throughout this thesis, I have utilised next-generation sequencing approaches to open the ‘black-box’ regarding plant-microbial interactions. Such culture-independent techniques provide quantitative and qualitative information about the microbial composition associated with plant roots and the bulk soil. However, from this information alone it is difficult to make reliable predictions regarding their ecological role in any given ecosystem (Peay, 2014). The reason for this is twofold. Firstly, fungal OTU presence is not directly related to metabolic state, i.e. the presence of rich N-fixating bacteria is not definitive of increased N-fixation at the time of sampling. To overcome this, more reliable predictions could be made by sequencing functional genes (i.e. amoB and cblh) or messenger RNA (Anderson & Parkin, 2007). Secondly, functional guild assignment based on OTUs also impedes analysis of OTU community data. However, this problem is not so easily resolved. Metagenomic datasets yield vast quantities of unique DNA sequences that are grouped by percentage similarity into OTUs. It is not uncommon to yield in excess of 5000 OTUs from soil samples (Wang *et al.*, 2018). However, many of these OTUs are defined ‘uncultured’ and therefore lack a true functional description. Functional assignment across all studies in this thesis ranged from 3 % - 56 %, with agricultural sites (Europe and South East Asia) being the most unrepresented ecosystems in the FunGuild database. This aligns with results in literature – 39 % (Mommer et al., 2018) and 10% (Tedersoo *et al.*, 2014). Moreover, in light of the theme in this thesis it is important to note functional assignment to ‘pathotroph’ or ‘plant pathogen’ does not necessarily infer pathogenicity

of the particular plant species of interest. This is stressed even more so by neighbour identity effects in which plant species can be non-symptomatic hosts of certain 'pathogenic' microbes. Therefore, whilst NGS has helped to answer some otherwise intractable questions, we have to be careful when it depends on addressing hypotheses relating to fungal guild. This is because guild coverage is not species-specific and guild coverage remains sparse in some ecosystems; both of these reasons could dampen the results use to test any hypotheses. Accordingly, a considerable amount of further studies are required if we want to infer true ecological function from guild assignment, this could include transcriptomic, or microbial isolation, taxonomic assignment and bioassays to infer function. However, this does not solve the issue of taxa that cannot be cultured (Pham & Kim, 2012).

7.2.2 | Fungal-mediated regulation of aboveground vegetation dynamics.

Data from Chapter 3 provided evidence to validate the two underlying assumptions of the 'pathogen-hypothesis'. Whilst evidence for host-specificity (i.e. Gilbert & Webb, 2007; Mills & Bever, 2014; Bev*er et al*., 2015) and NDD effects (Liang *et al*., 2016; Mommer *et al*., 2018) have been met in literature, to my knowledge, this study is the first to explore the strength of this relationship from a purely observational perspective and closes the 'black-box' of microbial mediated PSF in true natural grassland ecosystems. However, due to the spatio-temporal dynamic nature of grassland ecosystems and the ideas synthesised throughout this thesis, the next logical step would be to develop an understanding of pathogen load and plant density, i.e. does pathogen load linearly increase with plant density and is this persistent when plant fitness decreases? To address such questions, an experimental approach to allow plant populations to naturally fluctuate whilst pathogen populations were simultaneously monitored in a time dependent manor would be required. Furthermore,

another question to ask would be - why do grasslands differ in their aboveground diversity, both globally and regionally? There are a number of factors to consider when addressing this question, such as latitude, species-pool effect and microbial assembly. Data from Chapter 3 and 4 suggest the prevalence and direction of PSF might play a role in governing overall plant species diversity. However, the driving force that determines the presence of host-specificity within an ecosystem remains unknown. It is likely there a number of factors at interplay here. One possible reason could be nutrient availability. There is empirical evidence that shows nutrient poor soils drives plant species diversity (Wisheu *et al.*, 2000). Therefore, when key macronutrients such as N and P are in high abundance, it is likely interspecific competition is non-paramount and plants do not develop specialised natural enemies. To explore the effects of this, experiments that create nutrient limited niche space for the development of specialised natural enemies or symbionts would be required. As such, this is just one potential avenue for exploration, but the data from this thesis certainly opens a further gap in knowledge and provides a basis for further research.

7.2.3 | A new understanding of plant density - density as a function of neighbouring identity.

One of the overarching themes within this thesis is centred on plant density effects. Conspecific NDD implies diversity is maintained by specialised interactions between plant species and their natural enemies which results in increased mortality at high conspecific densities (Peters, 2003; LaManna *et al.*, 2017). Earlier studies exploring aboveground diversity effects characterise all plant species as a separate entity and this assumes all species harbour their own root-associated microbial communities. More recently, host-specific effects at the plant functional group level have received more attention (Wubs & Bezemer, 2016; Ma *et al.*, 2017; Emilia Hannula *et al.*, 2019), and thus implies that some plant species share the same microbial

consortia. This raises the question, how should we calculate plant density? For example, should plant density be calculated on a plant species basis, or should ecologists begin to make calculations based on heterospecific plant-microbe overlap. Density is not merely an outcome of host abundance, but is a more complex interaction that must take into account neighbour identity. The theory of neighbouring identity and disease transmission was recognised as early as the 1990's, termed as the 'root-camouflage' hypothesis (Gilbert, 1994) and later revisited by Mommer *et al*., (2018). Mommer *et al.*, (2018) describes this as a 'hide and seek' game in which neighbouring identity has the means to either dampen or accelerate pathogenic fungal transmission. Understanding the rules of host-density effects between plants and pathogens is important for biodiversity conservation, ecosystem functioning, and also has direct implications that may improve the design of mixed cropping systems (Li *et al.*, 2014). Genetic variants for agriculture could be selected in order to influence their overall 'identity' with the aim to reduce the rate of disease transmission. Understanding the rules of 'hide and seek' mediated by neighbouring identity will help inform the design of more productive mixed cropping systems (Li *et al.*, 2014; Brooker *et al.*, 2015). However, the control we have over creating genetic variants and manipulating the identity of each variant is beyond the scope of an ecologist and therefore relies on the expertise of geneticises and plant physiologists. Experiments that map neighbour identity (i.e. host, non-host, or asymptomatic host), complete with plant growth bioassays and quantitative measurements of rate of transmission is essential to validate the effects of neighbouring identity. In summary, this thesis advocates an alternative approach to measuring diversity-density effects, and as such, implementation of a modified means to calculate plant density might see that future studies might provide a more empirical body of evidence that implies host-specific pathogen mediated regulation of vegetation dynamics.

7.2.4 | Agriculture and sustainability – the future.

In contemporary agricultural ecosystems, nutrients are supplied through the application of synthetic fertilisers, this compensates for reduction in productivity as a consequence of soil-borne disease. However, this 'traditional' approach is contributing to shifts in key reservoirs within Earths major biogeochemical cycles through processes such as eutrophication and greenhouse gas emission during the manufacture process (Amundson *et al.*, 2015). Sustainable agriculture is thus attracting renewed interest. Understanding resource use efficiency and the trade-off between yield and environmental harm underpins sustainable agriculture. However, if the implementation of more environmentally friendly approaches reduces yield, then it is not true that the system is sustainable because the losses will have to be compensated elsewhere in the cycle. One possibility to increase the sustainability of agricultural ecosystems is to replace synthetic fertilisers with organic inputs, or supplement plants with beneficial microbes that drive the mineralisation of organic matter. While crop rotation, cover cropping, organic manure, and zero-till systems have increased resource efficiency in agricultural ecosystems (Krupins*ky et al*., 2002; Hubbard *et al*., 2013; Wang *et al*., 2017), more recently, the field of sustainable agriculture has achieved further success from the incorporation of a microbial perspective. This has largely been fuelled by developing molecular and high-throughput technologies. One example of microbial driven sustainable agriculture is vermicomposting, a process by which organic matter is broken down through the metabolic activity of microbes and earthworms (Dominguez & Edwards, 2010). Such methods of bioactive organic enrichment can be selected for based on mechanisms by which vermicompost influence plant growth (Gómez-Brandón et al., 2020), this provides an alternative to inorganic fertilisers. Another way to utilise microbes is for their host-specific plant-microbial associations, both positive and negative. However, the full range of plant-microbial interactions and their potential to replace external

synthetic chemical inputs remains in its infancy. The data from this thesis suggests it is possible to manipulate soil-borne and root-associated microbial diversity through multiple practices such as informed species selection for cover cropping, increasing aboveground plant diversity, and manipulating habitat complexity. One interesting avenue, based on results from Chapters 3 and 5, alongside the skills of plant geneticists, would be to explore the ability to create genetic variants that differ in their neighbouring identity, i.e. they do not share pathogenic suites. This could dampen disease transmission in mixed cultivar plots. The success of all these approaches is the ability of plants to shape the structure of their microbiomes and as such there is a growing consensus that biodiversity and genetic variation in crop plants is the key to sustainable agriculture (Belhaj *et al.*, 2015). The knowledge obtained from this study could inform the basis for a new era of microbiome assisted crop production.

The concept of plant nutrition and its direct link to the rhizosphere microbiome is an emerging field (Jacoby *et al.*, 2017). Breeding and modifying plants for cultivation (as in Chapter 5) has a number of applications for sustainable agriculture, this includes drought resistance and biocontrol to name a few (Bell *et al.*, 2001; Bechtold *et al.*, 2013). However, now we know plants have some control over their microbiome (Bulgarelli *et al.*, 2012; Zgadzaj *et al.*, 2016), geneticists and agriculturalists are now exploring the potential to breed plants with traction to increase associations with beneficial microbes. The next goal of this innovative area of research is to determine the genetic determinants that underpin how different plant genotypes interact with the species specific microbiome (Jacoby *et al.*, 2017). Genome wide association studies (GWAS) as opposed to sequencing analysis provides more information as it takes into account the full microbial genome and addresses the function of these microbes. GWAS analysis has led to the discovery of several candidate genes (demonstrated by correlations between phenotypic differences and positive SNPs) thus suggesting this is an appropriate approach to identify genetic-loci that control phenotypic variation in

plant-microbe interactions (Wintermans *et al.*, 2016). Moreover, root exudates that are known to influence microbial composition provide a further potential target for plant-microbial engineering. To be truly successful, in both of the above approaches it must be proven that beneficial microbes 1) can be inoculated onto plants, 2) proliferate and 3) have growth promoting properties on the target plant. This can be tested in relatively simple terms by means of plant growth bioassays. In summary, the data in this thesis and in literature, coupled with the acceleration in complex molecular analytical tools, provides vast scope to explore more sustainable management strategies for agri-ecosystems. While soil is a major determinant of microbial community assembly, plant identity with respect to genetic trait differences is also a significant driver of microbial assembly. In particular, genes involved in root exudation profiles that are important for microbial structuring processes are of most interest. Therefore, the implantation of more comparative genetic approaches such as GWAS will serve as an invaluable tool to explore plant nutrition. This knowledge could inform and guide promising approaches to exploit plant-microbial interactions for a future of sustainable agriculture.

References

Aarssen LW (1983) Ecological Combining Ability and Competitive Combining Ability in Plants: Toward a General Evolutionary Theory of Coexistence in Systems of Competition. *The American Naturalist*, **122**, 707–731.

Aarssen LW (1984) On the distinction between niche and competitive ability: Implications for coexistence theory. *Acta Biotheoretica*, **33**, 67–83.

Aarssen LW (1989) Competitive Ability and Species Coexistence - a Plants-Eye View. *Oikos*, **56**, 386–401.

Agtmaal M van, Straathof A, Termorshuizen A et al. (2017) Exploring the reservoir of potential fungal plant pathogens in agricultural soil. *Applied Soil Ecology*.

Aira M, Gómez-Brandón M, Lazcano C, Bååth E, Domínguez J (2010) Plant genotype strongly modifies the structure and growth of maize rhizosphere microbial communities. *Soil Biology and Biochemistry*.

Albornoz FE, Burgess TI, Lambers H, Etchells H, Laliberté E (2017) Native soilborne pathogens equalize differences in competitive ability between plants of contrasting nutrient-acquisition strategies. *Journal of Ecology*, **105**, 549–557.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *Journal of Molecular Biology*, **215**, 403–410.

Ampt EA, van Ruijven J, Raaijmakers JM, Termorshuizen AJ, Mommer L (2019) Linking ecology and plant pathology to unravel the importance of soil-borne fungal pathogens in species-rich grasslands. *European Journal of Plant Pathology*, **154**, 141–156.

Amundson R, Berhe AA, Hopmans JW, Olson C, Sztein AE, Sparks DL (2015) Soil and human security in the 21st century. *Science*.

Anderson IC, Parkin PI (2007) Detection of active soil fungi by RT-PCR amplification of precursor rRNA molecules. *Journal of Microbiological Methods*.

Ashton-Butt A, Aryawan AAK, Hood ASC et al. (2018) Understory Vegetation in Oil Palm Plantations Benefits Soil Biodiversity and Decomposition Rates. *Frontiers in Forests and Global Change*, **1**, 10.

Asplund J, Kauserud H, Bokhorst S, Lie MH, Ohlson M, Nybakken L (2018) Fungal communities influence decomposition rates of plant litter from two dominant tree species. *Fungal Ecology*.

Ayres E, Steltzer H, Simmons BL et al. (2009) Home-field advantage accelerates leaf litter decomposition in forests. *Soil Biology and Biochemistry*.

Badri D V., Vivanco JM (2009) Regulation and function of root exudates. *Plant, Cell and Environment*.

Bagchi R, Swinfield T, Gallery RE, Lewis OT, Gripenberg S, Narayan L, Freckleton RP (2010) Testing the Janzen-Connell mechanism: Pathogens cause overcompensating density dependence in a tropical tree. *Ecology Letters*, **13**,

1262–1269.

Bagchi R, Gallery RE, Gripenberg S et al. (2014a) Pathogens and insect herbivores drive rainforest plant diversity and composition. *Nature*, **506**, 85–8.

Bagchi S, Vlaeminck SE, Sauder LA, Mosquera M, Neufeld JD, Boon N, Poulain A (2014) Temporal and spatial stability of ammonia-oxidizing archaea and bacteria in aquarium biofilters. *PLoS ONE*.

Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM (2006) The role of root exudates in rhizosphere interactions with plants and other organisms. *Annual Review of Plant Biology*.

Bani A, Pioli S, Ventura M et al. (2018) The role of microbial community in the decomposition of leaf litter and deadwood. *Applied Soil Ecology*.

Barnes AD, Jochum M, Mumme S, Haneda NF, Farajallah A, Widarto TH, Brose U (2014) Consequences of tropical land use for multitrophic biodiversity and ecosystem functioning. *Nature Communications*.

Basiron Y (2007) Palm oil production through sustainable plantations. *European Journal of Lipid Science and Technology*.

Bates D, Mächler M, Bolker BM, Walker SC (2015) Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*.

Bechtold U, Albihlal WS, Lawson T et al. (2013) Arabidopsis heat shock transcription factor A1b overexpression enhances water productivity, resistance to drought, and infection. *Journal of Experimental Botany*.

Beketov MA, Kefford BJ, Schäfer RB, Liess M (2013) Pesticides reduce regional biodiversity of stream invertebrates. *Proceedings of the National Academy of Sciences of the United States of America*.

Belhaj K, Chaparro-Garcia A, Kamoun S, Patron NJ, Nekrasov V (2015) Editing plant genomes with CRISPR/Cas9. *Current Opinion in Biotechnology*, **32**, 76–84.

Bell HA, Fitches EC, Marris GC, Bell J, Edwards JP, Gatehouse JA, Gatehouse AMR (2001) Transgenic GNA expressing potato plants augment the beneficial biocontrol of Lacanobia oleracea (Lepidoptera; Noctuidae) by the parasitoid Eulophus pennicornis (Hymenoptera; Eulophidae). *Transgenic Research*.

Bent SJ, Forney LJ (2008) The tragedy of the uncommon: understanding limitations in the analysis of microbial diversity. *The ISME Journal*, **2**, 689–695.

Benton TG, Vickery JA, Wilson JD (2003) Farmland biodiversity: is habitat heterogeneity the key? *Trends in Ecology & Evolution*, **18**, 182–188.

Bergsma-Vlami M, Prins ME, Raaijmakers JM (2005) Influence of plant species on population dynamics, genotypic diversity and antibiotic production in the rhizosphere by indigenous Pseudomonas spp. *FEMS Microbiology Ecology*.

Berkelmann D, Schneider D, Engelhaupt M et al. (2018) How rainforest conversion to agricultural systems in Sumatra (Indonesia) affects active soil bacterial communities. *Frontiers in Microbiology*.

Bever JD (1994) Feeback between Plants and Their Soil Communities in an Old Field Community. *Ecology*, **75**, 1965–1977.

Bever JD, Dickie IA, Facelli E et al. (2010) Rooting theories of plant community ecology in microbial interactions. *Trends in Ecology and Evolution*, **25**, 468–478.

Bever JD, Mangan SA, Alexander HM (2015) Maintenance of Plant Species Diversity by Pathogens. *Annual Review of Ecology, Evolution, and Systematics*, **46**, 305–325.

Bhatti AA, Haq S, Bhat RA (2017) Actinomycetes benefaction role in soil and plant health. *Microbial Pathogenesis*.

Bihn JH, Gebauer G, Brandl R (2010) Loss of functional diversity of ant assemblages in secondary tropical forests. *Ecology*, **91**, 782–92.

Bintrim SB, Donohue TJ, Handelsman J, Roberts GP, Goodman RM (1997) Molecular phylogeny of Archaea from soil. *Proceedings of the National Academy of Sciences of the United States of America*.

Bonanomi G, Giannino F, Mazzoleni S (2005) Negative plant soil feedback and species coexistence. *Oikos*, **2**, 311–321.

Bonkowski M (2004) Protozoa and plant growth: The microbial loop in soil revisited. *New Phytologist*.

Börner H (1960) Liberation of organic substances from higher plants and their role in the soil sickness problem. *The Botanical Review*, **26**, 393–424.

Bouskill NJ, Lim HC, Borglin S, Salve R, Wood TE, Silver WL, Brodie EL (2013) Pre-exposure to drought increases the resistance of tropical forest soil bacterial communities to extended drought. *ISME Journal*.

Bradley DJ, Gilbert GS, Martiny JBH (2008) Pathogens promote plant diversity through a compensatory response. *Ecology Letters*, **11**, 461–469.

Bragg L, Tyson GW (2014) Metagenomics using next-generation sequencing. *Methods in Molecular Biology*.

Brochier-Armanet C, Boussau B, Gribaldo S, Forterre P (2008) Mesophilic crenarchaeota: Proposal for a third archaeal phylum, the Thaumarchaeota. *Nature Reviews Microbiology*.

Brooker RW, Bennett AE, Cong WF et al. (2015) Improving intercropping: A synthesis of research in agronomy, plant physiology and ecology. *New Phytologist*.

Brownlee G, Sanger F (1967) Nucleotide sequences from the low molecular weight ribosomal RNA of Escherichia coli. *Journal of molecular biology*, **23**, 337–353.

Buckley DH, Graber JR, Schmidt TM (1998) Phylogenetic analysis of nonthermophilic members of the kingdom Crenarchaeota and their diversity and abundance in soils. *Applied and Environmental Microbiology*.

Buée M, de Boer W, Martin F, van Overbeek L, Jurkevitch E (2009) The rhizosphere zoo: An overview of plant-associated communities of microorganisms, including

phages, bacteria, archaea, and fungi, and of some of their structuring factors. *Plant and Soil*.

Bulgarelli D, Rott M, Schlaeppi K et al. (2012) Revealing structure and assembly cues for Arabidopsis root-inhabiting bacterial microbiota. *Nature*.

Bullock DG (1992) Crop rotation. *Critical Reviews in Plant Sciences*.

Callahan BJ, McMurdie PJ, Holmes SP (2017) Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. *ISME Journal*.

Carlier L, Rotar I, Vlahova M, Vidican R (2009) Importance and functions of grasslands. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*.

Carney KM, Matson PA (2006) The influence of tropical plant diversity and composition on soil microbial communities. *Microbial Ecology*, **52**, 226–238.

Chapelle FH, Methé B (2002) A hydrogen-based subsurface microbial community dominated by methanogens Cooperative Water Program in South Carolina View project National Water Quality Assessment Mercury Topical Study View project.

Charles S. Elton (1958) *the Ecology of Invasions By Animals and Plants*, Vol. 53. 1689–1699 pp.

Chase JM, Abrams PA, Grover JP et al. (2002) The interaction between predation and competition: A review and synthesis. *Ecology Letters*, **5**, 302–315.

Chen H, Boutros PC (2011) VennDiagram: A package for the generation of highly-customizable Venn and Euler diagrams in R. *BMC Bioinformatics*.

Chesson P, Huntly N (1997) The roles of harsh and fluctuating conditions in the dynamics of ecological communities. *American Naturalist*.

Chiariello N, Hickman JC, Mooney HA (1982) Endomycorrhizal role for interspecific transfer of phosphorus in a community of annual plants. *Science*.

Chung AYC, Eggleton P, Speight MR, Hammond PM, Chey VK (2000) The diversity of beetle assemblages in different habitat types in Sabah, Malaysia. *Bulletin of Entomological Research*.

Churchland C, Grayston SJ (2014) Specificity of plant-microbe interactions in the tree mycorrhizosphere biome and consequences for soil C cycling. *Frontiers in Microbiology*.

Claire Horner-Devine M, Leibold MA, Smith VH, Bohannan BJM (2003) Bacterial diversity patterns along a gradient of primary productivity. *Ecology Letters*, **6**, 613–622.

Clark IM, Hirsch PR (2008) Survival of bacterial DNA and culturable bacteria in archived soils from the Rothamsted Broadbalk experiment. *Soil Biology and Biochemistry*.

Clark DR, Ferguson RMW, Harris DN et al. (2018) Streams of data from drops of water: 21st century molecular microbial ecology. *Wiley Interdisciplinary Reviews: Water*, **5**, e1280.

Clarke MA, Walsh RPD (2006) Long-term erosion and surface roughness change of rain-forest terrain following selective logging, Danum Valley, Sabah, Malaysia. *Catena*.

Coley PD, Barone JA (1996) Herbivory and plant defenses in tropical forests. *Annual Review of Ecology and Systematics*.

Comita LS, Muller-Landau HC, Aguilar S, Hubbell SP (2010) Asymmetric Density Dependence Shapes Species Abundances in a Tropical Tree Community. *Science*, **329**, 330–332.

Comte I, Colin F, Whalen JK, Grünberger O, Caliman JP (2012) Agricultural Practices in Oil Palm Plantations and Their Impact on Hydrological Changes, Nutrient Fluxes and Water Quality in Indonesia. A Review. In: *Advances in Agronomy*.

Connell JH (1971) On the role of natural enemies in preventing competitive exclusion in some marine animals and in rain forest trees. In: *Dynamics of populations. Proceeding of the advanced study institute, Osterbeek, 1970.*, Vol. 298, pp. 298–312.

Connell JH (1978) Diversity in tropical rain forests and coral reefs. *Science*.

Corley RHV, Tinker PB (2015) Care and Maintenance of Oil Palms. In: *The Oil Palm*.

Cortois R, Schröder-Georgi T, Weigelt A, van der Putten WH, De Deyn GB (2016) Plant–soil feedbacks: role of plant functional group and plant traits. *Journal of Ecology*.

Dabney SM, Delgado JA, Reeves DW (2001) Using winter cover crops to improve soil and water quality. *Communications in Soil Science and Plant Analysis*.

Daily G (1997) *Nature's Services: Societal Dependence On Natural Ecosystems: Gretchen Daily: Island Press*, Vol. 6. Island Press, 412 pp.

Dassen S, Cortois R, Martens H, de Hollander M, Kowalchuk GA, van der Putten WH, De Deyn GB (2017) Differential responses of soil bacteria, fungi, archaea and protists to plant species richness and plant functional group identity. *Molecular Ecology*, **26**.

Delgado-Baquerizo M, Oliverio AM, Brewer TE et al. (2018) A global atlas of the dominant bacteria found in soil. *Science*.

Denslow JS (1980) Gap Partitioning among Tropical Rainforest Trees. *Biotropica*.

Dominguez J, Edwards CA (2010) Biology and ecology of earthworm species used for vermicomposting. In: *Vermiculture Technology: Earthworms, Organic Wastes, and Environmental Management*.

Dorn B, Jossi W, van der Heijden MGA (2015) Weed suppression by cover crops: Comparative on-farm experiments under integrated and organic conservation tillage. *Weed Research*.

Drescher J, Rembold K, Allen K et al. (2016) Ecological and socio-economic functions across tropical land use systems after rainforest conversion. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, **231**,

1–7.

Dumbrell AJ, Nelson M, Helgason T, Dytham C, Fitter AH (2010) Relative roles of niche and neutral processes in structuring a soil microbial community. *ISME Journal*, **4**, 337–345.

Dumbrell AJ, Ashton PD, Aziz N et al. (2011) Distinct seasonal assemblages of arbuscular mycorrhizal fungi revealed by massively parallel pyrosequencing. *New Phytologist*, **190**, 794–804.

Dumbrell AJ, Ferguson RMW, Clark DR (2016) Microbial Community Analysis by Single-Amplicon High-Throughput Next Generation Sequencing: Data Analysis – From Raw Output to Ecology.

Eberl L, Vandamme P (2016) Members of the genus Burkholderia: good and bad guys. *F1000Research*.

Ehrenfeld JG, Ravit B, Elgersma K (2005) FEEDBACK IN THE PLANT-SOIL SYSTEM. *Annual Review of Environment and Resources*.

Emilia Hannula S, Kielak AM, Steinauer K et al. (2019) Time after time: Temporal variation in the effects of grass and forb species on soil bacterial and fungal communities. *mBio*.

Fierer N (2017) Embracing the unknown: Disentangling the complexities of the soil microbiome. *Nature Reviews Microbiology*.

Fierer N, Leff JW, Adams BJ et al. (2012) Cross-biome metagenomic analyses of soil microbial communities and their functional attributes. *Proceedings of the National Academy of Sciences of the United States of America*.

Fiore-Donno AM, Richter-Heitmann T, Degrune F et al. (2019) Functional traits and spatio-temporal structure of a major group of soil protists (rhizaria: Cercozoa) in a temperate grassland. *Frontiers in Microbiology*.

Fitzpatrick CR, Copeland J, Wang PW, Guttman DS, Kotanen PM, Johnson MTJ (2018) Assembly and ecological function of the root microbiome across angiosperm plant species. *Proceedings of the National Academy of Sciences*, 201717617.

Flynn JM, Brown EA, Chain FJJ, Macisaac HJ, Cristescu ME (2015) Toward accurate molecular identification of species in complex environmental samples: Testing the performance of sequence filtering and clustering methods. *Ecology and Evolution*.

Foley JA, Asner GP, Costa MH et al. (2007) Amazonia revealed: forest degradation and loss of ecoystem goods and services in the Amazon Basin. *Frontiers in Ecology and the Environment*, **5**, 25–32.

Folman LB, Postma J, Veen JA (2001) Ecophysiological characterization of rhizosphere bacterial communities at different root locations and plant developmental stages of cucumber grown on rockwool. *Microbial Ecology*.

Foster WA, Snaddon JL, Advento AD et al. (2014) The Biodiversity and Ecosystem Function in Tropical Agriculture (BEFTA) Project . *Planter*, **90**, 581–591.

Francioli D, van Ruijven J, Bakker L, Mommer L (2020) Drivers of total and pathogenic soil-borne fungal communities in grassland plant species. *Fungal Ecology*.

Francis R, Read DJ (1984) Direct transfer of carbon between plants connected by vesicular-arbuscular mycorrhizal mycelium. *Nature*.

Garciá-Orenes F, Morugań-Coronado A, Zornoza R, Scow K (2013) Changes in soil microbial community structure influenced by agricultural management practices in a Mediterranean agro-ecosystem. *PLoS ONE*.

Gause GF (1932) Experimental studies on the struggle for existence. *Journal of Experimental Biology*, **9**, 389–402.

Geiger F, Bengtsson J, Berendse F et al. (2010) Persistent negative effects of pesticides on biodiversity and biological control potential on European farmland. *Basic and Applied Ecology*.

Gibbs HK, Ruesch AS, Achard F, Clayton MK, Holmgren P, Ramankutty N, Foley JA (2010) Tropical forests were the primary sources of new agricultural land in the 1980s and 1990s. *Proceedings of the National Academy of Sciences of the United States of America*.

Gilbert (1994) Root Camouflage and Disease Control. *Phytopathology*.

Gilbert GS, Parker IM (2016) The Evolutionary Ecology of Plant Disease: A Phylogenetic Perspective. *Annual Review of Phytopathology*.

Gilbert GS, Webb CO (2007) Phylogenetic signal in plant pathogen-host range. *Proceedings of the National Academy of Sciences of the United States of America*.

Gilbert GS, Magarey R, Suiter K, Webb CO (2012) Evolutionary tools for phytosanitary risk analysis: phylogenetic signal as a predictor of host range of plant pests and pathogens. *Evolutionary Applications*, **5**, 869–878.

Givnish TJ (1999) On the causes of gradients in tropical tree diversity. *Journal of Ecology*, **87**, 193–210.

Godfray HCJ, Crute IR, Haddad L et al. (2010) The future of the global food system. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **365**, 2769–2777.

Gómez-Brandón M, Aira M, Domínguez J (2020) Vermicomposts Are Biologically Different: Microbial and Functional Diversity of Green Vermicomposts. In: *Earthworm Assisted Remediation of Effluents and Wastes*, pp. 125–140. Springer Singapore, Singapore.

Griffiths RI, Whiteley AS, O'Donnell AG, Bailey MJ (2000) Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. *Applied and Environmental Microbiology*.

Grime JP (1981) Plant strategies and vegetation processes. *Agro-Ecosystems*, **7**, 89.

Grubb PJ (1977) the Maintenance of Species-Richness in Plant Communities: the

Importance of the Regeneration Niche. *Biological Reviews*, **52**, 107–145.

Guillou L, Bachar D, Audic S et al. (2013) The Protist Ribosomal Reference database (PR2): A catalog of unicellular eukaryote Small Sub-Unit rRNA sequences with curated taxonomy. *Nucleic Acids Research*.

Hardwick SR, Toumi R, Pfeifer M, Turner EC, Nilus R, Ewers RM (2015) The relationship between leaf area index and microclimate in tropical forest and oil palm plantation: Forest disturbance drives changes in microclimate. *Agricultural and Forest Meteorology*.

Hartwig NL, Ammon HU (2002) Cover crops and living mulches. *Weed Science*.

Hawksworth DL (2001) The magnitude of fungal diversity: the 1.5 million species estimate revisited. *Mycological Research*, **105**, 1422–1432.

Heather JM, Chain B (2016) The sequence of sequencers: The history of sequencing DNA. *Genomics*, **107**, 1–8.

Hector A, Schmid B, Beierkuhnlein C et al. (1999) Plant diversity and productivity experiments in European grasslands. *Science*.

Heijden M Van der, Klironomos J, Ursic M (1998) Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. *Nature*.

Heinen R, Hannula SE, De Long JR et al. (2020) Plant community composition steers grassland vegetation via soil legacy effects. *Ecology Letters*.

Hendriks M, Ravenek JM, Smit-Tiekstra AE et al. (2015) Spatial heterogeneity of plant-soil feedback affects root interactions and interspecific competition. *New Phytologist*, **207**.

Herlemann DP, Labrenz M, Jürgens K, Bertilsson S, Waniek JJ, Andersson AF (2011) Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. *The ISME journal*, **5**, 1571–9.

Hille Ris Lambers J, Clark JS, Beckage B (2002) Density-dependent mortality and the latitudinal gradient in species diversity. *Nature*, **417**, 732–735.

Hillocks RJ (2012) Farming with fewer pesticides: EU pesticide review and resulting challenges for UK agriculture. *Crop Protection*.

Hoekstra JM, Boucher TM, Ricketts TH, Roberts C (2005) Confronting a biome crisis: Global disparities of habitat loss and protection. *Ecology Letters*, **8**, 23–29.

Hooper DU, Chapin FS, Ewel JJ et al. (2005) Effects of biodiversity on ecosystem functioning: A consensus of current knowledge. *Ecological Monographs*.

Hopwood D (2007) An introduction to the actinobacteria. *Microbiology Today*.

Howe AT, Bass D, Vickerman K, Chao EE, Cavalier-Smith T (2009) Phylogeny, Taxonomy, and Astounding Genetic Diversity of Glissomonadida ord. nov., The Dominant Gliding Zooflagellates in Soil (Protozoa: Cercozoa). *Protist*.

Huang LF, Song LX, Xia XJ, Mao WH, Shi K, Zhou YH, Yu JQ (2013) Plant-Soil Feedbacks and Soil Sickness: From Mechanisms to Application in Agriculture.

Journal of Chemical Ecology, **39**, 232–242.

Hubbard RK, Strickland TC, Phatak S (2013) Effects of cover crop systems on soil physical properties and carbon/nitrogen relationships in the coastal plain of southeastern USA. *Soil and Tillage Research*.

Hubbell SP (1997) A unified theory of biogeography and relative species abundance and its application to tropical rain forests and coral reefs. *Coral Reefs*.

Hugerth LW, Muller EEL, Hu YOO et al. (2014) Systematic Design of 18S rRNA Gene Primers for Determining Eukaryotic Diversity in Microbial Consortia (ed Voolstra CR). *PLoS ONE*, **9**, e95567.

Illumina An Introduction to Next-Generation Sequencing Technology [Materiały informacyjne].

Illumina (2013) *16S Metagenomic Sequencing Library Preparation. Part #15044223*. 1–28 pp.

Jacoby R, Peukert M, Succurro A, Koprivova A, Kopriva S (2017) The role of soil microorganisms in plant mineral nutrition—current knowledge and future directions. *Frontiers in Plant Science*.

Janzen DH (1970) Herbivores and the Number of Tree Species in Tropical Forests. *The American Naturalist*, **104**, 501–528.

Johnston-Monje D, Raizada MN (2011) Conservation and diversity of seed associated endophytes in Zea across boundaries of evolution, ethnography and ecology. *PLoS ONE*.

Jurgens G, Lindström K, Saano A (1997) Novel group within the kingdom Crenarchaeota from boreal forest soil. *Applied and Environmental Microbiology*.

Kardol P, Martijn Bezemer T, Van Der Putten WH (2006) Temporal variation in plant-soil feedback controls succession. *Ecology Letters*.

Kempel A, Rindisbacher A, Fischer M, Allan E (2018) Plant soil feedback strength in relation to large-scale plant rarity and phylogenetic relatedness. *Ecology*.

Khatun R, Reza MIH, Moniruzzaman M, Yaakob Z (2017) Sustainable oil palm industry: The possibilities. *Renewable and Sustainable Energy Reviews*.

Kimmerer RW, Young CC (1996) Effect of gap size and regeneration niche on species coexistence in bryophyte communities. *Journal of the Torrey Botanical Society*.

Kleppe K, Ohtsuka E, Kleppe R, Molineux I, Khorana HG (1971) Studies on polynucleotides. XCVI. Repair replication of short synthetic DNA's as catalyzed by DNA polymerases. *Journal of Molecular Biology*, **56**, 341–361.

Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, Glöckner FO (2013) Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research*, **41**, e1–e1.

Klironomos JN (2002) Feedback with soil biota contributes to plant rarity and

invasiveness in communities. *Nature*, **417**, 67–70.

Koh LP, Wilcove DS (2007) Cashing in palm oil for conservation. *Nature*, **448**, 993–994.

Kõljalg U, Nilsson RH, Abarenkov K et al. (2013) Towards a unified paradigm for sequence-based identification of fungi. *Molecular Ecology*.

Kowalchuk GA, Stephen JR (2001) Ammonia-Oxidizing Bacteria: A Model for Molecular Microbial Ecology. *Annual Review of Microbiology*, **55**, 485–529.

Kowalchuk GA, Buma DS, De Boer W, Klinkhamer PGL, Van Veen JA (2002) Effects of above-ground plant species composition and diversity on the diversity of soil-borne microorganisms. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*.

Koziol L, Crews TE, Bever JD (2020) Native plant abundance, diversity, and richness increases in prairie restoration with field inoculation density of native mycorrhizal amendments. *Restoration Ecology*.

Kremen C, Williams NM, Thorp RW (2002) Crop pollination from native bees at risk from agricultural intensification. *Proceedings of the National Academy of Sciences*, **99**, 16812–16816.

Krupinsky JM, Bailey KL, McMullen MP, Gossen BD, Kelly Turkington T (2002) Managing plant disease risk in diversified cropping systems. In: *Agronomy Journal*.

Kulmatiski A, Beard KH, Heavilin J (2012) Plant-soil feedbacks provide an additional explanation for diversity-productivity relationships. *Proceedings of the Royal Society B: Biological Sciences*.

LaManna JA, Mangan SA, Alonso A et al. (2017) Plant diversity increases with the strength of negative density dependence at the global scale. *Science*, **356**, 1389–1392.

Lauber CL, Strickland MS, Bradford MA, Fierer N (2008) The influence of soil properties on the structure of bacterial and fungal communities across land-use types. *Soil Biology and Biochemistry*.

Leake J, Johnson D, Donnelly D, Muckle G, Boddy L, Read D (2004) Networks of power and influence: the role of mycorrhizal mycelium in controlling plant communities and agroecosystem functioning. *Canadian Journal of Botany*, **82**, 1016–1045.

Lehtovirta LE, Prosser JI, Nicol GW (2009) Soil pH regulates the abundance and diversity of Group 1.1c Crenarchaeota. *FEMS Microbiology Ecology*.

Leininger S, Urich T, Schloter M et al. (2006) Archaea predominate among ammonia-oxidizing prokaryotes in soils. *Nature*.

Leveau JHJ, Preston GM (2008) Bacterial mycophagy: Definition and diagnosis of a unique bacterial-fungal interaction. *New Phytologist*.

Leveau JHJ, Uroz S, de Boer W (2010) The bacterial genus Collimonas: Mycophagy,

weathering and other adaptive solutions to life in oligotrophic soil environments. *Environmental Microbiology*.

Li L, Tilman D, Lambers H, Zhang FS (2014) Plant diversity and overyielding: Insights from belowground facilitation of intercropping in agriculture. *New Phytologist*.

Liang M, Liu X, Etienne RS, Huang F, Wang Y, Yu S (2015) Arbuscular mycorrhizal fungi counteract the Janzen-Connell effect of soil pathogens. *Ecology*, **96**, 562–574.

Liang M, Liu X, Gilbert GS, Zheng Y, Luo S, Huang F, Yu S (2016) Adult trees cause density-dependent mortality in conspecific seedlings by regulating the frequency of pathogenic soil fungi. *Ecology Letters*, **19**, 1448–1456.

Liu X, Liang M, Etienne RS, Wang Y, Staehelin C, Yu S (2012) Experimental evidence for a phylogenetic Janzen-Connell effect in a subtropical forest. *Ecology Letters*, **15**, 111–118.

Liu Y, Fang S, Chesson P, He F (2015) The effect of soil-borne pathogens depends on the abundance of host tree species. *Nature Communications*, **6**, 10017.

Luke SH, Advento AD, Aryawan AAK et al. (2020) Managing Oil Palm Plantations More Sustainably: Large-Scale Experiments Within the Biodiversity and Ecosystem Function in Tropical Agriculture (BEFTA) Programme. *Frontiers in Forests and Global Change*.

Luskin MS, Potts MD (2011) Microclimate and habitat heterogeneity through the oil palm lifecycle. *Basic and Applied Ecology*, **12**, 540–551.

Ma H-K, Pineda A, van der Wurff AWG, Raaijmakers C, Bezemer TM (2017) Plant–Soil Feedback Effects on Growth, Defense and Susceptibility to a Soil-Borne Disease in a Cut Flower Crop: Species and Functional Group Effects. *Frontiers in Plant Science*, **8**.

MacArthur R (1960) On the Relative Abundance of Species. *The American Naturalist*.

MacArthur RH, Wilson EO (1963) An Equilibrium Theory of Insular Zoogeography. *Evolution*.

Mackey RL, Currie DJ (2001) The diversity-disturbance relationship: Is it generally strong and peaked? *Ecology*, **82**, 3479–3492.

Mangan SA, Schnitzer SA, Herre EA, Mack KML, Valencia MC, Sanchez EI, Bever JD (2010) Negative plant–soil feedback predicts tree-species relative abundance in a tropical forest. *Nature*, **466**, 752–755.

Maron JL, Marler M, Klironomos JN, Cleveland CC (2011) Soil fungal pathogens and the relationship between plant diversity and productivity. *Ecology Letters*, **14**, 36–41.

Martin F, Cullen D, Hibbett D, Pisabarro A, Spatafora JW, Baker SE, Grigoriev I V. (2011) Sequencing the fungal tree of life. *New Phytologist*, **190**, 818–821.

Matson PA, Parton WJ, Power GA, Swift MJ (1997) Agricultural intensification and ecosystem properties. *Science (New York, N.Y.)*, **277**, 504–509.

May CY, Ngan MA, Yoo CK, Majid RA (1992) Palm Diesel : Green and Renewable Fuel from Palm Oil. *Palm Oil Developments*, 3–7.

McGuire KL, D'Angelo H, Brearley FQ et al. (2015) Responses of Soil Fungi to Logging and Oil Palm Agriculture in Southeast Asian Tropical Forests. *Microbial Ecology*, **69**, 733–747.

McMurdie PJ, Holmes S (2013) Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS ONE*.

McMurdie PJ, Holmes S (2014) Waste Not, Want Not: Why Rarefying Microbiome Data Is Inadmissible (ed McHardy AC). *PLoS Computational Biology*, **10**, e1003531.

McNear Jr. DH (2013) The Rhizosphere - Roots, Soil and Everything In Between. *Nature Education Knowledge*, **4**, 1.

Meijide A, Badu CS, Moyano F, Tiralla N, Gunawan D, Knohl A (2018) Impact of forest conversion to oil palm and rubber plantations on microclimate and the role of the 2015 ENSO event. *Agricultural and Forest Meteorology*, **252**, 208–219.

Merzendorfer H, Zimoch L (2003) Chitin metabolism in insects: Structure, function and regulation of chitin synthases and chitinases. *Journal of Experimental Biology*.

Miki T (2012) Microbe-mediated plant-soil feedback and its roles in a changing world. *Ecological Research*.

Miller RS (1967) Pattern and Process in Competition. *Advances in Ecological Research*, **4**, 1–74.

Mills KE, Bever JD (1998) Maintenance of diversity within plant communities: Soil pathogens as agents of negative feedback. *Ecology*, **79**, 1595–1601.

Mommer L, van Ruijven J, de Caluwe H et al. (2010) Unveiling below-ground species abundance in a biodiversity experiment: A test of vertical niche differentiation among grassland species. *Journal of Ecology*, **98**, 1117–1127.

Mommer L, Dumbrell AJ, Wagemaker CAM, Ouborg NJ (2011) Belowground DNA-based techniques: Untangling the network of plant root interactions. *Plant and Soil*, **348**, 115–121.

Mommer L, Cotton TEA, Raaijmakers JM et al. (2018a) Lost in diversity: the interactions between soil-borne fungi, biodiversity and plant productivity. *New Phytologist*, **218**, 542–553.

Mommer L, Cotton TEA, Raaijmakers JM et al. (2018b) Lost in diversity: the interactions between soil-borne fungi, biodiversity and plant productivity. *New Phytologist*, **218**, 542–553.

Morgulis A, Coulouris G, Raytselis Y, Madden TL, Agarwala R, Schäffer AA (2008) Database indexing for production MegaBLAST searches. In: *Bioinformatics*.

Morris EK, Caruso T, Buscot F et al. (2014) Choosing and using diversity indices: Insights for ecological applications from the German Biodiversity Exploratories. *Ecology and Evolution*, **4**, 3514–3524.

Mueller GM, Schmit JP, Leacock PR et al. (2007) Global diversity and distribution of macrofungi. *Biodiversity and Conservation*, **16**, 37–48.

Mullis K, Faloona F, Scharf S, Saiki R, Horn G, Erlich H (1986) Specific enzymatic amplification of DNA in vitro: The polymerase chain reaction. *Cold Spring Harbor Symposia on Quantitative Biology*, **51**, 263–273.

Myers N, Mittermeler RA, Mittermeler CG, Da Fonseca GAB, Kent J (2000) Biodiversity hotspots for conservation priorities. *Nature*.

Nájera A, Simonetti JA (2010) Can oil palm plantations become bird friendly? *Agroforestry Systems*.

Navrátilová D, Tláskalová P, Kohout P, Dřevojan P, Fajmon K, Chytrý M, Baldrian P (2018) Diversity of fungi and bacteria in species-rich grasslands increases with plant diversity in shoots but not in roots and soil. *FEMS Microbiology Ecology*.

Nguyen NH, Song Z, Bates ST et al. (2016) FUNGuild: An open annotation tool for parsing fungal community datasets by ecological guild. *Fungal Ecology*, **20**, 241–248.

Nikolenko SI, Korobeynikov AI, Alekseyev MA (2013) BayesHammer: Bayesian clustering for error correction in single-cell sequencing. *BMC Genomics*, **14**, S7.

Nilsson RH, Larsson KH, Taylor AFS et al. (2019) The UNITE database for molecular identification of fungi: Handling dark taxa and parallel taxonomic classifications. *Nucleic Acids Research*, **47**, D259–D264.

Normander B, Prosser JI (2000) Bacterial origin and community composition in the barley phytosphere as a function of habitat and presowing conditions. *Applied and Environmental Microbiology*.

Nunes MR, van Es HM, Schindelbeck R, Ristow AJ, Ryan M (2018) No-till and cropping system diversification improve soil health and crop yield. *Geoderma*.

Nur MMZ, Rosli BM, Kamaruzaman S, Md MM, Yahya A (2013) Effects of selected herbicides on soil microbial populations in oil palm plantation of Malaysia: A microcosm experiment. *African Journal of Microbiology Research*.

Nurk S, Bankevich A, Antipov D et al. (2013) Assembling single-cell genomes and mini-metagenomes from chimeric MDA products. *Journal of Computational Biology*.

Ochsenreiter T, Selezi D, Quaiser A, Bonch-Osmolovskaya L, Schleper C (2003) Diversity and abundance of Crenarchaeota in terrestrial habitats studied by 16S RNA surveys and real time PCR. *Environmental Microbiology*.

Ohene-Adjei S, Teather RM, Ivan M, Forster RJ (2007) Postinoculation protozoan establishment and association patterns of methanogenic archaea in the ovine rumen. *Applied and Environmental Microbiology*, **73**, 4609–4618.

Oksanen J, Blanchet FG, Friendly M et al. (2018) vegan: Community Ecology Package. R package version 2.5-2. *CRAN R*.

Olff H, Hoorens B, De Goede RGM, Van der Putten WH, Gleichman JM (2000) Small-

scale shifting mosaics of two dominant grassland species: The possible role of soil-borne pathogens. *Oecologia*, **125**, 45–54.

Van Overbeek L, Van Elsas JD (2008) Effects of plant genotype and growth stage on the structure of bacterial communities associated with potato (Solanum tuberosum L.). *FEMS Microbiology Ecology*.

Overton M (1996) Agricultural Revolution in England: The Transformation of the Agrarian Economy 1500-1850. 258.

Packer A, Clay K (2000) Soil pathogens and spatial patterns of seedling mortality in a temperatetree. *Nature*, **404**, 278–281.

Pärtel M, Zobel M, Zobel K, van der Maarel E, Partel M (1996) The Species Pool and Its Relation to Species Richness: Evidence from Estonian Plant Communities. *Oikos*, **75**, 111.

Peay KG (2014) Back to the future: Natural history and the way forward in modern fungal ecology. *Fungal Ecology*.

Petermann JS, Fergus AJF, Turnbull LA, Schmid B (2008) Janzen-Connell effects are widespread and strong enough to maintain diversity in grasslands. *Ecology*, **89**, 2399–2406.

Peters HA (2003) Neighbour-regulated mortality: The influence of positive and negative density dependence on tree populations in species-rich tropical forests. *Ecology Letters*, **6**, 757–765.

Pham VHT, Kim J (2012) Cultivation of unculturable soil bacteria. *Trends in Biotechnology*.

Philippot L, Raaijmakers JM, Lemanceau P, Van Der Putten WH (2013) Going back to the roots: The microbial ecology of the rhizosphere. *Nature Reviews Microbiology*, **11**, 789–799.

Pickett S (1980) Non-Equilibrium Coexistence of Plants. *Bulletin of the Torrey Botanical Club*, **107**, 238–248.

Prober SM, Leff JW, Bates ST et al. (2015) Plant diversity predicts beta but not alpha diversity of soil microbes across grasslands worldwide. *Ecology Letters*, **18**, 85–95.

Putten WHV Der, Dijk C Van, Peters BAM (1993) Plant-specific soil-borne diseases contribute to succession in foredune vegetation. *Nature*.

van der Putten WH (2017) Belowground drivers of plant diversity. *Science (New York, N.Y.)*, **355**, 134–135.

R Core Team (2020) (2020) R: A language and environment for statistical computing. *R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria*.

Raaijmakers JM, Paulitz TC, Steinberg C, Alabouvette C, Moënne-Loccoz Y (2009) The rhizosphere: A playground and battlefield for soilborne pathogens and beneficial microorganisms. *Plant and Soil*, **321**, 341–361.

Reinhart KO (2012) The organization of plant communities: Negative plant-soil feedbacks and semiarid grasslands. *Ecology*.

Rigg JL, Offord CA, Singh BK, Anderson I, Clarke S, Powell JR (2016) Soil microbial communities influence seedling growth of a rare conifer independent of plant-soil feedback. *Ecology*, **97**, 3346–3358.

Rillig MC, Mummey DL (2006) Mycorrhizas and soil structure. *New Phytologist*, **171**, 41–53.

Ripley MB (2011) *R: Package ' MASS .'*

Robertson CE, Harris JK, Spear JR, Pace NR (2005) Phylogenetic diversity and ecology of environmental Archaea. *Current Opinion in Microbiology*.

Rognes T, Flouri T, Nichols B, Quince C, Mahé F (2016) VSEARCH: A versatile open source tool for metagenomics. *PeerJ*.

Romdhane S, Spor A, Busset H et al. (2019) Cover Crop Management Practices Rather Than Composition of Cover Crop Mixtures Affect Bacterial Communities in No-Till Agroecosystems. *Frontiers in Microbiology*, **10**, 1618.

Ronaghi M, Karamohamed S, Pettersson B, Uhlén M, Nyrén PL (1996) Real-Time DNA Sequencing Using Detection of Pyrophosphate Release. *Analytical Biochemistry*, **242**, 84–89.

Rønn R, McCaig AE, Griffiths BS, Prosser JI (2002) Impact of protozoan grazing on bacterial community structure in soil microcosms. *Applied and Environmental Microbiology*.

Rousk J, Bååth E, Brookes PC et al. (2010) Soil bacterial and fungal communities across a pH gradient in an arable soil. *ISME Journal*, **4**, 1340–1351.

Roxburgh SH, Shea K, Wilson JB (2004) The intermediate disturbance hypothesis: Patch dynamics and mechanisms of species coexistence. *Ecology*, **85**, 359–371.

Sanger F, Brownlee GG, Barrell BG (1965) A Two-dimensional Fractionation Procedure for Radioactive N ucleotides. *Journal of Molecular Biology*, **13**, 373–398, IN1–IN4.

Sanger F, Nicklen S, Coulson a R (1977) DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America*, **74**, 5463–7.

Schiesari L, Grillitsch B (2011) Pesticides meet megadiversity in the expansion of biofuel crops. *Frontiers in Ecology and the Environment*.

Schneider D, Engelhaupt M, Allen K et al. (2015) Impact of Lowland Rainforest Transformation on Diversity and Composition of Soil Prokaryotic Communities in Sumatra (Indonesia). *Frontiers in Microbiology*, **6**, 1339.

Schnitzer SA, Klironomos JN, HilleRisLambers J et al. (2011) Soil microbes drive the classic plant diversity-productivity pattern. *Ecology*, **92**, 296–303.

Schoch CL, Seifert KA, Huhndorf S et al. (2012) Nuclear ribosomal internal transcribed

spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences of the United States of America*.

Sekercioglu CH (2012) Bird functional diversity and ecosystem services in tropical forests, agroforests and agricultural areas. *Journal of Ornithology*, **153**, 153–161.

Semchenko M, Leff JW, Lozano YM et al. (2018) Fungal diversity regulates plant-soil feedbacks in temperate grassland. *Science Advances*, **4**, eaau4578.

Sessitsch A, Coenye T, Sturz A V. et al. (2005) Burkholderia phytofirmans sp. nov., a novel plant-associated bacterium with plant-beneficial properties. *International Journal of Systematic and Evolutionary Microbiology*.

Shi LL, Mortimer PE, Ferry Slik JW, Zou XM, Xu J, Feng WT, Qiao L (2014) Variation in forest soil fungal diversity along a latitudinal gradient. *Fungal Diversity*.

Shmida A, Ellner S (1984) Coexistence of plant species with similar niches. *Vegetatio*, **58**, 29–55.

Silvertown J, Law R (1987) Do plants need niches? Some recent developments in plant community ecology. *Trends in Ecology and Evolution*.

Silvertown J, Gause GF, Chase JM et al. (2004) Plant coexistence and the niche. *Trends in Ecology & Evolution*, **19**, 605–611.

Simpson AGB, Slamovits CH, Archibald JM (2017) Protist Diversity and Eukaryote Phylogeny. In: *Handbook of the Protists*, pp. 1–21. Springer International Publishing.

Smith SE, Read D (2008a) Mycorrhizal Symbiosis. *Mycorrhizal Symbiosis*, 611–XVIII.

Smith S, Read D (2008b) *Mycorrhizal Symbiosis*.

Somers E, Vanderleyden J, Srinivasan M (2004) Rhizosphere bacterial signalling: A love parade beneath our feet. *Critical Reviews in Microbiology*.

Stieglmeier M, Alves RJE, Schleper C (2014) The phylum thaumarchaeota. In: *The Prokaryotes: Other Major Lineages of Bacteria and The Archaea*, pp. 347–362. Springer-Verlag Berlin Heidelberg.

Tao HH, Snaddon JL, Slade EM, Henneron L, Caliman JP, Willis KJ (2018) Application of oil palm empty fruit bunch effects on soil biota and functions: A case study in Sumatra, Indonesia. *Agriculture, Ecosystems and Environment*.

Taylor DR, Aarssen LW, Loehle C (1990) On the relationship between r/K selection and environmental carrying capacity: A new habitat templet for plant life history strategies. *Oikos*, **58**, 239–250.

Taylor DL, Walters WA, Lennon NJ, Bochicchio J, Krohn A, Caporaso JG, Pennanen T (2016) Accurate Estimation of Fungal Diversity and Abundance through Improved Lineage-Specific Primers Optimized for Illumina Amplicon Sequencing. *Applied and environmental microbiology*, **82**, 7217–7226.

Team RC (2016) R: A Language and Environment for Statistical Computing. *R Foundation for Statistical Computing*.

Tedersoo L, Bahram M, Polme S et al. (2014) Global diversity and geography of soil fungi. *Science*, **346**.

Tilman D (1982) Resource competition and community structure. *Monographs in population biology*, **17**, 1–296.

Tilman D, Wedin D, Knops J (1996) Productivity and sustainability influenced by biodiversity in grassland ecosystems. *Nature*, **379**, 718–720.

Tilman D, Fargione J, Wolff B et al. (2001) Forecasting agriculturally driven global environmental change. *Science*, **292**, 281–4.

Toju H, Tanabe AS, Yamamoto S et al. (2012) High-Coverage ITS Primers for the DNA-Based Identification of Ascomycetes and Basidiomycetes in Environmental Samples (ed Lespinet O). *PLoS ONE*, **7**, e40863.

Tripathi BM, Edwards DP, Mendes LW, Kim M, Dong K, Kim H, Adams JM (2016) The impact of tropical forest logging and oil palm agriculture on the soil microbiome. *Molecular Ecology*.

Tscharntke T, Sekercioglu CH, Dietsch T V., Sodhi NS, Hoehn P, Tylianakis JM (2008) Landscape constraints on functional diversity of birds and insects in tropical agroecosystems. *Ecology*, **89**, 944–951.

Tupinambá DD, Cantão ME, Costa OYA et al. (2016) Archaeal Community Changes Associated with Cultivation of Amazon Forest Soil with Oil Palm. *Archaea*, **2016**.

Uryu Y, Mott C, Foead N et al. (2008) *Deforestation, Forest Degradation, Biodiversity Loss and CO 2 Emissions in Riau, Sumatra, Indonesia One Indonesian Province's Forest and Peat Soil Carbon Loss over a Quarter Century and its Plans for the Future*. Jakarta, Indonesia.

Volkov I, Banavar JR, Hubbell SP, Maritan A (2003) Neutral theory and relative species abundance in ecology. *Nature*.

Vukicevich E, Lowery T, Bowen P, Úrbez-Torres JR, Hart M (2016) Cover crops to increase soil microbial diversity and mitigate decline in perennial agriculture. A review. *Agronomy for Sustainable Development*.

Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology*.

Wang Y, Naumann U, Wright ST, Warton DI (2012) Mvabund- an R package for model-based analysis of multivariate abundance data. *Methods in Ecology and Evolution*, **3**, 471–474.

Wang S, Liu Y, Xu J (2017a) Comparison of different drying methods for recovery of mushroom DNA. *Scientific Reports*.

Wang Z, Li T, Wen X, Liu Y, Han J, Liao Y, DeBruyn JM (2017b) Fungal communities in rhizosphere soil under conservation tillage shift in response to plant growth. *Frontiers in Microbiology*.

Wang B, Adachi Y, Sugiyama S (2018) Soil productivity and structure of bacterial and

fungal communities in unfertilized arable soil. *PLoS ONE*.

Wardle DA, Bardgett RD, Klironomos JN, Setälä H, van der Putten WH, Wall DH (2004) Ecological linkages between aboveground and belowground biota. *Science (New York, N.Y.)*, **304**, 1629–33.

Warton DI, Hui FKC (2011) The arcsine is asinine: The analysis of proportions in ecology. *Ecology*, **92**, 3–10.

Warton DI, Foster SD, De'ath G, Stoklosa J, Dunstan PK (2015) Model-based thinking for community ecology. *Plant Ecology*.

Weisser WW, Roscher C, Meyer ST et al. (2017) Biodiversity effects on ecosystem functioning in a 15-year grassland experiment: Patterns, mechanisms, and open questions. *Basic and Applied Ecology*, **23**.

Wensley RN (1956) The peach replant problem in ontario: iv. fungi associated with replant failure and their importance in fumigated and nonfumigated soils. *Canadian Journal of Botany*, **34**, 967–981.

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal rna genes for phylogenetics. In: *PCR Protocols*.

White R, Murray S, Rohweder M (2000) *Pilot Analysis of Global Ecosystems: Grassland Ecosystems.*

Wickham H (2009) *ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York*.

Wieczorek AS, Schmidt O, Chatzinotas A, Von Bergen M, Gorissen A, Kolb S (2019) Ecological functions of agricultural soil bacteria and microeukaryotes in chitin degradation: A case study. *Frontiers in Microbiology*.

Wilson JD, Whittingham MJ, Bradbury RB (2005) The management of crop structure: a general approach to reversing the impacts of agricultural intensification on birds? *Ibis*, **147**, 453–463.

Wilson JB, Peet RK, Dengler J, Pärtel M (2012) Plant species richness: The world records (ed Palmer M). *Journal of Vegetation Science*, **23**, 796–802.

Wintermans PCA, Bakker PAHM, Pieterse CMJ (2016) Natural genetic variation in Arabidopsis for responsiveness to plant growth-promoting rhizobacteria. *Plant Molecular Biology*.

Wisheu IC, Rosenzweig ML, Olsvig-Whittaker L, Shmida A (2000) What makes nutrient-poor mediterranean heathlands so rich in plant diversity? *Evolutionary Ecology Research*.

Wubs ERJ, Bezemer TM (2016) Effects of spatial plant-soil feedback heterogeneity on plant performance in monocultures (ed Austin A). *Journal of Ecology*, **104**, 364–376.

Yang T, Adams JM, Shi Y et al. (2017) Soil fungal diversity in natural grasslands of the Tibetan Plateau: associations with plant diversity and productivity. *New Phytologist*.

Yu Y, Stomph TJ, Makowski D, van der Werf W (2015) Temporal niche differentiation increases the land equivalent ratio of annual intercrops: A meta-analysis. *Field Crops Research*.

Zgadzaj R, Garrido-Oter R, Jensen DB, Koprivova A, Schulze-Lefert P, Radutoiu S (2016) Root nodule symbiosis in Lotus japonicus drives the establishment of distinctive rhizosphere, root, and nodule bacterial communities. *Proceedings of the National Academy of Sciences of the United States of America*.

Zhang Z, Schwartz S, Wagner L, Miller W (2000) A greedy algorithm for aligning DNA sequences. *Journal of Computational Biology*.

Zhang J, Kobert K, Flouri T, Stamatakis A (2014) PEAR: a fast and accurate Illumina Paired-End reAd mergeR. *Bioinformatics*, **30**, 614–620.

Zimmer Y (2010) *Competitiveness of rapeseed, soybeans and palm oil*, Vol. 1. 84–90 pp.

Zobel M (1992) Plant Species Coexistence: The Role of Historical, Evolutionary and Ecological Factors. *Oikos*, **65**, 314.

Appendices

Chapter 3 appendix

Appendix 3.1. Linear Modelling analysis of fungal richness, diversity and evenness as an effect of plant species and plant functional group (i.e. forbs and grasses).

Plant species effects			
Group	Richness	Diversity	Evenness
Ascomytota	$LR_{38,56} = 71.26$, $P < 0.001$	$F_{38,56} = 2.18$, $P < 0.05$	$F_{38,56} = 2.17$, $P < 0.05$
Basidiomycota	$LR_{37,55} = 59.74$, $P < 0.001$	$F_{37,55} = 2.16$, $P < 0.05$	$F_{37,55} = 2.0$, $P < 0.05$
Chytridiomycota	$LR_{36,54} = 31.98$, $P < 0.05$	$F_{30,48} = 1.9$, $P = 0.06$	$F_{30,48} = 1.91$, $P = 0.057$
Zygomycota	$LR_{36,54} = 31.98$, $P < 0.05$	$F_{33,51} = 1.51$, $P = 0.15$	$F_{33,51} = 1.32$, $P = 0.24$
Glomeromycota	$LR_{37,55} = 37.97$, $P < 0.05$	$F_{34,52} = 1.68$, $P = 0.09$	$F_{34,52} = 1.4$, $P = 0.19$
Pathogens	$LR_{38,56} = 76.55$, $P < 0.001$	$F_{38,56} = 3.89$, $P < 0.001$	$F_{38,56} = 3.79$, $P < 0.001$
Symbiotrophs	$LR_{38,56} = 76.55$, $P < 0.001$	$F_{38,56} = 1.96$, $P < 0.05$	$F_{37,55} = 1.95$, $P < 0.05$
Saptrotrophs	$LR_{38,56} = 78.88$, $P < 0.001$	$F_{38,56} = 2.86$, $P < 0.001$	$F_{38,55} = 3.09$, $P < 0.05$
Plant functional group			
Ascomytota	$LR_{55,56} = 9.14$, $P < 0.01$	$F_{55,56} = 2.68$, $P = 0.11$	$F_{55,56} = 2.14$, $P = 0.14$

Basidiomycota	$LR_{54,55}$ = 6.22, P < 0.05	$F_{54,55}$ = 0.64, P = 0.43	$F_{54,55}$ = 0.25, P = 0.62
Chytridiomycota	$LR_{53,54}$ = 2.12, P = 0.14	$F_{47,48}$ = 0.81, P = 0.37	$F_{47,48}$ = 0.33, P = 0.57
Zygomycota	$LR_{53,54}$ = 1.08, P = 0.29	$F_{50,51}$ = 0.67, P = 0.42	$F_{50,51}$ = 0.32, P = 0.57
Glomeromycota	$LR_{54,55}$ = 1.99, P = 0.16	$F51_{,52}$ = 0.45, P = 0.51	$F_{51,52}$ = 0.16, P = 0.69
Pathogens	$LR_{55,56}$ = 0.38, P = 0.53	$F_{55,56}$ = 0.02, P = 0.9	$F_{55,56}$ = 0.15, P = 0.69
Symbiotrophs	$LR_{55,56}$ = 1.23, P = 0.27	$F_{54,55}$ = 0.14, P = 0.71	$F_{54,55}$ = 0.31, P = 0.58
Saptrotrophs	$LR_{55,56}$ = 78.88, P < 0.001	$F_{55,56}$ = 0.48, P = 0.49	$F_{55,56}$ = 0.22, P = 0.63

Appendix 3.2. MV-GLM modelled shifts of pathogenic taxa occurring in significantly higher abundance in forbs when compared to grasses.

OTU.ID	Phylum	Species
OTU_81	Ascomycota	Mycocentrospora sp
OTU_70	Ascomycota	Pleosporaceae sp
OTU_125	Ascomycota	Trichothecium roseum
OTU_103	Ascomycota	Eutypa leptoplaca
OTU_43	Ascomycota	Clonostachys rosea f. catenulata

OTU_144	Ascomycota	Cylindrocarpon sp W5 1
OTU_1	Ascomycota	Acremonium sp
OTU_148	Ascomycota	Colletotrichum fuscum
OTU_119	Ascomycota	Phaeoacremonium prunicolum
OTU_110	Ascomycota	Xylariaceae sp
OTU_6	Ascomycota	Coniosporium sp
OTU_35	Ascomycota	Dissoconium proteae
OTU_15	Ascomycota	Phoma sp
OTU_64	Ascomycota	Botryosphaeriaceae sp
OTU_113	Ascomycota	Cyphellophora fusarioides
OTU_158	Ascomycota	Coniella sp
OTU_78	Ascomycota	Aureobasidium sp
OTU_60	Ascomycota	Xylariaceae sp
OTU_146	Ascomycota	Botryosphaeria corticis
OTU_26	Ascomycota	Monochaetia sp
OTU_87	Ascomycota	Acremonium rutilum

OTU_83	Ascomycota	Phoma brasiliensis
OTU_41	Ascomycota	Phaeoacremonium prunicolum
OTU_139	Ascomycota	Alternaria metachromatica
OTU_121	Basidiomycota	Corticiaceae sp
OTU_2	Basidiomycota	Typhula sp UW98 G4
OTU_116	Basidiomycota	Thanatephorus cucumeris
OTU_115	Basidiomycota	Ceratobasidiaceae sp
OTU_75	Basidiomycota	Helicobasidiaceae sp
OTU_140	Basidiomycota	Peniophora sp
OTU_27	Basidiomycota	Marasmius scorodonius
OTU_123	Basidiomycota	Ceratobasidiaceae sp
OTU_4	Basidiomycota	Ceratobasidium sp 2 MV 2011
OTU_16	Basidiomycota	Ceratobasidiaceae sp
OTU_90	Basidiomycota	uncultured Ceratobasidium
OTU_91	Basidiomycota	Ceratobasidiaceae sp
OTU_62	Basidiomycota	Thanatephorus sp

OTU_69	Basidiomycota	Ceratobasidiaceae sp
OTU_3	Basidiomycota	Athelia bombacina
OTU_155	Basidiomycota	Ceratobasidium sp FPUB 156
OTU_153	Basidiomycota	Ceratobasidiaceae sp
OTU_143	Basidiomycota	Athelia rolfsii
OTU_21	Basidiomycota	Mycena flavoalba
OTU_5	Basidiomycota	Peniophora sp
OTU_66	Basidiomycota	Mycena sp
OTU_94	Basidiomycota	Ustilago striiformis
OTU_111	Basidiomycota	Ceratobasidium sp 2 MV 2011
OTU_152	Basidiomycota	Thanatephorus cucumeris
OTU_56	Basidiomycota	Thanatephorus sp
OTU_77	Basidiomycota	Ceratobasidiaceae sp
OTU_68	Basidiomycota	Ceratobasidium sp
OTU_88	Chytridiomycota	Rhizophydium sp
OTU_89	Chytridiomycota	Spizellomyces acuminatus

Chapter 4 appendix

Appendix 4.1. PERMANOVA analysis of assemblies of fungal OTUs in Norway (including; Ascomycota, Basidiomycota, Chytridiomycota, Zygomycota and Glomeromycota) across differing plant taxonomic levels and between plant functional group. Significant results are highlighted in bold.

	Total	Asco-mycota	Basio-domycota	Chytridio-mycota	Zygo-mycota	Glomero-mycota
Plant sp.	$F_{12,26}$ = 1.01, P = 0.36	$F_{13,35}$ = 1.04, P = 0.28	$F_{13,29}$ = 1.06, P = 0.04	$F_{6,13}$ = 1.08, P = 0.33	$F_{10,22}$ = 1.07, P = 0.29	$F_{8,17}$ = 0.91, P = 0.76
Plant genus	$F_{12,26}$ = 1.04, P = 0.24	$F_{12,35}$ = 1.06, P = 0.19	$F_{12,29}$ = 1.09, P = 0.07	$F_{6,13}$ = 1.08, P = 0.33	$F_{9,22}$ = 1.14, P = 0.13	$F_{8,17}$ = 0.91, P = 0.76
Plant family	$F_{7,26}$ = 1.05, P = 0.23	$F_{8,35}$ = 1.11, P = 0.12	$F_{7,29}$ = 1.09, P = 0.07	$F_{5,13}$ = 1.19, P = 0.16	**$F_{7,22}$ = 1.27, P < 0.05**	$F_{5,17}$ = 0.98, P = 0.52
Plant order	$F_{7,26}$ = 1.05, P = 0.23	$F_{8,35}$ = 1.11, P = 0.12	$F_{7,29}$ = 1.09, P = 0.07	$F_{5,13}$ = 1.19, P = 0.16	**$F_{7,22}$ = 1.27, P < 0.05**	$F_{5,17}$ = 0.98, P = 0.52
Plant group	**$F_{1,26}$ = 1.27, P = 0.06**	$F_{1,35}$ = 1.26, P = 0.11	**$F_{1,29}$ = 1.29, P = 0.058**	$F_{1,13}$ = 1.03, P = 0.42	$F_{1,22}$ = 1.46, P = 0.07	$F_{1,17}$ = 0.99, P = 0.45

Appendix 4.2. PERMANOVA analysis of assemblies of fungal OTUs in the UK (including; Ascomycota, Basidiomycota, Chytridiomycota, Zygomycota and Glomeromycota) across differing plant taxonomic levels and between plant functional group. Significant results are highlighted in bold.

UK	Total	Asco-mycota	Basiodo-mycota	Chytridio-mycota	Zygo-mycota	Glomero-mycota
Plant sp.	**$F_{21,31}$ = 1.26, P <0.01**	**$F_{21,33}$ = 1.28, P <0.01**	$F_{21,29}$ = 1.05, P = 0.13	$F_{12,19}$ = 1.18, P = 0.13	$F_{14,20}$ = 1.17, P = 0.17	$F_{12,18}$ = 1.24, P = 0.08
Plant genus	**$F_{17,31}$ = 1.3, P <0.001**	**$F_{17,33}$ = 1.33, P <0.001**	**$F_{17,29}$ = 1.09, P < 0.05**	$F_{11,19}$ = 1.2, P = 0.11	$F_{13,20}$ = 1.36, P < 0.05	$F_{11,18}$ = 1.13, P = 0.19
Plant family	**$F_{7,31}$ = 1.66, P <0.001**	**$F_{7,33}$ = 1.94, P <0.001**	**$F_{7,29}$ = 1.19, P < 0.01**	**$F_{5,19}$ = 1.5, P < 0.01**	$F_{7,20}$ = 1.68, P < 0.001	**$F_{5,18}$ = 1.33, P < 0.05**
Plant order	**$F_{7,31}$ = 1.66, P <0.001**	**$F_{7,33}$ = 1.94, P <0.001**	**$F_{7,29}$ = 1.19, P < 0.01**	**$F_{5,19}$ = 1.5, P < 0.01**	$F_{7,20}$ = 1.68, P < 0.001	**$F_{5,18}$ = 1.33, P < 0.05**
Plant group	**$F_{1,31}$ = 3.44, P <0.001**	**$F_{1,33}$ = 4.39, P <0.001**	**$F_{1,29}$ = 1.72, P < 0.001**	$F_{1,19}$ = 1.56, P = 0.08	**$F_{1,20}$ = 2.54, P <0.01**	**$F_{1,18}$ = 1.78, P <0.05**

Appendix 4.3. MV-GLM modelled shifts of pathogenic taxa occurring in significantly higher abundance in the UK in comparison to Norway. SH refers to 'species hypothesis' by where more information can be found by entering the respective SH number within the website address at: https://unite.ut.ee/sh/SHxxxxxx.xxFU.#fndtn-panel3

OTU.ID	Phylum	Species
OTU_3252	Ascomycota	*Phoma sp*|*SH205841.06FU*
OTU_723	Ascomycota	*Alternaria eichhorniae*|*SH224789.06FU*
OTU_1327	Ascomycota	*Verticillium sp*|*SH223647.06FU*
OTU_2465	Ascomycota	*Drechslera poae*|*SH224852.06FU*
OTU_1276	Ascomycota	*Acremonium dichromosporum*|*SH211528.06FU*
OTU_1160	Ascomycota	*Glomerella tucumanensis*|*SH229539.06FU*
OTU_1766	Ascomycota	*Acremonium sp*|*SH220977.06FU*
OTU_304	Ascomycota	*Alternaria metachromatica*|*SH224792.06FU*
OTU_2104	Ascomycota	*Diaporthaceae sp*|*SH194745.06FU*
OTU_2770	Ascomycota	*Acremonium sp*|*SH235788.06FU*
OTU_1478	Ascomycota	*Fusarium sp*|*SH225201.06FU*
OTU_1055	Ascomycota	*Pyrenophora erythrospila*|*SH212472.06FU*
OTU_3111	Ascomycota	*Diplodia allocellula*|*SH236108.06FU*
OTU_1986	Ascomycota	*Drechslera triseptata*|*SH212476.06FU*

OTU_1143	Ascomycota	*Acremonium rutilum*|*SH240964.06FU*
OTU_3151	Ascomycota	*Pilidium sp*|*SH197203.06FU*
OTU_3259	Ascomycota	*Pleospora herbarum*|*SH224791.06FU*
OTU_1146	Ascomycota	*Microdochium bolleyi*|*SH216929.06FU*
OTU_2981	Ascomycota	*Lectera longa*|*SH219644.06FU*
OTU_1805	Ascomycota	*Magnaporthe griffinii*|*SH230187.06FU*
OTU_1498	Ascomycota	*Coniochaeta cateniformis*|*SH226460.06FU*
OTU_2548	Ascomycota	*unclassified Diaporthe*
OTU_3128	Ascomycota	*Acremonium sp 64 OA 2013*|*SH191584.06FU*
OTU_1188	Ascomycota	*Pleosporaceae sp*|*SH212470.06FU*
OTU_2039	Ascomycota	*Magnaporthe griffinii*|*SH230187.06FU*
OTU_2043	Ascomycota	*Stemphylium loti*|*SH224886.06FU*
OTU_1101	Ascomycota	*Acremonium rutilum*|*SH240964.06FU*
OTU.884	Basidiomycota	*Rhodotorula ferulica*|*SH215113.06FU*
OTU_213	Basidiomycota	*Ceratobasidium sp*|*SH227164.06FU*
OTU_747	Basidiomycota	*unclassified Ceratobasidiaceae*
OTU_1970	Basidiomycota	*Ceratobasidiaceae sp*|*SH207631.06FU*
OTU_2443	Basidiomycota	*unclassified Mycena*
OTU_1324	Basidiomycota	*Ustilago striiformis*|*SH237191.06FU*

OTU_1257	Basidiomycota	*unclassified Rhodotorula*
OTU_3586	Basidiomycota	*Rhodotorula ferulica|SH215113.06FU*
OTU_1829	Basidiomycota	*Ceratobasidiaceae sp|SH209053.06FU*
OTU_3434	Basidiomycota	*Eocronartium sp|SH237966.06FU*
OTU_1208	Basidiomycota	*Rhodotorula lamellibrachiae|SH227552.06FU*
OTU_1968	Basidiomycota	*Helicobasidiaceae sp|SH224111.06FU*
OTU_3613	Basidiomycota	*Rhodotorula nothofagi|SH223682.06FU*
OTU_3312	Basidiomycota	*unclassified Ceratobasidiaceae unidentified*
OTU_3147	Basidiomycota	*unclassified Ceratobasidiaceae*
OTU_1708	Basidiomycota	*Rhodotorula lamellibrachiae|SH227552.06FU*
OTU_2085	Basidiomycota	*Rickenella swartzii|SH201023.06FU*
OTU_1486	Basidiomycota	*Rhodotorula cresolica|SH212267.06FU*
OTU_1126	Basidiomycota	*Ganoderma applanatum|SH232520.06FU*
OTU_2217	Basidiomycota	*Marasmius oreades|SH234273.06FU*
OTU_2282	Basidiomycota	*unclassified Marasmiaceae*
OTU_2450	Basidiomycota	*Tilletia bromi|SH192499.06FU*
OTU_3562	Basidiomycota	*unclassified Rhodotorula*
OTU_1454	Basidiomycota	*unclassified Mycena*
OTU_2042	Basidiomycota	*Ceratobasidiaceae sp|SH199615.06FU*

OTU_3067	Chytridiomycota	*unclassified Rhizophydium*
OTU_2299	Chytridiomycota	*unclassified Rhizophydium*

Chapter 5 appendix

Appendix 5.1. Bray-Curtis dissimilatory was calculated for microbial community composition, and Euclidean distance was calculated for the environmental variables. The numbers indicate the Mantel statistic (r) value and significance (P).

	Environmental variable	Bacteria				Fungi			
		Roots		Soil		Roots		Soil	
		Stat	P	Stat	P	Stat	P	Stat	P
Anions	Acetate	-0.13	0.84	-0.02	0.53	-0.24	0.98	-0.08	0.66
	Butyrate	0.13	0.13	-0.09	0.72	-0.04	0.63	-0.02	0.53
	Chloride	-0.05	0.61	0.03	0.39	0.04	0.37	0.00	0.47
	Chlorite	0.06	0.24	-0.20	0.88	-0.08	0.66	-0.14	0.72
	Flouride	0.13	0.07	-0.07	0.77	-0.05	0.70	-0.07	0.78
	Formate	0.04	0.35	-0.14	0.83	-0.08	0.72	-0.15	0.87
	Lactate	-0.19	0.95	-0.21	0.91	-0.23	0.95	-0.24	0.94
	Nitrate	0.15	0.06	-0.09	0.81	-0.19	0.98	0.04	0.33

	Nitrite	-0.01	0.51	-0.07	0.74	-0.07	0.79	-0.08	0.79
	Phosphate	-0.04	0.64	0.01	0.46	0.10	0.14	0.03	0.36
	Phosphite	0.36	0.08	-0.14	0.80	NA	NA	0.15	0.22
	Sulphate	0.00	0.45	-0.04	0.57	-0.07	0.68	-0.10	0.75
Cations	Calcium	0.10	0.22	0.05	0.35	0.01	0.46	-0.12	0.78
	Magnesium	-0.03	0.56	-0.02	0.51	-0.10	0.75	-0.14	0.82
	Potassium	0.07	0.27	-0.01	0.50	-0.03	0.58	-0.18	0.92
	Sodium	0.15	0.13	-0.06	0.62	0.04	0.35	-0.13	0.81
Abiotic	pH	-0.11	0.82	0.03	0.39	-0.15	0.89	0.07	0.28
	Total carbon	-0.01	0.50	0.11	0.20	-0.03	0.59	-0.16	0.92
	Moisture (%)	0.23	**0.04**	-0.12	0.78	0.03	0.38	-0.03	0.55

Appendix 5.2. ANOVA results for bacteria OTU richness. Tukey-post hoc analysis of log(lib.size) included as a factor in ANOVA.

Treatment	richness	log(lib.size)	groups
Mixed.5	1851	10.13	a
Ringo.5	1734	9.94	a
Mixed.4	1702	10.58	a
Mixed.1	1693	10.03	ab
Mixed.3	1467	10.10	abc
Bronze.1	1346	10.44	abc
Bronze.3	1251	10.87	abc

Bronze.2	1183	10.15	abc
Ringo.2	1176	10.72	abc
Mixed.2	1124	10.16	abc
Terra.1	1116	10.82	abc
Terra.3	1101	10.73	abc
Terra.2	1064	10.68	abc
Bronze.4	1041	9.96	abc
Ringo.3	1031	10.47	abc
Exito.1	1028	10.10	abc
Terra.5	952	10.12	abc
Bronze.5	927	10.34	abc
Exito.2	879	10.09	abc
Ringo.1	828	9.53	abc
Terra.4	699	10.30	abc
Ringo.4	491	8.82	abc
Exito.4	374	7.71	abc
Exito.3	210	7.62	bc
Exito.5	75	5.04	c

Appendix 5.3. ANOVA results for fungal OTU richness. Tukey-post hoc analysis of log(lib.size) included as a factor in ANOVA.

Treatment	richness	log(lib.size)	groups
Terra.2	115	8.49	a
Ringo.3	113	8.46	ab
Ringo.2	111	7.68	ab
Terra.1	107	8.23	abc

Terra.5	87	7.04	abcd
Terra.3	86	7.94	abcd
Terra.4	86	8.03	abcd
Ringo.5	76	7.01	abcd
Ringo.1	75	7.25	abcd
Mixed.5	55	5.37	abcd
Bronze.2	43	5.61	abcd
Mixed.5	40	5.92	abcd
Exito.2	39	7.76	abcd
Exito.1	37	6.13	abcd
Ringo.4	37	7.04	abcd
Mixed.4	37	7.54	abcd
Exito.4	36	6.72	abcd
Mixed.3	31	4.88	abcd
Bronze.3	28	4.87	bcd
Bronze.4	24	5.10	cd
Bronze.5	23	4.25	cd
Bronze.1	23	4.57	cd
Exito.1	22	5.70	cd
Mixed.1	20	4.06	d
Exito.5	9	3.37	d