Producing better sports turf through soil microbial management

A thesis submitted to the University of London for the degree of Doctor of Philosophy

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Declaration

I hereby declare that this thesis, titled "Producing better sports turf through soil microbial management", submitted to Royal Holloway University of London as a requirement of the degree Doctor of Philosophy represents my own work and has not been previously submitted to this or any other institution for any degree, diploma or other qualification.

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Abstract

Sports turf is an integral part of the UK economy, contributing valuable public green space, employment and £20.29 billion of economic activity. This thesis aimed to examine how to reduce the reliance of turf on pesticides and fertilizers, and to create sustainable high quality turf in the UK, by soil microbial management. The four main outcomes of this research were:

1. The microbial communities of golf courses were found to differ between greens and between courses, through both Phospholipid Fatty Acid (PLFA) analysis and Illumina sequencing. A significant proportion of those sequences could not be identified to a species level and so suggested the communities are unique with possible undescribed species.

2. Soil type, proximity to the coast, age, location, biostimulant use, microbial inoculant use and number of fungicide active ingredients used were all shown to influence microbial populations. However, application of individual pesticides had no effect on microbial communities in field trials. Garlic products (currently favoured as a nematode control method) were shown to reduce arbuscular mycorrhizal fungal (AMF) colonisation.

3. Microbial inoculants must be tailored to the management regime and sward composition to ensure compatibility. Higher fertiliser use reduced AMF colonisation though this did not always result in reduced growth effects. Ideal inoculant dose rates were determined but it is possible to overdose an inoculum, thereby reducing benefits.

4. Common benefits found from mycorrhizal colonisation of turfgrasses were tested in a simulated golf putting green environment. Drought and waterlogging tolerance were shown in multiple grass species, as well as a reduced need for fertiliser. *Poa annua* was suppressed through AMF inoculation, though it could be encouraged using PGPR.

Overall microbial inoculants display a range of benefits for use in an integrated pest management scheme, though they must be tailored to management regime and sward composition for maximal effect.

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Table of Contents

Abstract 3
Acknowledgements 5
Table of Contents 6
Glossary 10
List of Figures 11
List of Tables 19
Chapter 1: Introduction 21
1.1 Building a golf green and the considerations involved 23
1.2 Turfgrass diseases and current treatments..25
1.3 Microbial Inoculants 27
1.3.1 Mycorrhiza..28
1.3.2 PGPR 32
1.3.3 Biostimulants...33
1.4 Current standings 36
1.5 Overview of research aims and chapters...39
Chapter 2: General Methods 41
2.1 Phospholipid Fatty Acid Analysis (PLFA)...42
2.1.1 Introduction 42
2.1.2 Method: 43
2.2 Mycorrhizal Staining...48
2.2.1 Introduction 48
2.2.2 Method: 48
2.3 DNA analysis 49
2.3.1 Introduction 49
2.3.2 Method: 50
2.3.3 Analysis...51
2.4 General pot set up 51
2.4.1 Method 51
Chapter 3: What microbes are already in the soil?...................................53
3.1 Introduction 54
3.1.1 Summary of hypotheses:...59
3.2 Analysis of golf courses across the UK by PLFA 59
3.2.1 Method 59
3.2.2 Results 61
3.3 Identification of specific species in golf courses...................................69
3.3.1 Method 69

3.3.2 Results 69
3.4 Discussion 78
3.4.1 PLFA 78
3.4.2 Sequencing 81
3.5 Conclusions 84
Chapter 4: What current practises change existing microbial populations? 85
4.1 Introduction 86
4.1.1 Summary of hypotheses..89
4.2 Pesticide and Growth Regulator Field Trials..90
4.2.1 Methods 90
4.2.2 Results 95
4.2.3 Discussion..99
4.3 Biostimulant Field Trials..102
4.3.1 Methods 102
4.3.2 Results 104
4.3.3 Discussion..107
4.4 Garlic Products 108
4.4.1 Methods 108
4.4.2 Results 109
4.4.3 Discussion:..111
4.5 General discussion 112
4.6 Conclusions 114
Chapter 5: Factors affecting the application of inoculants to sports turf 115
5.1 Introduction 116
5.1.1 Summary of hypotheses..121
5.2 Methods..121
5.2.1 Grass Species Mycorrhizal Consortium trial..121
5.2.2 Grass Species PGPR trial 122
5.2.3 Mycorrhizal monocultures trial..122
5.2.4 Fertiliser trial..122
5.2.5 Mycorrhiza dose rate trial 123
5.2.6 Germination trial..123
5.2.7 Statistical analysis..125
5.2.8 Calculation of Inequality 126
5.3 Results..128

5.3.1 Grass Species Mycorrhizal Consortium trial 128
5.3.2 Grass Species PGPR trial 132
5.3.3 Mycorrhizal monocultures trial 135
5.3.4 Fertiliser trial 140
5.3.5 Dose rate trial 143
5.3.6 Germination trial 145
5.4 Discussion 148
5.4.1 Grass species mycorrhizal consortium and mycorrhizal monocultures trial 148
5.4.2 Grass PGPR trial 150
5.4.3 Fertiliser trial 151
5.4.4 Dose rate trial 152
5.4.5 Germination trial 153
5.5 Conclusions 155
Chapter 6: Benefits gained from using microbial inoculants in sports turf 156
6.1 Introduction 157
6.1.1 Mycorrhiza and thatch 157
6.1.2 Microbial inoculants and disease 158
6.1.3 Mycorrhiza, waterlogging and drought tolerance 164
6.1.4 Mycorrhiza and *P. annua* 166
6.1.5 Summary of hypotheses 167
6.2 Methods 168
6.2.1 Thatch trial 168
6.2.2 Disease trial 170
6.2.3 Turf Quality and Colour trial 173
6.2.4 Drought trial 173
6.2.5 *Poa Annua* trial 174
6.2.6 Statistical Analysis 174
6.3 Results 175
6.3.1 Thatch trial 175
6.3.2 Disease trial 178
6.3.3 Turf Quality and Colour trial 180
6.3.4 Drought trial 182
6.3.5 *Poa Annua* trial 185
6.4 Discussion 189
6.4.1 Thatch trial 189

6.4.2 Disease trial 190
6.4.3 Turf Quality and Colour trial 192
6.4.4 Drought trial..192
6.4.5 *Poa Annua* trial..194
6.5 Conclusion 195
Chapter 7: General Discussion 196
7.1 Summary of outcomes 197
7.2 Existing microbial populations of golf putting greens 199
7.3 How applicable will microbial inoculants be on golf putting greens....200
7.4 Could microbial inoculants feasibly replace any existing products.....205
7.5 Conclusions 208
Chapter 8: References: 209
Chapter 9: Appendices...249
9.1 FAMES/BAMES List for PLFA 249
9.2 Grass Species Application Rates...252
9.3 Golf Course Management Survey Questions.....................................253
9.4 Golf Course Characteristics 254
9.4.1 PLFA golf course characteristics 254
9.4.2 Sequencing golf course characteristics 255
9.5 Graphs for NDVI, Chlorophyll and Ground hardness of different applications of fungicide and herbicide..257
9.6 The ingredients of the J.Arthur Bower Liquid Lawn food 263
9.7 Remaining graphs for changes in dry biomass of different grass species treated with PGPR...263
9.8 The Gini Coefficients and Lorenz asymmetry coefficients for different grass species treated with PGPR 266
9.9 The Gini Coefficients and Lorenz asymmetry coefficients for different grass species treated with mycorrhizal monocultures 270

Glossary

AMF: Arbuscular mycorrhizal fungi

AUDPC: Area under disease progression curve

AUGPC: Area under germination progression curve

Biostimulant: A product that stimulates the biological elements of the soil

Clegg hammer: A tool used to measure ground hardness by measuring the impact force of a dropped weight from a specific height

GM: Gravities, the unit of measurement for a Clegg hammer

Inoculum: something containing microbes designed to add them to the soil

Integrated pest management (IPM): a management strategy which utilises both cultural and chemical practises to reduce economic loss.

K: Potassium

N: Nitrogen

NDVI: Normalised difference vegetation index

P: Phosphorous

PGPR: Plant growth promoting rhizobacteria

Plant Protective Products: products designed to protect plants during growth and their products post harvesting

Rhizosphere: The area around the roots where root exudates permeate, including the root itself, root surface and surrounding soil

RLC: Root length colonisation

Sward: An area of turf, often with mixed grass species

Thatch: organic matter build-up on the surface of the soil from grass clippings

OUT: Operational Taxonomic Unit

List of Figures

Figure 1: The structure of a golf putting green, credit to the STRI.

Figure 2: A granular mycorrhizal inoculum, used throughout this thesis.

Figure 3: The change in number of results for "microbes and turf" on the TGIF database for turfgrass resources from 1950-2018.

Figure 4: Golf course sampling locations around the UK.

Figure 5: A 2.5cm diameter soil core and the sandwich bag it was stored in.

Figure 6: PCA plot of microbial biomass in all soil core replicates for all 18 greens across the 19 golf courses sampled.

Figure 7: PCA ordination plot of microbial PLFAs across 19 golf courses, grouped by golf course (A-S).

Figure 8: PCA ordination plot of microbial PLFAs across 19 golf courses, grouped by whether greens are sand-based, or soil-based

Figure 9: PCA ordination plot of microbial PLFAs across 19 golf courses, grouped by whether they are from the North, South or Midlands.

Figure 10: PCA ordination plot of microbial PLFAs across 19 golf courses, grouped by whether they are coastal or non-coastal.

Figure 11: PCA ordination plot of microbial PLFAs across 19 golf courses, grouped by course age.

Figure 12: PCA ordination plot of microbial PLFAs across 9 golf courses, grouped by golf course.

Figure 13: PCA ordination plot of microbial PLFAs across 9 golf courses, grouped by usage of microbial inoculants.

Figure 14: PCA ordination plot of microbial PLFAs across 19 golf courses, grouped by biostimulant usage.

Figure 15: PCA ordination plot of microbial PLFAs across 19 golf courses, grouped by number of active ingredients of fungicide applied each year.

Figure 16: The relative species abundance for different golf course (A-D) greens sequenced for fungal DNA using ITS2.

Figure 17: The phyla of the top ten fungal species discovered across all four golf courses sampled, grouped by green. The letters indicate the anonymised golf course and the numbers represent the green.

Figure 18: Unweighted Unifrac PCoA ordination plot for the fungal communities of 4 golf courses (A-D).

Figure 19: Weighted Unifrac PCoA ordination plot for the fungal communities of 4 golf courses (A-D).

Figure 20: The percentage of different AMF species identified across the four golf courses.

Figure 21: A phylogenetic tree showing sequences identified as unknown AMF compared to known sequences for different AMF species.

Figure 22: The relative species abundance for different golf course (A-D) greens sequenced for bacterial DNA using 16S.

Figure 23: The phyla of the top ten bacterial species identified across all four golf courses sampled, grouped by green. The letters indicate the anonymised golf course and the numbers represent the green.

Figure 24: Unweighted Unifrac PCoA ordination plot of bacteria community for different golf courses (A-D).

Figure 25: Weighted Unifrac PCoA ordination plot of bacteria community for different golf courses (A-D).

Figure 26: The trial site where assessments for fungicides, herbicides and growth regulators took place at STRI, Bingley UK.

Figure 27: The equipment used to assess pesticide and biostimulant trials. A is a chlorophyll meter (FieldScout, Illinois USA) B is a 0.5kg Clegg hammer (Sd Instrumentation, Bath, England), C is an NDVI meter (Trimble Greenseeker, California USA). Credit STRI.

Figure 28: The mean amount of phospholipid fatty acid methyl esters found for bacteria and fungi after treatment with different active ingredients of fungicide. The error bars shown are standard error.

Figure 29: The proportion of phospholipid fatty acid methyl esters found for bacteria, fungi and actinomycetes after treatment with different active ingredients of fungicide.

Figure 30: The mean amount of phospholipid fatty acid methyl esters found for bacteria and fungi after treatment with different active ingredients of herbicide. The error bars shown are standard error.

Figure 31: The proportion of phospholipid fatty acid methyl esters found for bacteria, fungi and actinomycetes after treatment with different active ingredients of herbicide.

Figure 32: The mean amount of phospholipid fatty acid methyl esters found for bacteria and fungi after treatment with different growth regulator products. The error bars shown are standard error.

Figure 33: The proportion of phospholipid fatty acid methyl esters found for bacteria, fungi and actinomycetes after treatment with different active ingredients of growth regulator.

Figure 34: The mean amount of phospholipid fatty acid methyl esters found for bacteria and fungi following treatment with different biostimulants. The error bars shown are standard error.

Figure 35:The proportion of phospholipid fatty acid methyl esters found for bacteria, fungi and actinomycetes after treatment with different biostimulants.

Figure 36: The impact of biostimulant treatments on the mean NDVI reading of trial plots. (L) represents liquid and (S) represents solid. The error bars shown are standard error.

Figure 37: The impact of biostimulant treatments on the mean ground hardness of trial plots. (L) represents liquid and (S) represents solid. The error bars shown are standard error.

Figure 38: The impact of biostimulant treatment on the mean chlorophyll content of trial plots. (L) represents liquid and (S) represents solid. The error bars shown are standard error.

Figure 39: The mean percentage root length colonisation for Festuca rubra trichophylla and Poa pratensis upon the addition of commercial garlic treatments and mycorrhizal inoculum. The error bars shown are standard error. Starred bars were significantly different from control.

Figure 40 The mean dry biomass of Poa pratensis and Festuca rubra trichophylla upon the addition of commercial garlic treatments and mycorrhiza inoculums. The error bars shown are standard error. Starred bars were significantly different from control.

Figure 41: Trial layout for germination trial run in the controlled temperature room at Royal Holloway.

Figure 42: Germinating *L. perenne* seeds treated with mycorrhiza.

Figure 43: Taken from Damgaard & Weiner (2000), a diagram of three Lorenz curves, the bold line showing a symmetrical case and the two unbold lines showing asymmetric cases.

Figure 44: The change in dry biomass for different grass species when treated with a mycorrhizal inoculant. The error bars show standard error. Starred bars were significantly different from the control.

Figure 45: The change in percentage root length colonisation for different grass species when inoculated with mycorrhiza. The error bars show standard error. Starred bars were significantly different from the control.

Figure 46: Lorenz curves showing the effect of mycorrhizal inoculum on the inequality of F. r. commutata and A. capillaris.

Figure 47: The changes in dry biomass when PGPR species were added to Lolium perenne (2n). The error bars show standard error. Starred bars were significantly different from the control.

Figure 48: The Lorenz curves showing the effect of Azospirillum spp and Rhizobacterium spp inoculation to L.perenne (2n).

Figure 49:The changes in dry biomass when PGPR species are added to Agrostis capillaris. The error bars show standard error.

Figure 50: The changes in dry biomass when PGPR species are added to Agrostis stolonifera. The error bars show standard error.

Figure 51: Lorenz curves showing the effect of treatment with Rhizobacterium spp and Pseudomonas spp 2 on A. stolonifera.

Figure 52: The change in dry biomass following inoculation with different mycorrhizal monocultures in F. r. rubra, A. capillaris and A. stolonifera. The error bars show standard error. Starred bars were significantly different from the control.

Figure 53: The change in percentage root length colonisation following inoculation with different mycorrhizal monoculture in F. r. rubra, A. capillaris and A. stolonifera. The error bars show standard error. Starred bars were significantly different from the control

Figure 54: The changes in the inequality of dry biomass per pot for different grass species when inoculated with Funneliformis spp A. The table shows Gini coefficients quantifying the difference from the line of equality.

Figure 55: The change in dry biomass in A. stolonifera from treatment with mycorrhizal inoculant and different fertiliser rates. Error bars show standard error.

Figure 56: The change in dry biomass in P. pratensis from treatment with mycorrhizal inoculant and different fertiliser rates. Error bars show standard error. Starred bars showed significant difference from the control.

Figure 57: The change in dry biomass in Festuca rubra trichophylla from treatment with mycorrhizal inoculant and different fertiliser rates. Error bars show standard error. Starred bars show significant difference from the control.

Figure 58: The change in dry biomass in Lolium perenne (2n) from treatment with mycorrhizal inoculant and different fertiliser rates. Error bars show standard error.

Figure 59: The change in % root length colonisation for pots treated with mycorrhizal inoculant and different fertiliser rates. Error bars show standard error. Starred bars show significant difference from the control.

Figure 60: The change in % root length colonisation in F. r. trichophylla for different dose rates of AMF inoculant. The error bars show standard error.

Figure 61: The dry biomass of F. r. trichophylla for different dose rates of mycorrhizal inoculant. The error bars show standard error.

Figure 62: The germination over time for different grass species treated with mycorrhizal inoculant.

Figure 63: The difference in average shoot length (mm) for different grass species following colonisation with a mycorrhizal inoculant. Error bars show standard error.

Figure 64: The difference in root length (mm) for different grass species following colonisation with a mycorrhizal inoculant. Error bars show standard error.

Figure 65: A hole changer used in golf courses to swap holes on a green.

Figure 66: *Microdochium nivale* on the disease trial plot at STRI Bingley.

Figure 67: The average percentage organic matter at different depths of cores taken from a naturally thatch-prone trial plot with different treatments.

Figure 68: The average percentage organic matter at different depths of cores taken from a trial plot not prone to thatch with different treatments.

Figure 69: The rate of organic matter decomposition for trial plots in thatch prone or non-thatch-prone areas with different microbial treatments. Error bars show standard error.

Figure 70: The proportion of the labile fraction remaining (S) shown for trial plots in thatch prone or non-thatch-prone areas with different microbial treatments. Error bars show standard error.

Figure 71: The disease progression curve of Microdochium nivale for different treatment plots over time, 2017/18.

Figure 72: Area under disease progression curve (AUDPC) for different treatments in the 2017/18 disease trial. Error bars show standard error.

Figure 73: The disease progression curve of Microdochium nivale for different treatment plots over time, 2018/2019. Starred points show significant difference from the control for that assessment date.

Figure 74: Area under disease progression curve (AUDPC) for different treatments in the 2018/19 disease trial. Error bars show standard error.

Figure 75: The turf quality of thatch prone and non-thatch prone trial plots treated with different microbial inoculants. Error bars show standard error.

Figure 76: The turf colour of thatch prone and non-thatch prone trial plots treated with different microbial inoculants. Error bars show standard error.

Figure 77: The difference in dry biomass for turf grass treated with mycorrhiza or control under different watering regimes. Error bars show standard error. Starred bars show significant difference from the control.

Figure 78: The difference in percentage root length colonisation for turf grass treated with mycorrhiza or control under different watering regimes. Error bars show standard error. Starred bars were significantly different from the control.

Figure 79: The dry biomass of Poa annua when treated with different monocultures of mycorrhiza. The error bars show standard error. Starred bars were significantly different from the control.

Figure 80: The percentage root length colonisation in P. annua following addition of mycorrhizal inoculant. Error bars show standard error. Starred bars were significantly different from the control.

Figure 81: Dry biomass of Poa annua when treated with different monocultures of PGPR. Error bars show standard error.

Figure 82: The effect of different fungicide active ingredients on the mean NDVI of trial plots. The error bars shown are one standard error.

Figure 83: The effect of different herbicide active ingredients on the mean NDVI of trial plots. The error bars shown are standard error.

Figure 84: The effect of fungicide active ingredients on the mean ground hardness of trial plots. The error bars shown are standard error.

Figure 85: The effect of different herbicide active ingredients on the mean ground hardness of trial plots. The error bars shown are standard error.

Figure 86: The effect of fungicide treatments on the mean chlorophyll content of trial plots. The error bars shown are standard error.

Figure 87: The effect of different herbicide active ingredients on the mean chlorophyll content of trial plots. The error bars shown are *standard error*.

Figure 88: The change in dry biomass when PGPR species were added to S.phoenix. Error bars show standard error

Figure 89: The change in dry biomass when PGPR species were added to F. r. commutata. Error bars show standard error

Figure 90: The change in dry biomass when PGPR species were added to F. r. rubra. Error bars show standard error

Figure 91: The change in dry biomass when PGPR species were added to P. pratensis Error bars show standard error

Figure 92: The change in dry biomass when PGPR species were added to F. r. trichophylla. Error bars show standard error

Figure 93: The change in dry biomass when PGPR species were added to L. perenne (4n). Error bars show standard error

List of Tables

Table 1: The main turfgrass diseases, their season and their causative pathogen, (Charbonneau et al 2015, Entwistle et al 2005, Mann 2015).

Table 2: The different categories of biostimulants, and some example ingredients for these. Table adapted from Yahkin et al 2016

Table 2 : Modified from Reuss & Chamberlain (2010). PLFAs which could be assigned to more than one organism were excluded to avoid misidentification.

Table 3: The primers used for amplicon sequencing to profile the bacterial and fungal communities.

Table 4: The different styles of management for golf courses, hybrid management styles consist of a mix of the organic and conventional styles (Allan-Perkins & Jung 2015, Rixon 2005).

Table 5: The application rates and schedule for the pesticide and growth regulator trial at STRI 2016/17.

Table 6: The application rate, calendar and method of application for the biostimulant trial at STRI in 2016.

Table 7: The amount of inoculum applied for each rate in the dose rate trial.

Table 8: The Gini Coefficient with 95% Confidence Interval and Lorenz asymmetry coefficient (LAC) for different grass species when treated with a consortium of AMF. Bold results represent treatments significantly different from their control.

Table 9: GLM outputs for the change in percentage root length colonisation for different grass species upon the addition of different mycorrhizal monocultures.

Table 10: The final germination percentage for different grass species when treated with mycorrhizal inoculant.

Table 11: A summary of the suppression effects shown with microbes to diseases.

Table 12: A summary of the suppression effects shown with AMF and diseases.

Table 13: The application rates for different treatments in the disease trial.

Table 14: The watering regimes used for the drought trial.

Table 15: The statistical results for turf quality and turf colour assessments following microbial treatments of thatch prone and non-thatch-prone trial plots.

Table 16: The statistical results for change in percentage root length colonisation following addition of mycorrhizal inoculants in different watering regimes.

Table 17: The Gini Coefficient with 95% Confidence Interval and Lorenz asymmetry coefficient (LAC) for P. annua when treated with different AMF. Bold results show significant differences from control.

Table 18: The Gini Coefficient with 95% Confidence Interval and Lorenz asymmetry coefficient (LAC) for P. annua when treated with different PGPR. Bold results show significant differences from control.

Table 19: A summary of the objectives and outcomes of this thesis.

Table 20: showing the different brands of grass species used and their respective sowing rates.

Table 21: The characteristics of different golf courses included in the PLFA trial

Table 22:The characteristics of different golf courses included in the sequencing trial

Table 22: The ingredients of the J. Arthur Bower liquid lawn food used for the fertiliser trial.

Table 23: The Gini coefficients (with 95% confidence intervals) and Lorenz asymmetry coefficients for different grass species treated with PGPR. Bold results represent treatments significantly different from their control.

Table 24: The Gini coefficients (with 95% confidence intervals) and Lorenz asymmetry coefficients (LAC) for different grass species treated with monocultures of AMF. Bold results represent treatments significantly different from their control.

Chapter 1: Introduction

Producing and maintaining high quality sports turf is a large industry across the world with varying needs. Different sports have specific requirements and countries have different preferences in colour and grass type, as well as different environmental conditions to face. The standard specifications for a modern (within the past 40 years) golf green in the USA follow the USGA (United States Golf Association (USGA 2016)) specifications and are largely made up of USGA grade sand (see section 1.1). In the UK, USGA specification golf greens are not as common. However, sand-based rootzones are seen frequently, especially in more recently built golf courses. While different rootzones are available, the key difference comes down to the amount of sand and soil. There is also a large variation in the grass types used, with different swards and variations of grass species commercially available to suit the different requirements of sports and local conditions

Public greenspace has been proven to provide a total economic value of £30.24 per individual per year, a wellbeing value of £34.2billion per year, and could save the NHS £111 million per year based purely on reduction in GP visits (Fields in Trust 2018). Golf courses are an important source of green space, especially in urban areas where open land is such a high value commodity. Greenspace was found to reduce the mortality effects of heatwaves and air pollution, and to encourage exercise which in turn reduced mortality (Taylor & Hochuli 2015). Greenspace can also benefit mental health and reduce stress, whether the greenspace is actively used by the individuals or not (Taylor & Hochuli 2015).

Golf is an integral part of the economy with 3.883 million adult golfers, 1.554 million of which are counted as regular golfers who participate once every 4 weeks or more. The total economic activity associated with golf in the UK in 2016 was £20.291 billion considering direct, indirect and induced effects. The industry employs 74,480 people, 54,190 of which are full time making up 0.21-0.24% of national employment (Sport Industry Research Centre 2016). With such a large economic and social impact, the golf industry has the potential to make huge changes to public perception and spending if greenkeepers strive to develop further management techniques providing ever more environmental benefits. Similarly, the use of golf and other sporting industries to educate players and spectators on how to be more environmentally friendly shows promise and is something many organisations like BASIS (British Association for Sustainability in Sport) are trying to implement. Current practises do have cultural remedies to diseases but there is still a focus on pesticide usage, which can have unintended effects on the environment. Knowing this, there is huge potential for management techniques such as microbial management, if they can be perfected.

1.1 Building a golf green and the considerations involved

In the USA, golf putting greens are designed under USGA specifications. In the UK the availability of USGA grade sands is low, therefore other sand mixes with peat are often used. Figure 1 shows the basic profile of a sand-based putting green in the UK, consisting of a granular layer for drainage, a rootzone layer made up of 80:20 sand to soil mix and a binding layer to separate the two. Older courses in the UK will often be ‘push-up’ greens constructed with local

soil and so will have different properties to modern golf courses (Bary et al 2005). However, some greens may have been renovated and so there could be variation within a course. For example, Pyecombe golf course which has 17 push-up greens and one USGA green because the members wanted to see what it was like to play on (A. Gange personal communication 24/7/19).

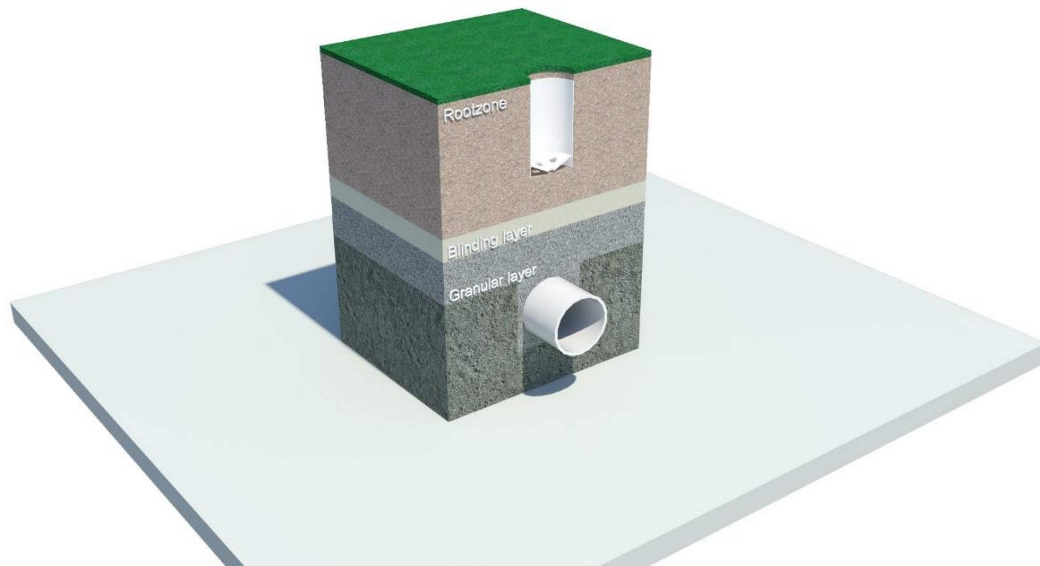


Figure 1: The structure of a golf putting green, credit to the STRI.

Sand-based greens provide drainage and a firm ground for golf, preventing common issues such as compaction and waterlogging. However, there are some problems that are still prevalent in sand-based greens. Sand rootzones require higher fertilisation due to the lack of nutrients present and the rapid use of those available in the peat mixes. While soil rootzones are more nutrient friendly, clay soils readily hold water making them prone to swelling and shrinking depending on the weather conditions, which can lead to the surface cracking (Stewart 1994). There is also a difference in the levels of cadmium, arsenic and lead, with higher levels of all three found in soil-based greens (Bary et al 2005). However, this difference was only just above the average levels for

the UK and may be due to the use of lead arsenate and cadmium chloride as pesticides up until the 1960s. Levels of copper were the same for sand or soil-based putting greens (Bary et al 2005).

There have been conflicting reports on whether sand-based or USGA specification greens can support a substantial microbial population (Hagley 2002, Zhang et al 2015). This is of interest as the golf industry grows in places like China, with 300 new golf courses built since 2003 to the USGA specifications (Zhang et al 2015). Although evidence suggests that soil microbial levels in USGA greens may reach the same as soil-based greens within 2 years, the lack of microbes before this can leave the course open to disease as it may weaken the turf's ability to overcome pathogens (Zhang et al 2015).

Turf grasses are normally sown as a mix of different grasses, for instance golf putting greens tend to favour a mix of fescue (*Festuca spp.)* and bentgrass (*Agrostis spp.).*The species of grass grown are highly dependent on the intended usage, maintenance standard available, climate and environmental conditions of the course or pitch itself, as well as the desired physical appearance (Charbonneau et al 2015).

1.2 Turfgrass diseases and current treatments

Turf grass diseases vary depending on the types of grass present in the sward and the time of year. The most prevalent turf diseases are shown in Table 1. The main consistent symptom of these diseases is that they cause

discolouration and ultimately dead patches of grass. These patches affect play by creating an uneven surface as well as not being aesthetically pleasing. Management strategies depend on the disease in question but can involve interrupting the disease cycle, often through competition for nutrients with another organism, moving to reduce leaf growth, minimising thatch or using resistant species (Charbonneau et al 2015). The most common turfgrass disease in the UK is Microdochium patch (TGA 2015a).

Turfgrass disease	Causative fungi	Season
Microdochium patch (AKA fusarium patch)	*Microdochium nivale*	Autumn/winter
Red thread	*Laetisaria fuciformis*	Spring/Autumn
Take-all patch	*Gaeumannomyces graminis*	Spring/Autumn
Anthracnose rot	*Colletotrichum cereale*	Autumn/winter
Anthracnose blight	*Colletotrichum cereale*	Summer
Yellow tuft	*Sclerophthora macrospora*	All year
Brown patch	*Rhizoctonia solani*	Summer
Fairy rings	*Marasmius oreades* and other mushrooms	All year
Superficial fairy rings	*Trechispora spp* and others	Autumn/winter
Dollar spot	*Sclerotinia homeocarpa*	Summer

Table 1: The main turfgrass diseases, their season and their causative pathogen, (Charbonneau et al 2015, Entwistle et al 2005, Mann 2015).

Microdochium patch, also known as fusarium patch, is caused by the pathogen *Microdochium nivale,* formally known as *Fusarium nivale.* Microdochium patch is observed as matting in the grass, water-soaked patches, orange or brown

rings with a pale dead centre and mycelium growth is observed around the edge of the patch (PennState College of Agricultural Sciences 2015, Mann 2015). The fungus lives as spores and mycelium in the thatch or soil during the summer months though is often not present unless there are extended wet periods, otherwise the optimal conditions are cool and wet (Charbonneau et al 2015). Current cultural management techniques include ensuring adequate drainage, controlling thatch and controlling the use of fertiliser and nitrogen. The invasive weed grass *Poa annua* is more susceptible to *M. nivale* and so encouragement of finer grasses can help to reduce disease incidence (Mann 2015).

1.3 Microbial Inoculants

Microbial inoculants are products applied containing live microorganisms in order to influence the soil ecology. An example of a mycorrhizal inoculum is shown in Figure 2. The main microbes included in commercial inoculants are arbuscular mycorrhizal fungi (AMF), or Plant Growth Promoting Rhizobacteria (PGPR). The types of species in these inoculums is very dependent on the brand and the intended use, but common species include *Bacillus spp, Pseudomonos spp, Rhizophagus spp* and *Glomus spp*.

Despite the regulation plant protective products undergo, the general public are still wary of pesticides and the harmful impacts they can have on wildlife and human health when used incorrectly. Through this there is a greater desire to use organic methods to manage golf courses, and microbial inoculants are an

excellent way to do this and still reap some of the benefits current chemicals provide.

Figure 2: A granular mycorrhizal inoculum, used throughout this thesis.

1.3.1 Mycorrhiza

Mycorrhiza are fungi that form symbiotic, and mostly beneficial, relationships with plants. There are six major types of mycorrhiza, Monotropoid, Arbutoid, Ecto, Orchid, Ericoid and Arbuscular. Monotropoid is identified by its fungal pegs, while an Arbutoid mycorrhiza is identified by its intracellular penetration of the root cells (Read 2002). Ectomycorrhizal fungi are characterised by a sheath of fungal tissue that encloses the root, an inward growth of hyphae between epidermal and cortical cells, and an outward growing of hyphal elements (Smith & Read 2008). Orchid mycorrhizas have pelotons, which are coiled hyphae that

form inside the orchid root cells, and Ericoid mycorrhiza have hyphal complexes in the root hairs (Read 2002). Arbuscular mycorrhizal fungi (AMF), which are the most common type of mycorrhiza, form arbuscules in the plant root cortical cells and some mycothalli (Smith & Read 2008). The focus of this section will be on AMF as they associate with more than 80% of plant species; including turf grass species, despite only having around 120 species capable of forming such relationships (Jeffries et al 2003).

Some form of mycorrhiza is thought to have been around for over 1000 million years, and AMF specifically are assumed to be similarly ancient. It is thought that mycorrhiza aided in the colonisation of land by plants due to their role in added nutrient uptake, and some fossil evidence supports this theory (Brundrett 2002). There are three key constituents of an arbuscular mycorrhiza: the root, fungal structures and an extraradical mycelium. While AMF are typically identified by their formation of arbuscules in the plant roots, they have several structures that are recognised, including intracellular hyphal coils which can occur when the arbuscules are absent (Smith & Read 2008). The structure of the fungi is highly dependent on the plant species it is colonising. AMF are entirely dependent on the plant for their source of carbon in exchange for the uptake of minerals (mostly phosphate and nitrogen), meaning while the plant can function without the fungi; the fungi cannot survive without the plant. Therefore, when the carbon source stops the fungi will undergo stress and thus sporulate to ensure its survival (Smith & Read 2008). The plant will lose around 20% of their photosynthetic carbon through root exudates to the fungi, which is then used in respiration or stored in the external mycelium (Jakobsen &

Rosendahl 1990). This means the plant must have a real need of the mycorrhiza for phosphate or N uptake to make the relationship worthwhile. Mycorrhizal colonisation is less likely to occur in nutrient rich soils where the plant can easily access nutrients on its own although they will still lose carbon through root exudates (Jakobsen et al 2002).

AMF improve phosphate mobilisation in the soil. Inorganic phosphate is available to plants for uptake through the labile pool whereas organic phosphate is often not. Phosphate mobility mediation by AMF is linked to the hydrolysis of organic phosphate to inorganic P. This takes place either by relations of the fungi to P-solubilising organisms, secretion of H+ by hyphae thus changing soil pH and chelating inorganic phosphate ions, or by phosphatases that are produced either by the fungi themselves or by stimulating production in the root surface. The flow of phosphate in the soil must match the rate of uptake by the roots; otherwise a depletion zone may form around the rootzone. AMF hyphae provide a larger network to absorb phosphate as they take less carbon to produce than roots, on top of this they can absorb phosphorus from up to 7cm away, giving a far greater area for phosphate absorption on top of the greater size of the hyphal network. Similarly, hyphae are considerably smaller diameter than roots meaning their depletion zone will be less and they can penetrate soils not accessible to roots. This phosphate is then rapidly translocated through the fungal mycelium to the plant in order to exchange it for carbon (Smith & Read 2008).

Phosphorus is not the only nutrient increase associated with mycorrhizal symbiosis. AMF of the Glomales order were shown to acquire nitrogen from organic material and increase the rate of decomposition, thus making more nitrogen accessible. Inoculated plants showed no change in dry biomass but had three times as much nitrogen compared to uninoculated, as well as a change in the amino acid composition in the xylem (Hodge et al 2001, Matsumura et al 2013). A study of AMF colonisation of Chickpea found elevated manganese, potassium, copper and iron uptake also (Farzaneh et al 2011).

AMF can aid water uptake in plants independently of nutrient uptake through extension of the root absorptive-surface area using extraradical hyphae.
AMF have also been shown to aid drought resistance through the mechanism of drought avoidance, whereby the plant experiences less of the effects of drought such as necrosis and wilting and will wilt at a lower soil moisture content than un-colonised plants (Augé 2001). Drought tolerance would be very useful in the sports turf industry and others, as changing climate and increased water pressure on regions mean periods of drought are more likely.

Mycorrhiza aid soil structure and integrity. The networks of fungal hyphae and deposition of glycoproteins, hydrophobins and polysaccharides on surrounding soil particles by either the fungi or the roots aid the formation of microaggregates and macroaggregates (Rillig & Mummey 2006), crucial components of soil structure. This structural advantage is particularly useful in disturbed soils (Jeffries et al 2003) and so may be useful in agriculture or sports

turf where soil structure is often disturbed by management practices and foot traffic.

1.3.2 PGPR

Plant growth-promoting rhizobacteria (PGPR) encompass many different species of bacteria and form symbiotic relationships with plants through their roots in the rhizosphere. PGPR can be intracellular in specific root nodules or extracellular, living in the rhizosphere or between cells of the root cortex (Gouda et al 2018). While soil living bacteria can cause positive, neutral, or deleterious effects, PGPR tends to refer to those causing beneficial effects on plant growth (Beneduzi et al 2012). PGPR have been used in agriculture since ancient times where Greeks and Romans utilised legume crops to fertilise the soil, and so inadvertently PGPR (Bhattacharyya & Kha 2012).

PGPR have been shown to enhance plant growth through phosphate mobilisation like AMF, but also through nitrogen fixation, and solubilisation of nutrients such as phosphorus, potassium and iron. PGPR can influence growth through the production of phytohormones, especially plant growth regulators such as gibberellins, abscisic acid, cytokinins, ethylene and auxin. They can also improve growth through salinity tolerance, heavy metal detoxification and the biological control of phytopathogens and insects (Gouda et al 2018). Maize, wheat, soybean and sugar beet all showed increases in yield upon inoculation with PGPR, though the extent of increased yield depended on the host plant and the species of PGPR used (Gholami et al 2009).

While evidence for microbial inoculants containing AMF can be quite consistent, PGPR can be a lot more unpredictable. It is consistently found that while PGPR can be effective in controlled laboratory experiments, they often do not perform well in the field (Cordiki et al 2004, Herrmann and Lasueur 2013, Gadhave et al 2016). This is thought to be due to poor quality products or the application of species which have different life-history characteristics to the field conditions (Schwartz et al 2006, Gadhave et al 2016). This exemplifies the importance of trialling different PGPR species in the highly specific conditions of golf putting greens.

1.3.3 Biostimulants

Biostimulants are products designed to simulate the biological elements of the soil, whether this is the plants themselves or the microbial populations. These are increasingly popular in the sports turf industry as well as agriculture, where sales were estimated to grow to $2billion by 2018 (Brown & Saa 2015). This is especially important as greenskeepers begin to consider soil biology more in their management plans. Biostimulants can be classified in six categories summarised in *Table 2*. Microbial inoculants were separated from biostimulants in this thesis as they are the focus of the research. Biostimulants considered in this thesis include amino acid supplements, humic acids, seaweed products and compost teas.

Biostimulants have a range of reported effects, from increased germination and plant growth, plant quality, yield increases, root formation and vigour, to improved microbial colonisation. They also claim improvements in stress

tolerance of drought, diseases, salinity, iron deficiency, frost, nematodes and heavy metals (Yahkin et al 2016). This wide range of effects is largely due to the wide range of products described as biostimulants, but different types will show different benefits.

Biostimulant category	Sub-categories	Example ingredients
Bacteria	Preparations of living microorganisms Preparations derived from non-living microorganisms and their metabolites	Cytokinins, amino acids, gibberellins, peptides
Fungi	Preparations of living microorganisms Preparations derived from non-living microorganisms and their metabolites	Amino acids, auxin like compounds, minerals, nucleic acids, proteins, carbohydrates, cytokinins
Algae	-	Carbohydrates, lipids, minerals, auxins, sterols
Higher Plants	-	Amino acids, plant hormones, cellulose, elements,
Animal Raw Materials	-	Elements, fat, free amino acids, proteins
Humate-containing Raw Materials	-	Compost, amino acids, cellulose, saccharides, plant hormones, phenols

Table 2: The different categories of biostimulants, and some example ingredients for these. Table adapted from Yahkin et al 2016

One of the current issues surrounding biostimulants is that there are multiple definitions and terms used, which can include biogenic stimulators, phytostimulators, allelopathic preparation and metabolic enhancers (Yakhin et al 2016) and some of the definitions encompass inoculants while others don't. Biostimulants can also be difficult to classify due to the range of reported effects, meaning some biostimulants are often licensed as biofertilisers rather than plant protection products to reduce time and resources required for registration despite offering plant protection effects (Woo & Pepe 2018). In fact the EU regulatory definition for plant biostimulants defines "products stimulating plant nutrition processes independently of the products nutrient content, with the aim of improving one or more of the following characteristics of the plant: nutrient use efficiency, tolerance to abiotic stress, crop quality traits or availability of confined nutrients in the soil and rhizosphere" (Ricci et al 2019), which would include a wide range of different products but still exclude some of the other potential effects biostimulants are known for. This can make it quite difficult to regulate biostimulants for the effects they are colloquially sold known and lead to misunderstandings from consumers who don't realise the wide range of products that can be classed as biostimulants.

Currently most research on biostimulants is focused on agriculture, which has different management styles as well as desired outcomes to sports turf. Therefore, more research of biostimulant uses in sports turf is integral to ensure these products are effective in this environment.

1.4 Current standings

Currently the industry is reliant on integrated pest management programs, which include the use of plant protection products, particularly regarding disease control. Golf is a huge industry, accounting for 10.1% of all pesticides sprayed in the amenity sector in the UK in 2012 (Pesticides Forum 2013). In 2014 the EU updated legislation from 2009 on plant protection products (European Union 2015). This legislation provides a list of approved pesticides and fungicides, removing many older active ingredients from use in member countries. They also now require far stricter labelling, protection of nearby water and integrated management programmes for all usage (European Union 2013). Over the course of this PhD alone, iprodione and propiconazole, two popular fungicidal active ingredients, as well as the popular lumbricide carbendazim, have been removed from the market. And following the recent ban of longstanding herbicide glyphosate in Austria (August 2019), all pesticides are facing greater scrutiny by both the public and governing bodies. There is also uncertainty regarding what could happen to legislation following Brexit.

The use of plant protective products can enhance certain diseases by reducing biodiversity. The reduction of competition allows resistant strains of fungal diseases or resistant pests to outcompete other more susceptible strains and can cause more severe infections or infestations (van Elsas et al 2012). Pesticides can leach through the soil to water supplies if not applied correctly causing damage to marine life (Invertebrates: Bunzel et al 2015, Van Dievel et al 2019, Fish: Pérez-Parada et al 2018, Amphibians: Baker et al 2013), though this tends to be less in sports turf due to the thatch layer reducing leaching

(Fresenburg 2015). Bad spraying practises can also cause significant negative effects on pollinators (Sponsler et al 2019, Siviter et al 2018). There can also be effects on the endocrine system of animals even at small doses and a long time after exposure, the severity of the effects depends on the developmental stage of the organism in question (Vincelli & Munshaw 2014). On a microbiological level, pesticides can have detrimental effects on microbial diversity and the ecosystems services they provide (such as litter degradation, nutrient cycling, plant growth promotion and degradation of pollutants), regardless of whether the pesticide is aimed at microbes or not (Jacobsen & Hjelmsø 2014).

Public perception is also growing increasingly concerned about pesticide usage and the potential impacts on human health and the environment. Based on these findings it is no surprise that greenkeepers are worried about finding alternative methods to control diseases, but this also leaves the golf industry looking for viable alternatives. Integrated pest management (IPM) is a management strategy which utilises both cultural and chemical practises to reduce economic loss. IPM is already popular within the sports turf industry but some of the biological controls can lack the research needed to make their inclusion in IPM schemes beneficial.

There is a change occurring in the attitudes of the turf grass community. Pesticide usage in the amenity sector in 2012 had already fallen by 40% from 2006 (Pesticides Forum 2013). Results for “microbes” and “turf” on the Turf Grass Information File Database (TGIF) have increased rapidly in the last 30 years showing the increase in interest for both magazine articles and peer-

reviewed papers looking at microbes in turf (shown in Figure 3). There was a peak in the results in the late 1990s (potentially following a previous PhD thesis on this subject (Hagley 2002)) but then a slow decline in the last 20 years. This highlights the desperate need for more research on this topic, especially bringing it up to date with more modern techniques now that a lot more is known about soil ecology in other sectors. With the increasing interest in how microbial inoculants could help reduce pesticide usage, and the increasing legislative pressure upon the sports turf industry, there is a real potential to influence management practises and encourage a lower pesticide, more microbial approach.

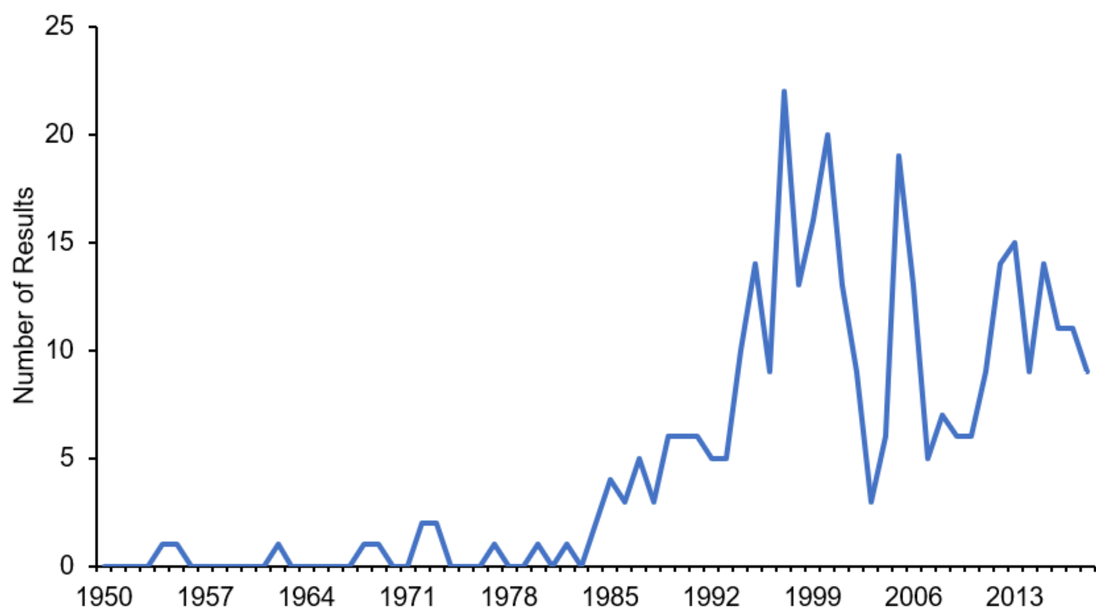


Figure 3: The change in number of results for "microbes and turf" on the TGIF database for turfgrass resources from 1950-2018.

1.5 Overview of research aims and chapters

Most of the current research in soil microbial management is either from the USA or not sports turf specific. Due to differing climates, golf course construction, licensed pesticides, disease pressures and popular grass species, a lot of research from the US is not applicable to the UK. The aims of this thesis were to begin to fill this knowledge gap and to understand how soil microbial management could be applicable specifically in UK golf putting greens. This thesis also wanted to consider any limitations to microbial management in UK golf putting greens as well as the potential benefits it could bring. The main methods utilised in this are outlined in chapter two.

Prior to manipulating the microbes in golf putting greens, chapter 3 aims to establish what microbes are already present in a range of golf courses around the UK. Based upon current research the trial tried to incorporate changes in location, management style, age and soil type. The aim of this research is to provide insight into how consistent the microbial community is between golf courses. Chapter four then specifically trialled different pesticides, biostimulants and a garlic product to determine the impacts these can have upon the indigenous microbes. The aim of this is to then be able to advise greenkeepers of which practises should be avoided following treatments with microbes, or to at least make them aware of limitations.

The factors affecting the application of inoculants to sports turf were assessed in chapter five to work out the most effective ways they can be applied. This included trialling both AMF and PGPR monoculture inoculants against different

grass species to check for colonisation and repeating this with a consortium of AMF. The impact of fertiliser on AMF colonisation was assessed to show how applicable inoculants would be alongside the high levels of fertilisation on a golf putting green. The ideal dose rate for the AMF inoculum was determined, and any impacts on germination were trialled to consider percentage gemination success, time to germination, root and shoot length.

Finally, the benefits that could be gained using microbial inoculants were determined and applied to the golf putting green system. This included any changes in organic matter and nutrient cycling which is integral to ensuring the playability of turf. The primary concern among greenkeepers is the current loss of pesticides, and so disease tolerance was trialled for the most common UK golf turf disease, microdochium patch (*Microdochium nivale).* Another important problem in sports turf is control of the pest grass *Poa annua*, and so previous results showing AMF reduce *P.* annua were replicated using different microbes. Drought tolerance affects were considered to help mitigate growing water stress in all sectors, and more general turf quality and colour effects were monitored.

It was hypothesised that microbial inoculants can provide benefits to sports turf and reduce the inputs required when included in an integrated pest management program.

Chapter 2: General Methods

2.1 Phospholipid Fatty Acid Analysis (PLFA)

2.1.1 Introduction

Phospholipids are a component of the membranes of cells, including those of different microbes within the soil. PLFA isolates these phospholipids and identifies them, to allow the identification of different microbes from their membrane phospholipids. Phospholipids which are between C14-C20 are most commonly associated with bacteria and fungi (Zelles 1999). However, phospholipids can differ depending on carbon chain length, presence and placement of double bonds, iso/anteiso and cis/trans isomerism. These specific phospholipids can then be associated with fungi, bacteria or, actinomyces (see Table 3). Archaea cannot be quantified with this method as they do not contain PLFAs (Quideau et al 2016). PLFA has been used since the 1980s to profile soil communities in a variety of environmental conditions (Frostegård et al 1993, Frostegård & Bååth 1996, Yong et al 2011, Reuss & Chamberlain 2010).

Despite PLFA being an incredibly important technique to profile soil microbial communities, it does have limitations. There are many papers using PLFA to identify microbial stress (Guckert et al (1986), White et al (1996), Kharlamenko et al (2009)) and response to environmental changes (Eiland et al (2001), Schoug et al (2008)) but these must be treated with caution (Frostegård et al 2011). Similarly, there is a lot of contradiction for which phospholipids are indicators of bacteria or fungi, with some finding the same lipids present in both, meaning a lot of these must be excluded from analysis. In mixed microbe environments such as soil, while PLFA cannot be used to identify specific

microbes present, it can be used to provide a fingerprint of the community as a whole, and the biomass of microbes present (Frostegård et al 2011).

2.1.2 Method:

Soil cores of 2.5cm diameter were taken from different golf course greens and stored in plastic bags at -20°C until analysis, 1g of each core was used for PLFA analysis taken from the top 2-3cm of the core. For all samples dry soil weight was calculated from wet weight using percentage water content. All samples were processed in batches of 32 including one control with no soil. All solvents used were HPLC grade. All glassware used was soaked in Decon and rinsed in water before being fired in a muffle furnace at 400°C for a minimum of 5 hours and rinsed with hexane. All pipette tips (both plastic and glass) were autoclaved at 121°C for 20 minutes. The following method was as described by Frostegård et al (1993) except for the extraction which was adapted from Buyer & Sasser (2012) and Gómez-Brandón et al (2009).

2.1.2.1 Lipid Extraction:

A sample of 1g of soil from each core was placed in a glass centrifuge tube with a PTFE lined cap and 7ml Bligh and Dyer extractant (ChCl3:MeOh:Citrate buffer, ratio 1:2:0.8) added. The sample was vortexed and then agitated for 2hours before centrifuging at 1500rpm for 2 minutes. Supernatant was pipetted off to a clean glass centrifuge tube using a glass disposable pipette, before 2.3ml of chloroform and 2.3ml citric acid buffer (0.15M, 7.88g citric acid powder in 250ml ultra-pure water adjusted to pH 4(+/-0,02) with NaOH (2M)) were

added. Samples were centrifuged at 1500rpm for 2 minutes and the lower organic phase transferred using a glass disposable pipette to a new clean centrifuge tube and evaporated off under nitrogen.

2.1.2.2 Lipid Fractionation:

SPE columns (Strata Phenomenex, Torrance CA, USA), 500mg, reservoir volume 6ml) were washed with 5ml chloroform into a waste tube. Dry lipid material was taken up in 600µl of chloroform before being transferred to columns in fractions of 200µl, mixing thoroughly to ensure all lipid material was taken up. Chloroform was passed slowly through the column releasing neutral fatty acids. The column was washed with 6ml of chloroform and 6ml of acetone into the waste tube, which was then emptied and replaced with a labelled centrifuge tube. The column was washed slowly with 2x4ml of methanol and the eluate collected and evaporated off under nitrogen

2.1.2.3 Alkaline Methanolysis

Each sample had 200µl of internal standard (C19:0, 6.26mg/250ml MeOH, kept in the fridge), then 1ml methanol/toluene (ratio 1:1) and 1ml 0.2M potassium hydroxide (1.8g/250ml methanol, made fresh daily) added. Samples were vortexed for 10 seconds and incubated at 37 °C for 15minutes. Samples were left to cool before 2ml hexane/chloroform mixture (ratio 4:1), 0.3ml acetic acid (1M, 5.7ml glacial acetic acid diluted to 100ml) and 2ml ultra-pure water were added. Samples were vortexed for 1minute, then centrifuged at 1500rpm for 2minutes. The upper organic phase was transferred to small glass centrifuge

tubes using disposable glass pipettes and the aqueous phase extracted by adding 2ml hexane/chloroform, vortexing and spinning as previously. Both supernatant fractions were combined and evaporated off under nitrogen. Residue was suspended with 3x100µl hexane chloroform (ratio 4:1) and draw up and down the pipette tip to mix before transferring into a gas chromatography vial insert.

2.1.2.4 Gas Chromatography

Samples were loaded in a Perkin Elmer autosampler. Analysis was performed using a Hewlett Packard (HP) 5890 series II GC, equipped with a flame ionisation detector and a DB-5 capillary column (30mm x 0.25mm i.d, film thickness 0.25µm. 1 µL samples were injected in splitless mode (injector temp 260°C, purge delay 1.0 min) on a J&W DB5 or Abel AB-5MS column (30 m x 0.25 mm dia x 0.25 µ film) using helium as the carrier gas at constant flow of 30 cm/s. The column oven was programmed from 100°C (2 min) to 160°C at 20°C /min, then to 160°C at 3.5°C/min, then to 320°C (hold 1.07min, run time 40.0min). The GC-MS interface temperature was 295°C.

In January 2019 the previous GC-MS was replaced and used the following protocol. Samples were loaded in a G4567A Agilent autosampler. Analysis was performed using an Agilent 7820A GC, equipped with a Agilent 5977B MSD mass spectrometer detector with EI mode and an Agilent 190915-433 HP-5ms capillary column (30 m x 250 µm i.d, film thickness 0.25 µm). Samples were injected in spitless pulsed mode.1 µL of sample was injected with 2 pumps. The inlet was kept at 250°C with an inlet pressure of 12.93 psi. The injection pulse

pressure was 25 psi for 30 secs and the purge flow to split vent was set at 100 ml/min at 1 min. Helium was used as the carrier gas at constant flow of 30 cm/s. The column oven was programmed from 100°C (2 min) to 160°C at 20°C /min, then to 270°C at 3.5°C/min, then to 320°C (hold 1.07min, run time 40.0min). The GC-MS interface temperature was 320°C.

2.1.2.5 Analysis

Peaks were isolated using OpenChrom then identified by chromatographic retention time. This was verified with a standard BAMES and FAMES mix of reference PLFAs (Sigma-Aldrich, St Louis USA, see appendix 1 (section 9.1)). Some of these PLFAs were then used to determine bacteria and fungi from the PLFAs identified (see Table 3). PLFAs which could be assigned to more than one organism were excluded to avoid misidentification.Total microbial mass, and µg g^{-1}dry weight of soil was calculated from PLFAs by comparing to the internal standard using the equation below modified from Hedrick et al (2005).

$$\text{PLFA Biomass } (\mu g\ g^{-1}) = \frac{(sum\ A_{FA} / A_{IS}) \times IS \text{ added} \times X}{Y}$$

$sum\ A_{FA}$= sum of all the areas of identified PLFAs, excluding the internal standard

A_{IS}= Area of the internal standard

IS= Concentration of internal standard (µg μl^{-1})

X= volume of internal standard

Y= Mass of dry soil (g)

Saturation	Fatty acid type	Fatty acid	Predominant origin	Reference	Fatty acids used in this Study
Saturated	Straight	C20	Plants	Zelles (1999) Reuss et al (2007)	C22:0, C24:0
	Iso/anteiso methyl-branched	i, a in C14-C18	Bacteria	Zelles (1997, 1999)	C14:01, C15:0i, C15:0ai, C16:1i, C16:0i, C17:0i, C17:0ai
	10-Methyl-branched	10Me in C15-18	Actinomycetes	Brennan (1988)	16:0 (10Me), C17:0 (10Me), C18:0 (10Me)
	Cyclopropyl ring	-	Bacteria	Zelles (1997, 1999)	cy17:0, cy19:0
	Hydroxy Substituted	OH in C10-C18	Bacteria	Wakeham et al (2003), Lee et al (2004)	2OH-C12:0, 3OH-C12:0, 2OH-C14:0, 3OH-C14:0
Monounsaturated	Double bond C7	C16:1ω7	Bacteria	Guckert et al (1991), Zelles (1999)	C16:1ω7
	Double bond C8	C18:1ω8	Bacteria	Ringelberg et al (1989)	C18:1ω8
	Double bond C9	C20:1ω9	Fungi	Sakamoto et al (2004)	C20:1ω9
Polyunsaturated	ω6 family	C18:2ω6	Fungi	Frostegård & Bååth (1996), Zelles (1999)	C18:2ω6
	ω3 family	C18:3ω3,9,12	Fungi	Vestal and White (1989), van der Westhuizen et al (1994)	C18:3ω3,9,12

Table 3 : Modified from Reuss & Chamberlain (2010). PLFAs which could be assigned to more than one organism were excluded to avoid misidentification.

2.2 Mycorrhizal Staining

2.2.1 Introduction

Mycorrhizal staining remains the predominant method of quantifying mycorrhizal colonisation within roots. The Quink method was selected for these trials due to the safety and reliability of the staining, as trypan blue has been identified as a possible carcinogen (Vierheilig et al 1998). Modern techniques can use genetic sequencing to identify the presence of mycorrhiza however this is costly for large numbers of samples and doesn’t distinguish between arbuscules, vesicles and hyphae.

2.2.2 Method:

Harvested roots were rinsed and then stored in 70% ethanol until used. Stored roots were rinsed before staining and were stained using the Parker Quink protocol of Vierheilig et al (1998). The only modifications were skipping the acetic acid step and de-staining the roots in KOH for 30 minutes, changing to fresh KOH after 15minutes. Roots were counted for visible arbuscules, vesicles and hyphae using the McGonigle et al (1990) magnified intersections method. This involves counting the number of arbuscules, vesicles and hyphae present at each intersection for a minimum of 200 intersections per slide. AMF were then reported as percentage root length colonisation based on the number of intersections with AMF against the number of negative without.

2.3 DNA analysis

2.3.1 Introduction

High-throughput sequencing, specifically Illumina, is commonly used to profile the specific species present in soil microbial communities (Robbins et al 2017, He et al 2017a, He et al 2017b, Zhu et al 2018). Illumina was selected from other next generation sequencing techniques as it is currently the most cost-efficient method of sequencing per base pair, allowing greater depth per sample. Illumina gives a good impression of the individual species present within the soil microbial community however as Illumina is a multistep process, there is the potential to create bias at each step leaving it inferior to methods such as qPCR or PLFA for quantification (Aird et al 2011). Despite this, insight into quantity can be gained from the number of reads per sample.

The primers 16S and ITS1 are both hyper-alternating ribosomal regions used to identify bacteria and fungi respectively. Another disadvantage of Illumina is that the accuracy of sequencing decreases with chain length, so primers were selected that were 466 (16s) and 307(ITS1) base pairs to ensure they remain a suitable quality. These primers have also been selected for similar studies in the past to profile rhizosphere communities (Robbins et al 2017). Sequences were compared to the BLAST database though this also presents some limitations. Despite the number of fungal submissions to GenBank increasing rapidly since 2007 (Begerow & Nilsson 2010), identifications are only possible with enough comparable sequences in the database and for certain environments, such as turfgrass and pastureland, a lot of the sequences are currently unidentified.

2.3.2 Method:

2.3.2.1 DNA Extraction

Soil cores were collected from golf course greens and stored in plastic bags. Bulk DNA was extracted from top 2-3cm of the core as soon as possible following soil core collection using the Qiagen DNeasy PowerSoil kit (Qiagen, Venlo, Netherlands). DNA was then checked using a nanodrop 1000 (Thermofisher, Waltham MA, USA) to determine concentration and quality. DNA samples with suitable content and quality were then shipped in eppendorfs on dry ice to Hong Kong for sequencing by NovoGene Technology Co., Ltd.

2.3.2.2 Illumina Sequencing (Performed by Novogene)

Samples were checked again using a Qubit (Thermofisher) to ensure quality had not degraded following shipping. Sequencing was completed for the 16S V3-V4 and ITS1 regions using Illumina HiSeq (Novogene Technology Co., Ltd, Hong Kong). The Primers used are shown in Table 4.

Target Region	Primer	Primer Sequence (5'-3')
Bacterial 16S rDNA V3-4	341F	CCTAYGGGRBGCASCAG
	806R	GGACTACNNGGGTATCTAAT
Fungal ITS1	ITS5-1737F	GGAAGTAAAAGTCGTAACAAGG
	ITS2-2043R	GCTGCGTTCTTCATCGATGC

Table 4: The primers used for amplicon sequencing to profile the bacterial and fungal communities.

2.3.3 Analysis

Illumina sequencing data was cleaned by Novogene Technology Co., Ltd, (Hong Kong) by removing unique barcodes and primers before paired-end reads were merged using FLASH (V1.2.7). Raw tags were filtered for quality using QIIME (V1.7.0 for quality control). OTUs were identified using BLAST (NCBI 2019) based upon a 97% confidence. These OTUs were then plotted as rank abundance and assessed for species evenness, before assessing the relationship between greens using PCoA (figures amended from plots by Novogene Technology Co., Ltd, (Hong Kong) using WGCNA package and ggplot2 package in R 2.15.3). This was done separately for both fungal ITS 2 and bacterial 16S.

For individual AMF species each sequence was checked in BLAST (NCBI 2019) to identify to the closest phylum and then unknown species were plotted in a phylogenetic tree against several known AMF sequences from GenBank (Benson et al 2005). The tree was plotted using MEGA (Kumar et al 2018) following the protocol by Hall (2013).

2.4 General pot set up

2.4.1 Method

Pot trials were conducted in 9cm^2 pots with a 70:30 sand: soil mix rootzone to replicate a golf putting green. Grass seed was applied at the manufacturer recommended rates shown in appendix 2 (section 9.2). Mycorrhizal inoculum (a consortium of 5 species *Glomus spp A, Glomus spp B, Rhizophagus spp,*

Funneliformis spp A, Funneliformis spp B) provided by Plantworks was applied at a dose rate of 128kg/ha or 0.1g per pot. There is currently no recommended rate of application for turfgrass for the inoculant, so this rate was chosen to ensure rapid colonisation. Controls without mycorrhizal treatment had autoclaved inoculum (121°C for 20minutes) added at the same rate. Control treatments also had microbial filtrate added, made by washing mycorrhizal inoculum at the same dose rate as the treated through a 20µm sieve to ensure no spores get through but any PGPR and bacteria that may be present do.

Grass was grown to a height of 10cm initially before being cut with scissors down to 5cm. The grass was then grown to 7cm and cut to 3cm, then grown to 5cm and cut to 10mm where it was maintained at this height. Cutting with scissors occurred every 3 days to simulate mowing on a golf putting green. While regular greens are often mown every day there was not enough growth to collect clippings for dry biomass in this time frame. All clippings were dried and weighed. No fertiliser was added other than what existed in the rootzone, but water was applied as needed to maintain soil moisture, normally every two days.

Trials ran for 4 weeks in a polytunnel before leaves were harvested and dried, and roots were harvested and stored in ethanol. Grass trials were only completed during April-October to coincide with the natural growing season. Roots were stained and counted by the Quink method (2.2.2: Mycorrhizal Staining Method) and leaves were harvested, dried to constant weight and combined with previous clippings to give dry biomass.

Chapter 3: What microbes are already in the soil?

3.1 Introduction

While there has been much research into soil microbial community structure in general, the structure and diversity of microbes in sports turf has large gaps, especially in the UK where the research that was undertaken is older and often used outdated techniques with a low resolution. Global assessments of AMF have found grassland to be a favourable habitat for these fungi with high species diversity (Davison et al 2015). In fact, a study by Öpik et al (2006) found that the average AMF taxa per plant was 8.3 in grasslands whereas sites with an anthropogenic influence such as arable fields had just 5.2 taxa per host plant species (Öpik et al 2006). Golf courses are managed extensively and so could see a reduction in taxa per plant similar to the arable fields.

A study of 42 turf grass locations in Southern Wisconsin showed the presence of four main groups of microorganisms (Lloyd et al 2009). These groups included gram-positive bacteria, gram-negative bacteria, saprophytic fungi and AMF. There was also a range of other bacteria and fungi that could not be grouped so broadly. Turf grass in general was found to be bacterially dominant, particularly in gram-positive bacteria, which is unusual of grasslands, with prairies containing predominantly AMF and gram-negative bacteria (Lloyd et al 2009). Similarly, total microbial biomass assessed through PLFA was found to be two to four times higher in grasslands than in cultivated soils (agricultural systems) and was affected by herbicide application, fertiliser and irrigation (Steenworth et al 2002). This suggests that comparisons to grasslands are not as useful when looking at microbial diversity as they can be for other metrics.

Management style	Organic	Conventional
Pesticides on roughs	None used	Herbicide and fungicide application
Height of grass on roughs	Mostly left to grow but cut down when needed to a longer length	Cut regularly to a shorter height (20-100mm)
Pesticides on fairways	None used	Herbicide and fungicide application
Height of grass on fairways	Cut regularly but to a longer length	Cut short on a regular basis to 6-30mm
Pesticides on greens	None used	Herbicide and Fungicide application
Height of grass on putting greens	Cut short to 2-8mm	Cut short to 2-8mm
Synthetic fertiliser	None used	Some can be used
Organic fertiliser (eg. compost or seaweed)	Only organic fertiliser used	Some can be used
Biocontrols (eg microbes, insects, mineral products or hot water/hand weeding)	Yes	No
Cultural practices	Yes	Yes

Table 5: The different styles of management for golf courses, hybrid management styles consist of a mix of the organic and conventional styles (Allan-Perkins & Jung 2015, Rixon 2005).

Looking more specifically at sports turf, an experiment in the USA found that microbial diversity was high in different nitrogen regimes and potassium treatments, but that bacteria and archaea diversity was consistently higher than fungi (Beirn et al 2015). However, this study did not consider organically managed courses (as shown in Table 5). A different experiment, also in the

USA, compared organic and conventionally managed golf courses, some features of conventional and organic course management can be seen in Table 5 (Allan-Perkins & Jung 2015). Conventionally managed courses showed higher bacterial species diversity and species evenness than organic courses, however fungal abundance was found to be higher in organic courses. Regardless of management technique, alphaproteobacteria were the dominant species across tested courses (Allan et al 2014). The time since establishment of the turf grass has a large influence on microbial populations. An initial increase in microbial population was observed after sward establishment, but then declined after 4months. A plateau followed this at 6 months but an overall increase on original microbial population was observed (Zhang et al 2015).

Different locations on a golf course differ also in their microbial populations. One study in the USA found that fungal abundance and species diversity is lower in putting greens than on roughs and fairways (Allan et al 2014). Further research by Allan et al (2015) showed the highest fungal abundance on the roughs of an organically managed course. The organic course showed greater fungal abundance in putting greens, though fungal diversity saw no difference between organic, hybrid or conventional management styles (Allan et al 2015). Alternatively, bacterial abundance and species diversity was found to be lowest in the organically managed course compared to the hybrid and conventional course. There is currently no published research of this nature on UK golf courses, which are managed very differently in terms of fertiliser and plant protection product input.

Soil type has a distinct effect on the establishment of microbial populations. Zhang et al (2015) compared the microbial populations of sports turf grown on native soil and sand using culture plating. The native soil had a significantly higher population than the sand up to 2 months, after which they had the same levels of microbes. The limitation to the population in the sand was linked to lower nutrient levels shorty after establishment. Despite the eventual balancing out of microbial biomass, the turf grown in native soil was of a higher quality throughout the study, hypothesised to be because it improved the amount of nutrients readily available. However, they did find that the root: shoot ratio was higher in a sand: soil mix than just native soil or pure sand alone (Zhang et al 2015).

Once microbes have been identified from a USGA specification sand-based golf green, they can be split into different functional groups. Wang & Skipper (2004) showed that multiple rhizobacteria were identified using FAME and 16S DNA analysis in sand-based putting greens. These were then broken down into 17% denitrifiers, 47% nitrate-reducers and 36% non-denitrifiers. Of those denitrifiers identified, most were *Bacillus* or *Pseudomonas*. The same study found little difference in the percentage of rhizobacteria species between bermudagrass and bentgrass at 19% and 15% respectively (Wang & Skipper 2004). However, this could be due to the similarity of the grass species used; different plant species are known to be associated with different microbial populations and a sward is unlikely to ever be only one species of turf grass. The specificity of microbes and plants is thought to originate from the different exudates of different plants. These exudates alter soil chemistry and allow different chemicals for the microbes to utilise (Bever et al 2012).

There is currently no published information regarding the effects of soil type, golf course management style or location of the golf course on the abundance or diversity of microbes in UK golf courses. A study in the UK by Gange & Hagley (2004) sampled greens of acidic, alkaline and neutral soils and one USGA course from the south of England, finding that the number of bacteria including *Bacillus* spp was far higher in neutral soils when measured with dilution plating. However, alkaline soils were shown to be most bacteria abundant when measured using PLFA. The common theme between both techniques was that USGA greens had the lowest bacterial abundance (Hagley 2002). Unfortunately, this research used culture plating to establish number of bacteria which is not as accurate as other molecular methods, as not all species are capable of being grown on culture plates.

Colonisation of grass species by mycorrhiza in UK putting greens has been observed previously by Gange (1994). They found that *Agrostis stolonifera* had up to 30% mean root length colonisation but the majority less than 10%, and *Poa annua* had mostly below 6% mean root length colonisation. While the colonisation for *A. stolonifera* is below what would be expected in grassland, it is still present suggesting that some mycorrhizal species can still survive in high fungicide and high fertiliser environments. The species observed were found to be comprised of 63% *Funneliformis mosseae* (formerly known as *Glomus mosseae*), 26% *G. fasciculatum* and 7% could not be identified through spore morphology with any certainty. (Gange 1994).

The objectives for this chapter were to analyse the microbial populations of golf greens in the UK through PLFA and Illumina sequencing. Following this, to

allow comparisons of these populations considering different environmental factors and cultural practises to identify any difference. Based upon current research, it was hypothesised that a low number of specific AMF species are found in golf courses. It was also thought that there is a difference in microbial population between soil types, course location and age, meaning microbial management may need to be tailored to these factors. .

3.1.1 Summary of hypotheses:

- Golf courses have a low number of specific AMF species
- Microbial populations differ between soil types, course location and age, meaning microbial management may need to be tailored to these factors.

3.2 Analysis of golf courses across the UK by PLFA

3.2.1 Method

Twenty-one golf courses around the UK and Ireland were selected for sampling based on management styles and location. Sampling sites are shown in Figure 4 and golf course characteristics summarised in appendix 9.4 . All eighteens greens were sampled by taking three 2.5cm diameter soil cores from three random points on the green. Water content was also measured using a soil moisture probe (Delta T devices, Cambridge UK) at the same points. A survey was sent to each golf course to record recent management or renovation and course history (see appendix 3, section 9.3). Soil cores were stored in plastic sandwich bags and frozen (see Figure 5) until they were processed using PLFA

(see Chapter 2: General Methods, section 2.1). From the sites sampled, 19 were processed for analysis based upon survey responses.

Statistical analysis of PLFA data were completed in R 3.6.0 using PCA through the packages “ggbiplot” and “vegan” (R Core Team 2019). Data was standardised by setting CENTRE and SCALE to TRUE in the prcomp function. PCAs were grouped by golf course, by course age, by location (North, Midlands or South) to examine climactic effects, by soil type (sand-based, or soil-based) and by whether they were coastal or non-coastal. For golf courses that responded to the survey, PCA was also performed and grouped by biostimulant usage, microbial inoculant usage, iron usage and number of fungicide active ingredients used per year. Ellipsoids representing 95% confidence intervals were added to PCA plots for better visualisation using either “vegan” or “ggbiplot” depending on which had been used to plot the PCA.

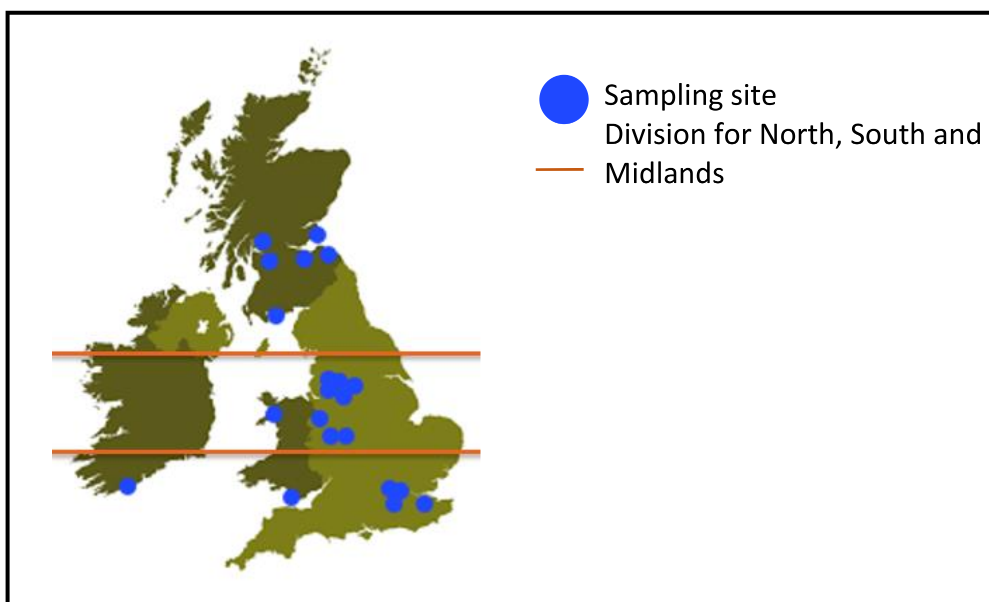


Figure 4: Golf course sampling locations around the UK.

Figure 5: A 2.5cm diameter soil core and the sandwich bag it was stored in.

3.2.2 Results

PCA ordination plots were constructed based upon the bacterial and fungal PLFAs for each sample. PC1 accounted for 82.1% of the variation and PC2 accounted for 16.5% of the variation, as shown in Figure 6.

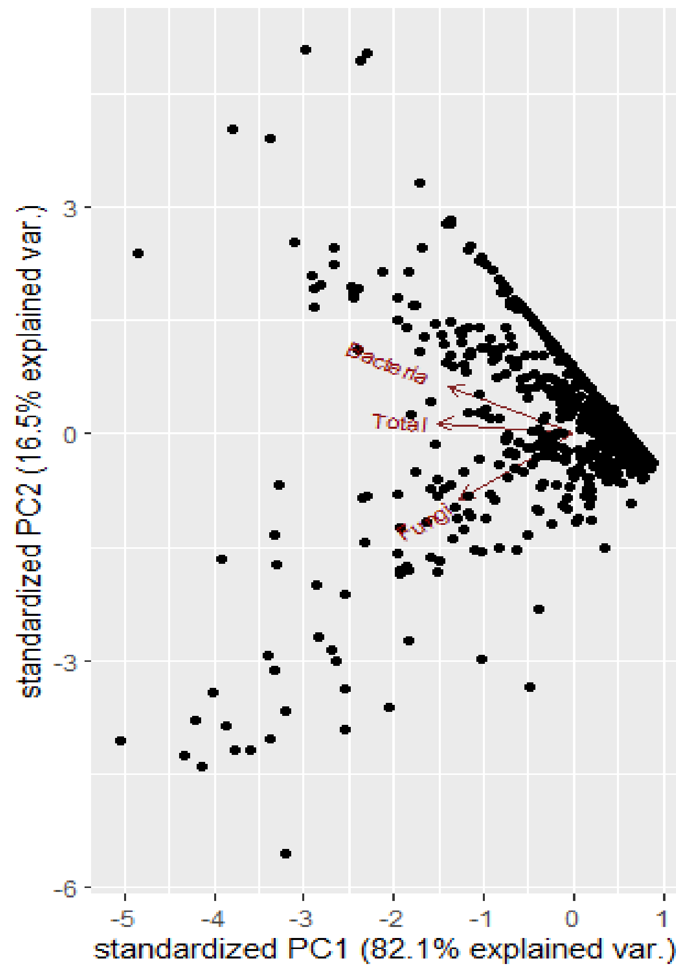


Figure 6: PCA plot of microbial biomass in all soil core replicates for all 18 greens across the 19 golf courses sampled.

PCA determined that different golf courses (anonymised as A-S) have different microbial biomass, but also that the range in microbial biomass between greens varies for different golf courses. This result was especially noticeable for golf course F (shown in Figure 7).

By grouping by soil type in Figure 8, there was overlap between soil-based and sand-based greens. However, there was greater variation in microbial biomass for sand-based greens than soil-based greens.

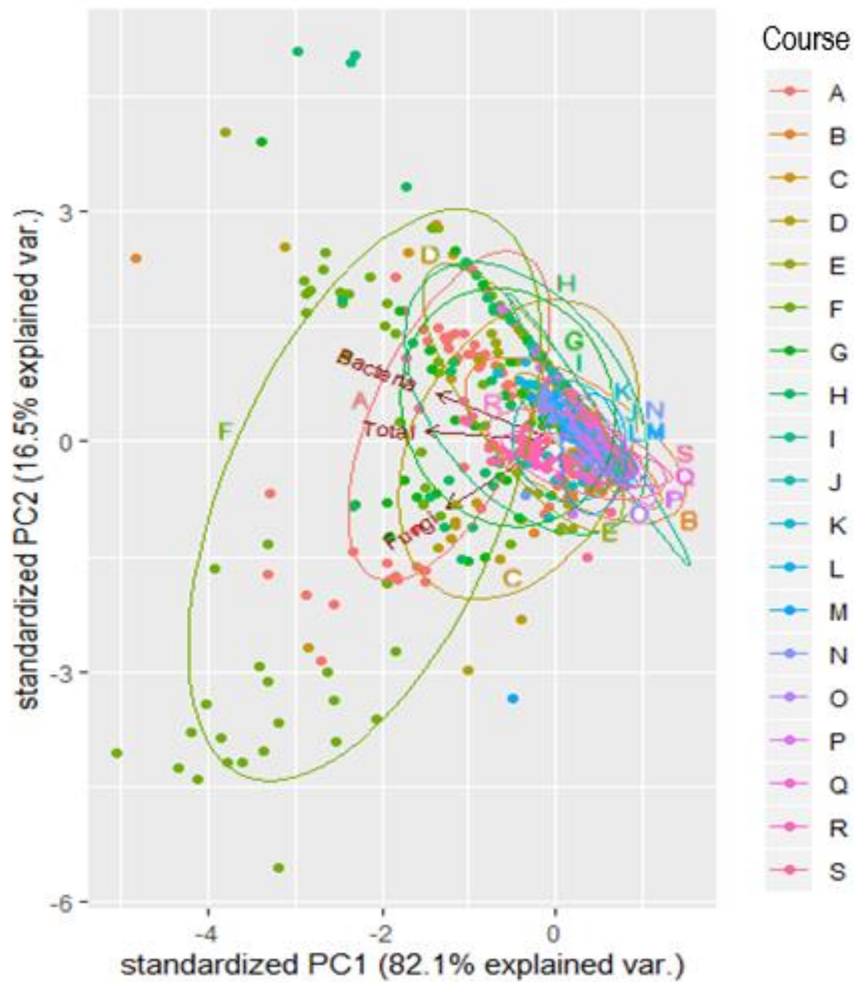


Figure 7: PCA ordination plot of microbial PLFAs across 19 golf courses, grouped by golf course (A-S).

Figure 8: PCA ordination plot of microbial PLFAs across 19 golf courses, grouped by whether greens are sand-based, or soil-based

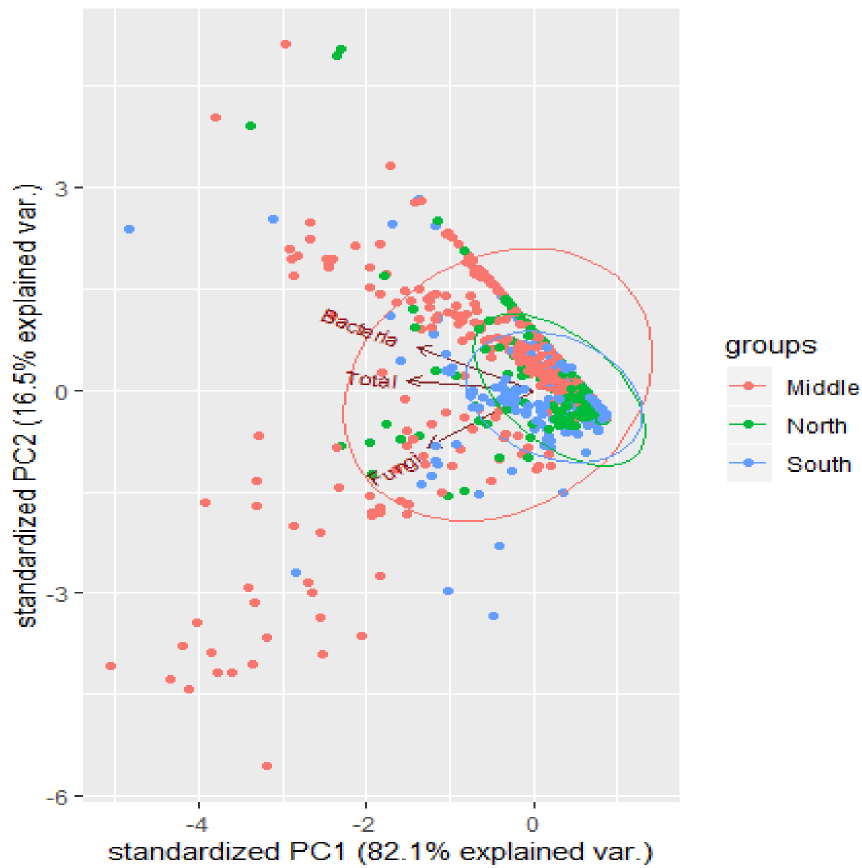


Figure 9: PCA ordination plot of microbial PLFAs across 19 golf courses, grouped by whether they are from the North, South or Midlands.

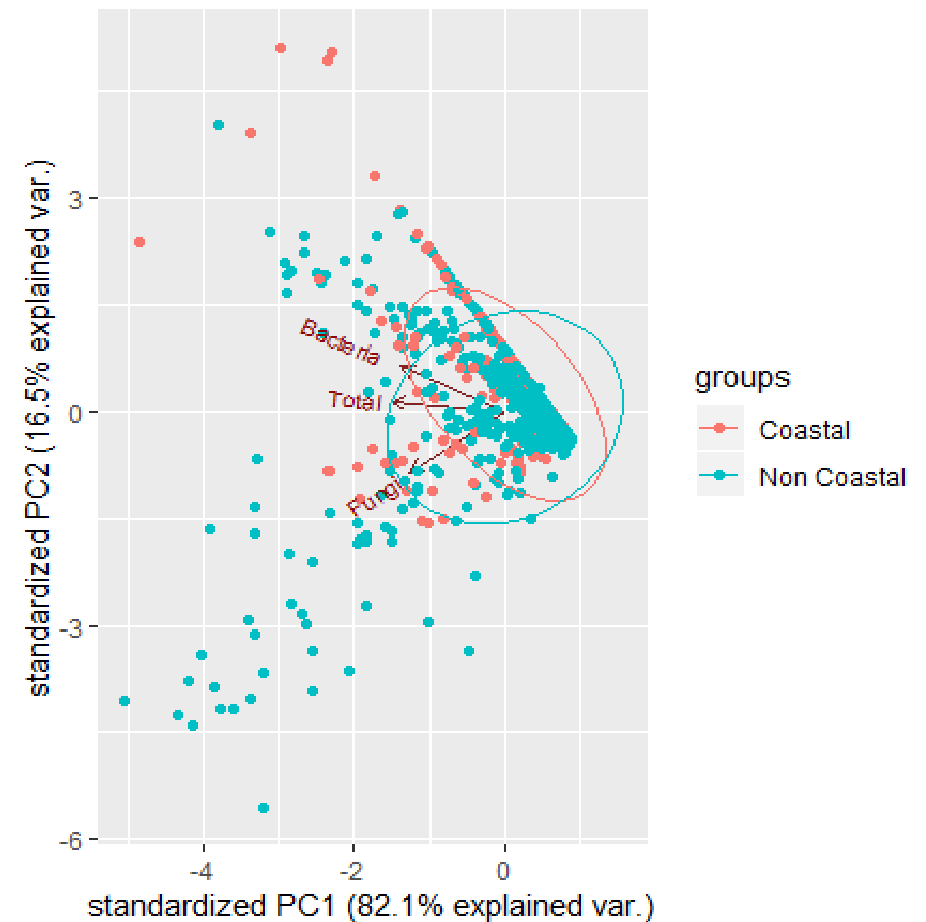


Figure 10: PCA ordination plot of microbial PLFAs across 19 golf courses, grouped by whether they are coastal or non-coastal.

PCA grouped by location showed greater variation in microbial biomass for courses in the midlands, than the north or south (Figure 9) though there was overlap for all locations. Coastal courses had more bacteria while non-coastal courses had more fungi. Non-coastal courses also had greater extremes of microbial biomass, especially in terms of fungal population (Figure 10).

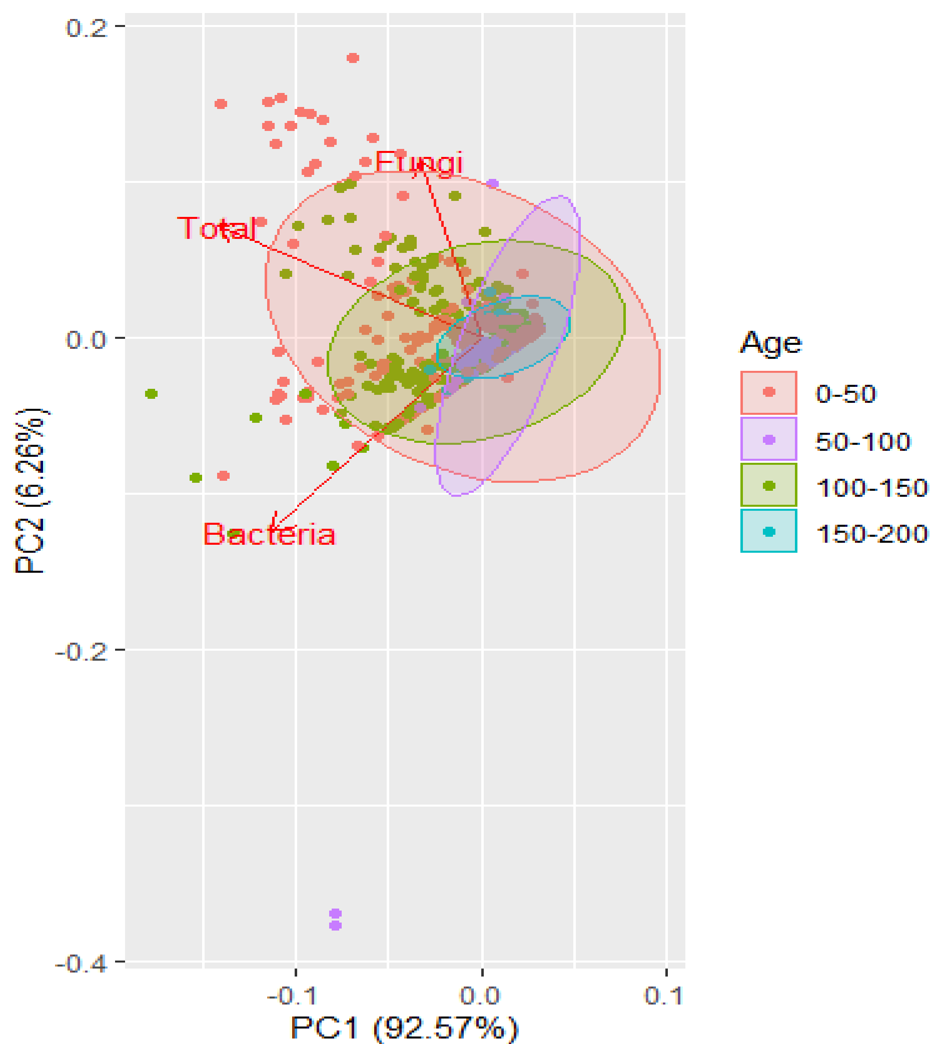


Figure 11: PCA ordination plot of microbial PLFAs across 19 golf courses, grouped by course age.

When comparing age of golf courses in Figure 11 course ages were grouped in groups of 50 years. There was a lot of overlap between the different age groups of courses, though variation in microbial biomass was highest for the youngest courses (under 50 years) tending towards extremes of total microbial biomass. The golf courses with the most consistent microbial population were the oldest courses (between 150 and 200 years old).

Only nine golf courses responded to the management technique survey and these are grouped by golf course in the PCA plot Figure 12. When comparing microbial inoculant usage in Figure 13, inoculant usage caused greater extremes in bacteria and fungi biomass but otherwise overlapped with courses which did not use microbial inoculants. Three types of biostimulant were compared in Figure 14; seaweed, humus and phosphites. PCA showed that humus increased bacterial biomass, phosphites increased fungal biomass and seaweed caused an increase in total biomass. Courses without any biostimulant application had far less variety in microbial biomass between greens.

Pesticide usage was considered by counting the number of different fungicide active ingredients applied to each course every year. Figure 15 showed that lower pesticide applications had greater amounts of bacteria and fungi. More application of different active ingredients caused less variation in microbial biomass between greens and favoured more bacteria than fungi.

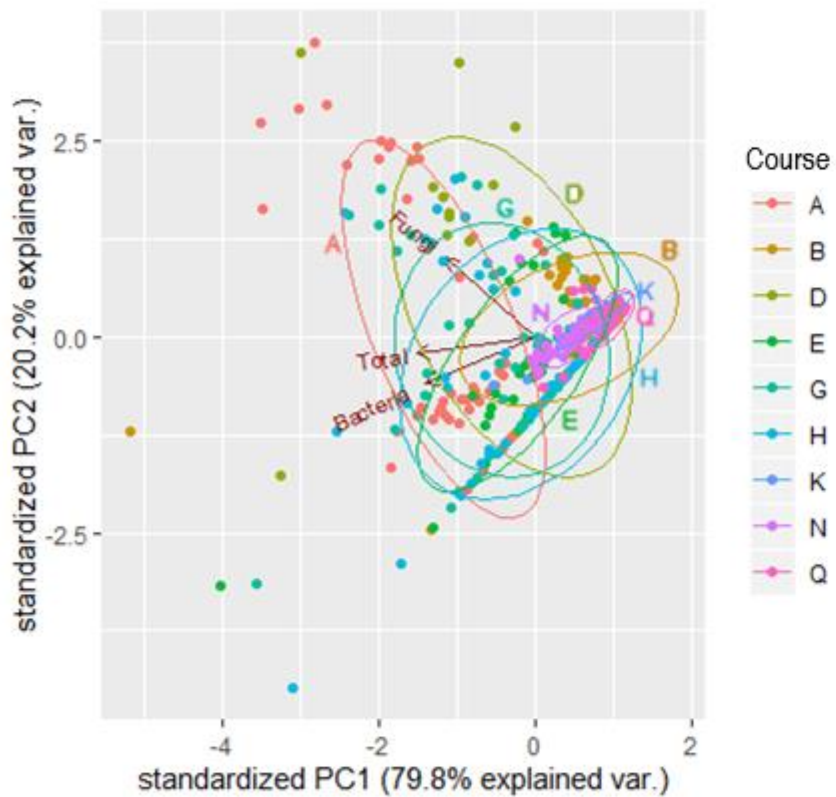


Figure 12: PCA ordination plot of microbial PLFAs across 9 golf courses, grouped by golf course.

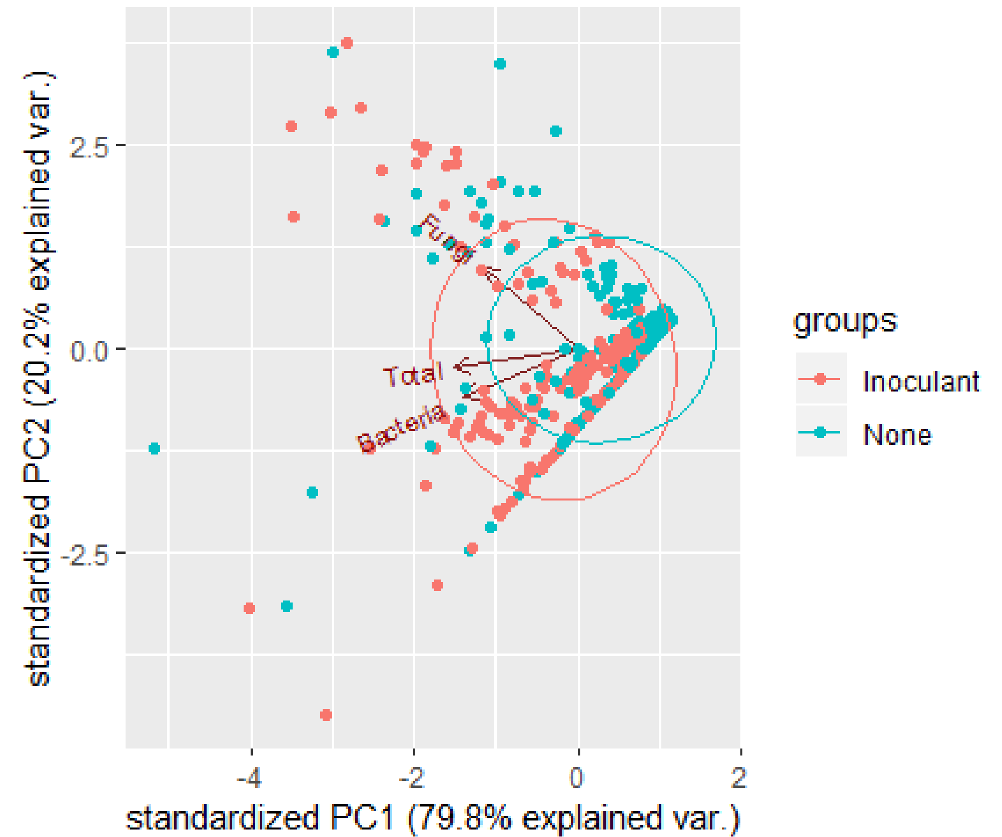


Figure 13: PCA ordination plot of microbial PLFAs across 9 golf courses, grouped by usage of microbial inoculants.

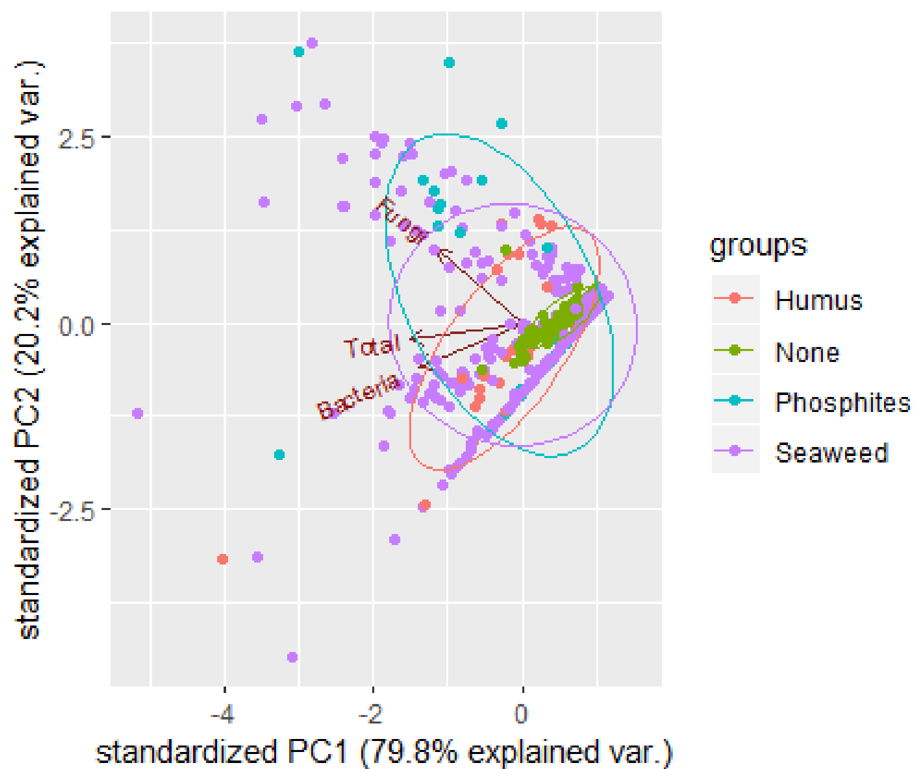


Figure 14: PCA ordination plot of microbial PLFAs across 19 golf courses, grouped by biostimulant usage.

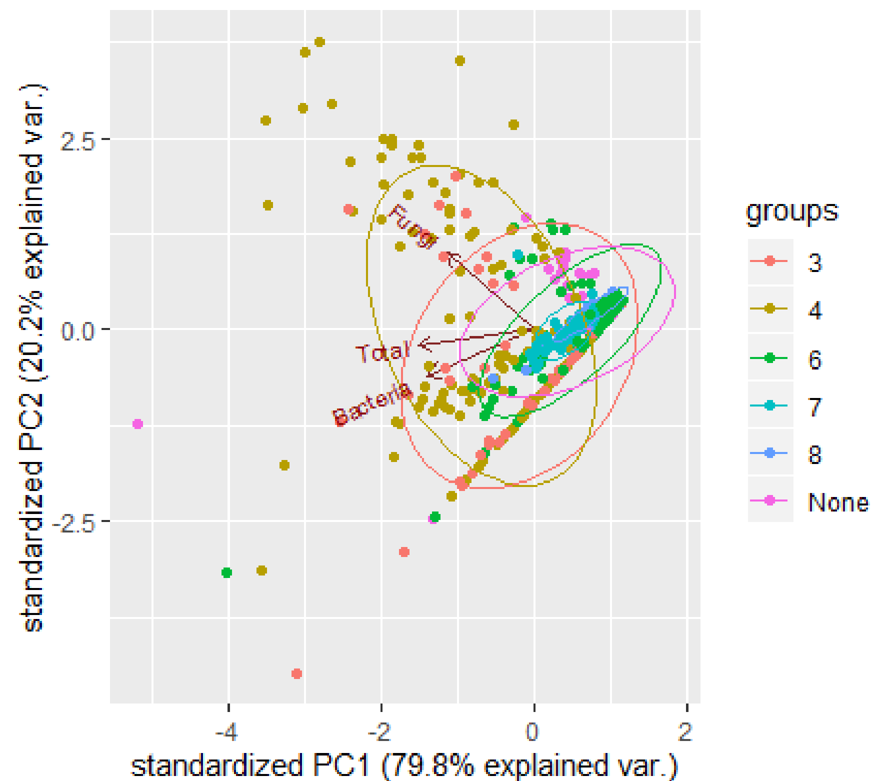


Figure 15: PCA ordination plot of microbial PLFAs across 19 golf courses, grouped by number of active ingredients of fungicide applied each year.

3.3 Identification of specific species in golf courses

3.3.1 Method

Golf courses near London (four in total) were selected for sampling based on age and management style to allow for comparisons (characteristics summarised in appendix 9.4.2). One golf course was the same as was sampled in the PLFA study (D for PLFA trial, A for sequencing trial). Nine greens were sampled by taking 2.5cm soil cores from three random points on the green. Soil cores were stored in plastic sandwich bags and immediately had DNA extracted using the Qiagen DNeasy PowerSoil kit (Qiagen, Venlo, Netherlands). DNA was identified and analysed as shown in Chapter 2 (section 2.3).

Illumina sequencing OTUs were identified using BLAST based upon a 97% confidence. These OTUs were then plotted as rank abundance and assessed for species evenness, before assessing the relationship between greens using PCoA and phylogenetic trees. This was done separately for both fungal ITS 2 and bacterial 16S.

3.3.2 Results

3.3.2.1 Fungi

Of the 108 samples, 91 were high enough quality for sequencing. The average number of tags per sample was 58,697, of which 49,766 were unclassified and 1479 were unique to that sample. This yielded an average 760 OTUs per sample. Through plotting rank abundance in Figure 16, species richness across the four golf courses is shown to be up to around 780 fungal species. However, Figure 16 also shows poor species evenness due to the steep nature of the

curve, which implies that there are a few species which are especially abundant and far fewer of the rare species present.

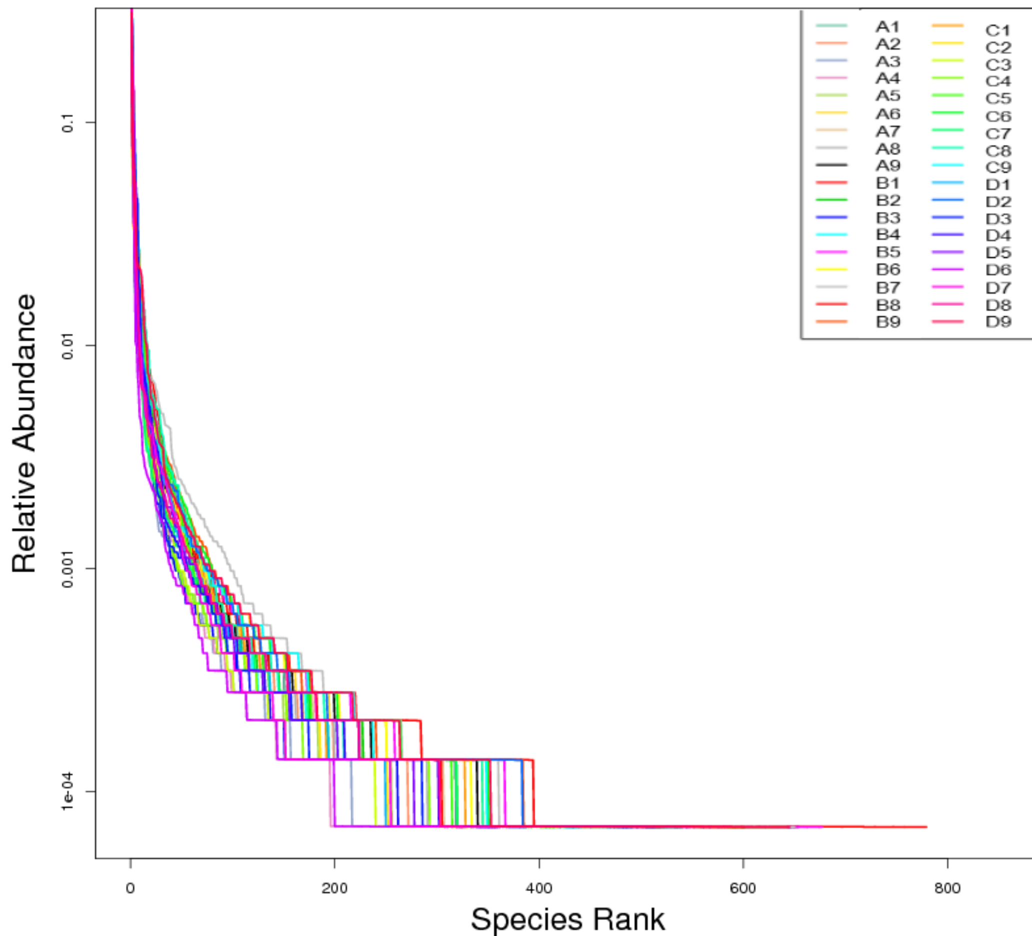


Figure 16: The relative species abundance for different golf course (A-D) greens sequenced for fungal DNA using ITS2.

The top ten species isolated across all four golf courses were grouped by phylum in Figure 17 to show their distribution across the greens sampled. The five more common phyla indentified were Basidiomycota, Glomeromycota, Zygomycota, Ascomycota and Chytridiomycota. As shown in Figure 17, golf course D had more Glomeromycota than other courses, although they were present in other greens.

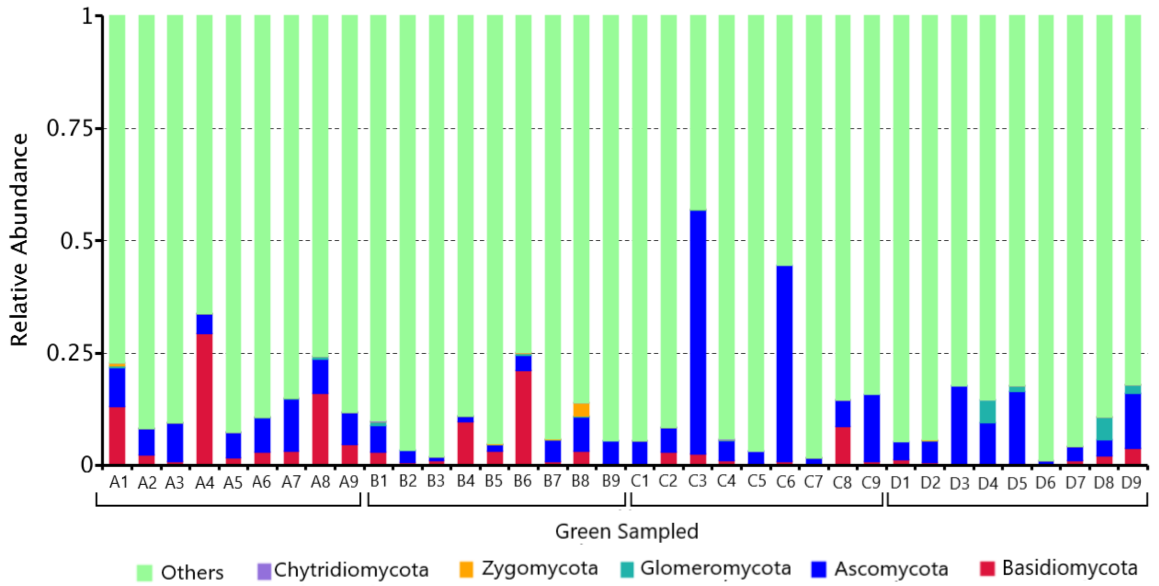


Figure 17: The phyla of the top ten fungal species discovered across all four golf courses sampled, grouped by green. The letters indicate the anonymised golf course and the numbers represent the green.

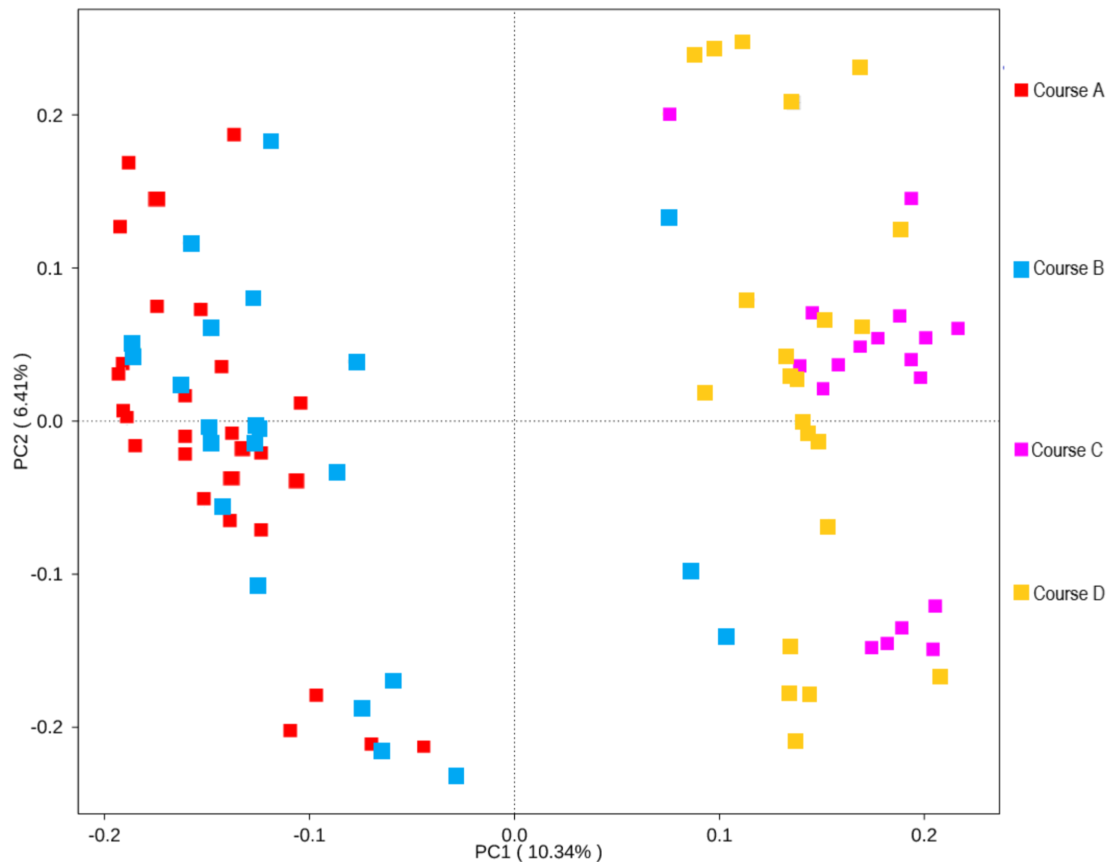


Figure 18: Unweighted Unifrac PCoA ordination plot for the fungal communities of 4 golf courses (A-D).

The fungal communities plotted in a PCoA ordination using unweighted unifrac consider species richness but not the abundance of each species. As shown in Figure 18, courses A and B grouped together, and courses C and D grouped together, suggesting similar fungal communities for these courses. However, when considering weighted unifrac in Figure 19 which does take abundance into account there is not a clear pattern between courses, suggesting little difference between communities when abundance is taken into account. The main difference in management style was that courses A and B used more biostimulants aimed at promoting bacteria biomass, whereas C and D applied amino acids. C and D were sand-based and built more recently whereas A and B were older and soil-based.

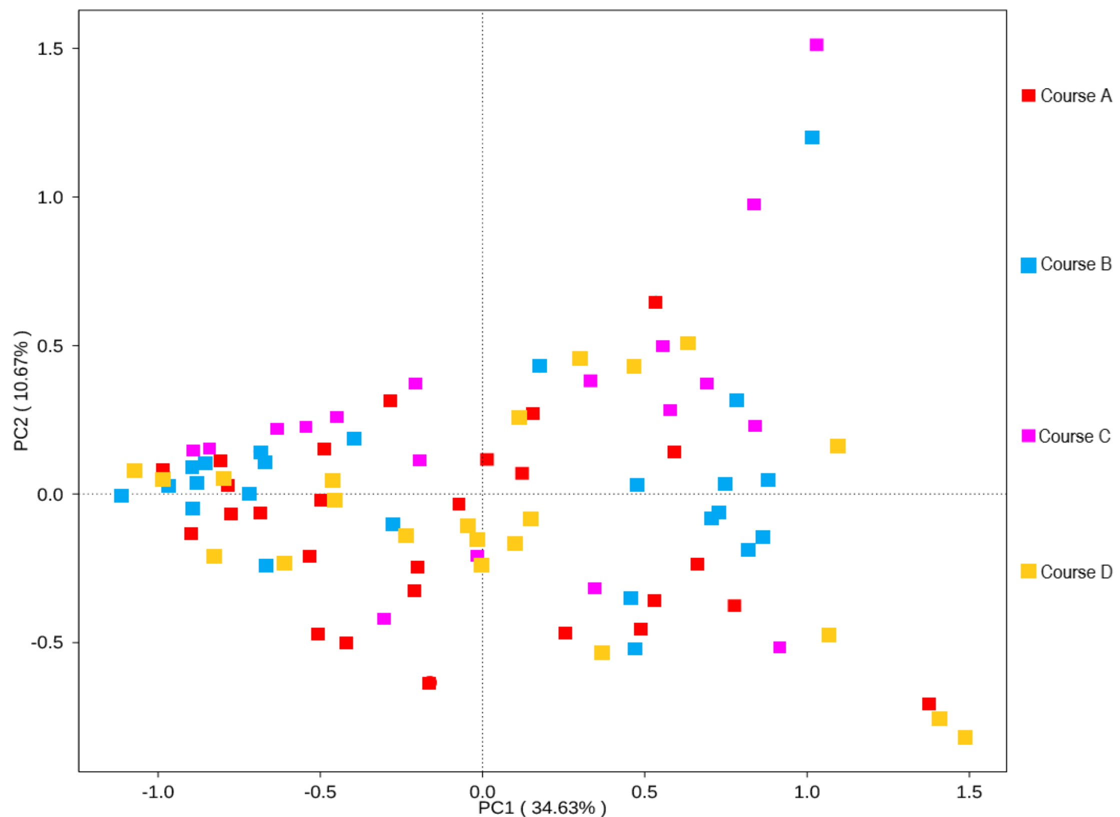


Figure 19: Weighted Unifrac PCoA ordination plot for the fungal communities of 4 golf courses (A-D).

Looking more specifically at AMF identified, each green had an average of 25.8 distinct OTUs of AMF although this varied greatly between greens. Of the sequences obtained, 68% of the OTUs identified as AMF could not be identified at a species level. The 32% that could be identified were predominantly *Glomus invermaium* and *Rhizophagus irregularis*, with four other species also identified (Figure 20). *Paraglomus laccatum* is an AMF that was originally found colonising *Festuca* spp by Blaszkowski (1988). The unknown sequences that were identified as AMF but could not be identified to a species level were plotted on a phylogenetic tree alongside several known AMF to produce the tree in Figure 21. This showed that while some of the unknown sequences were closely related to a known species, others were distinct.

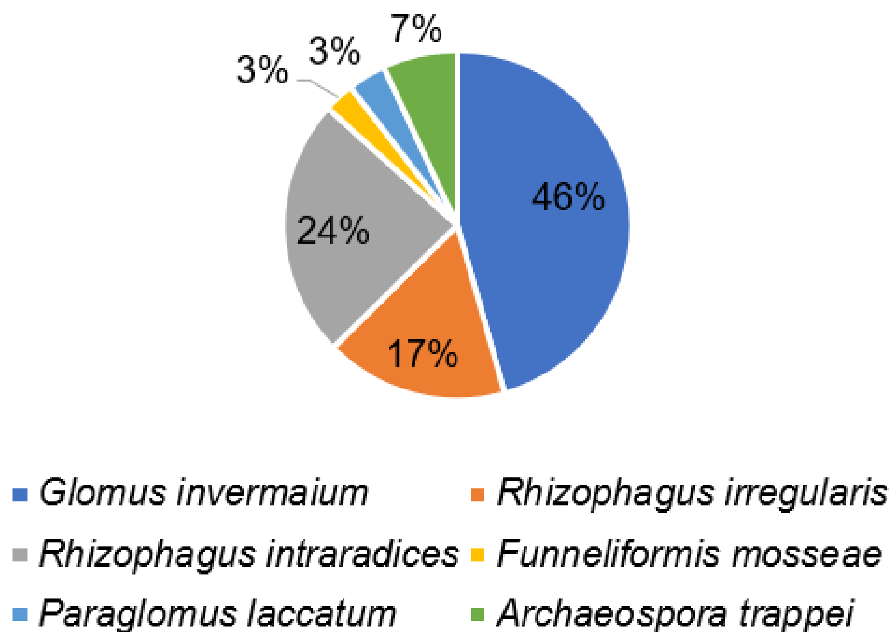


Figure 20: The percentage of different AMF species identified across the four golf courses.

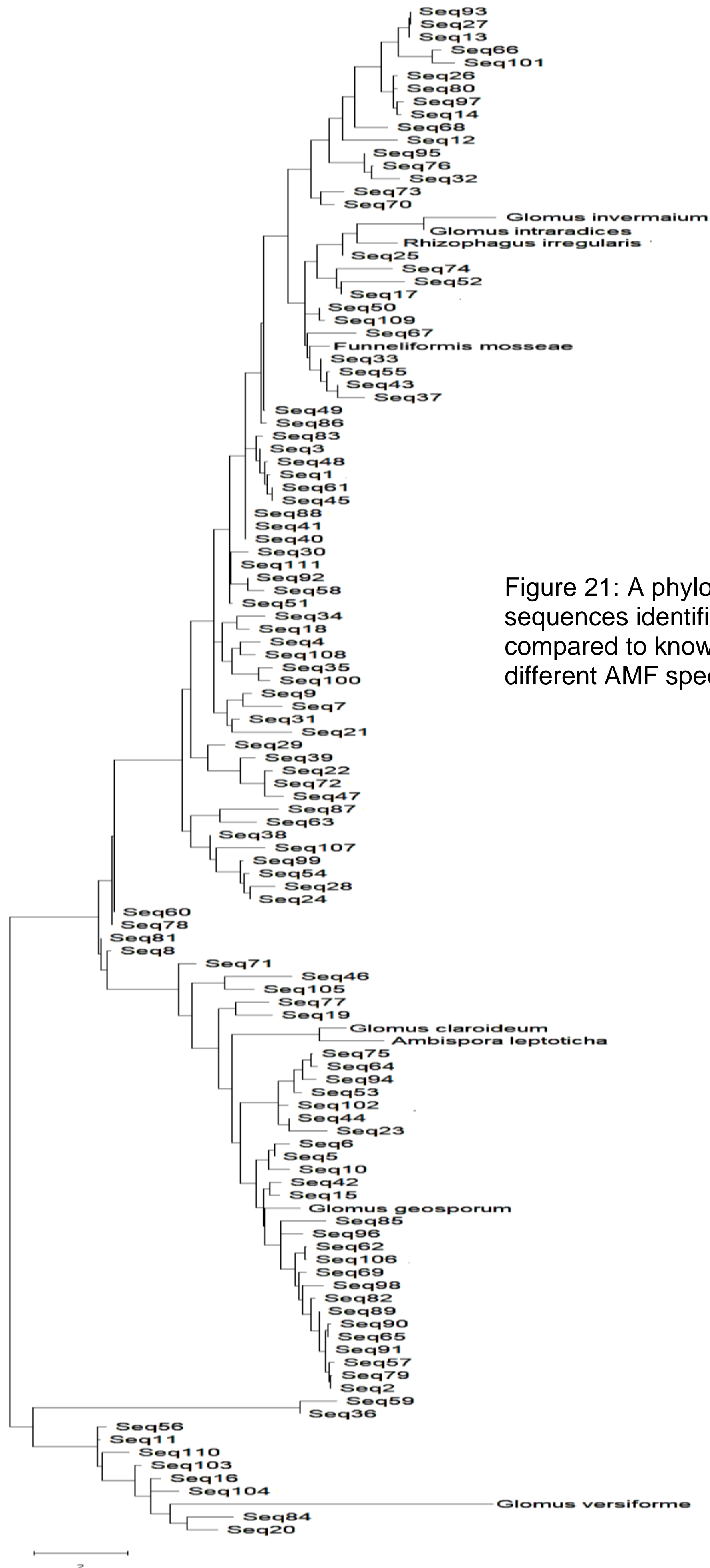


Figure 21: A phylogenetic tree showing sequences identified as unknown AMF compared to known sequences for different AMF species.

3.3.2.2 Bacteria

Of the 108 samples, 91 were high enough quality for sequencing. The average number of tags per sample was 57,695, of which on average all were classified and 21,148 were unique to that sample. This yielded an average 3,134 OTUs per sample and 36,547 taxon tags. Through plotting rank abundance in Figure 22, species richness across the four golf courses is shown to be up to around 36,500 bacterial species. However, Figure 22 also shows poor species evenness among bacteria though this is less pronounced than for fungi, which implies that there are still especially abundant species and some rarer less abundant species.

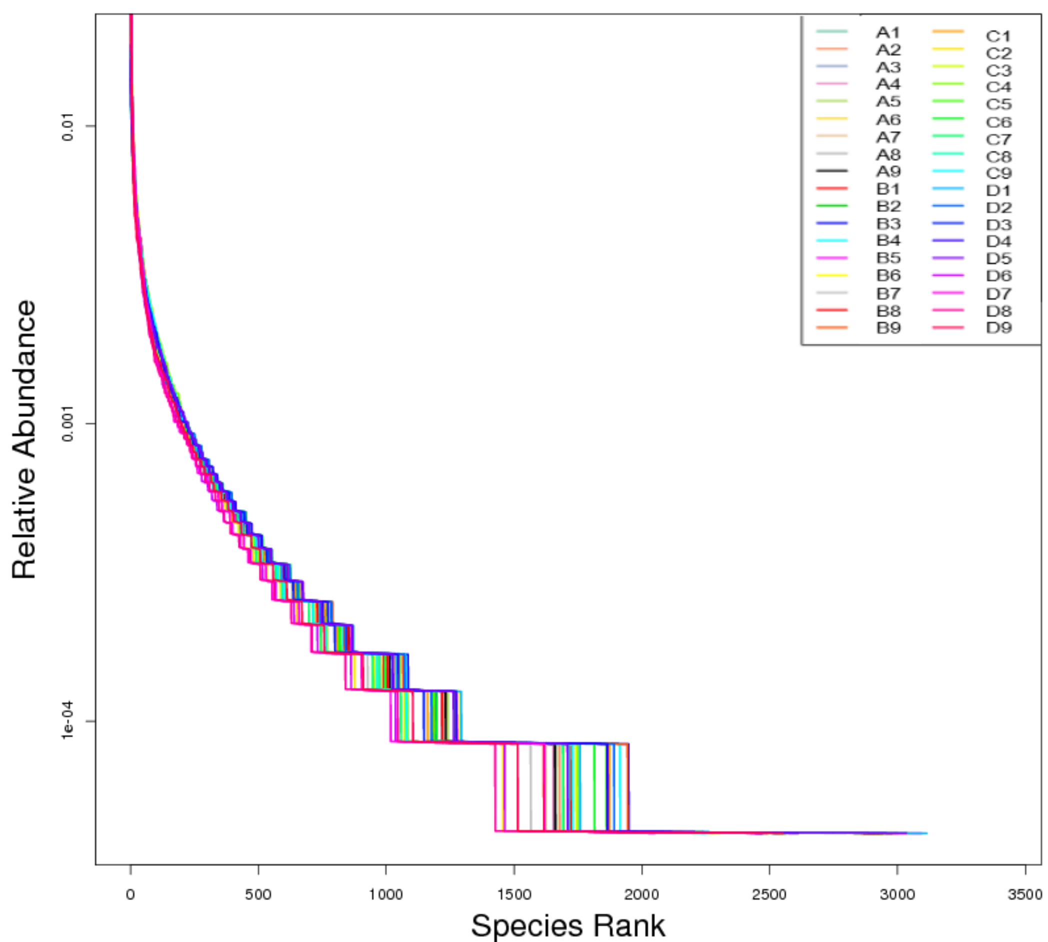


Figure 22: The relative species abundance for different golf course (A-D) greens sequenced for bacterial DNA using 16S.

The top ten species isolated across all four golf courses were identified and grouped by phylum in Figure 23. The most common phyla identified were Spirochaetes, Chloroflexi, Firbobacteres, Actinobacteria, Verrucamicrobia, Gemmatimonadetes, Bacteriodetes, Firmicutes, Acidiobacteria and Proteobacteria. Proteobacteria made up around half of the OTUs identified in each golf course and green, and include rhizobacteria. Rhizomicrobium were identified as one of the top ten genera across all samples, as was Candidatus Solibacter which was originally identified in pastureland (Ward et al 2009).

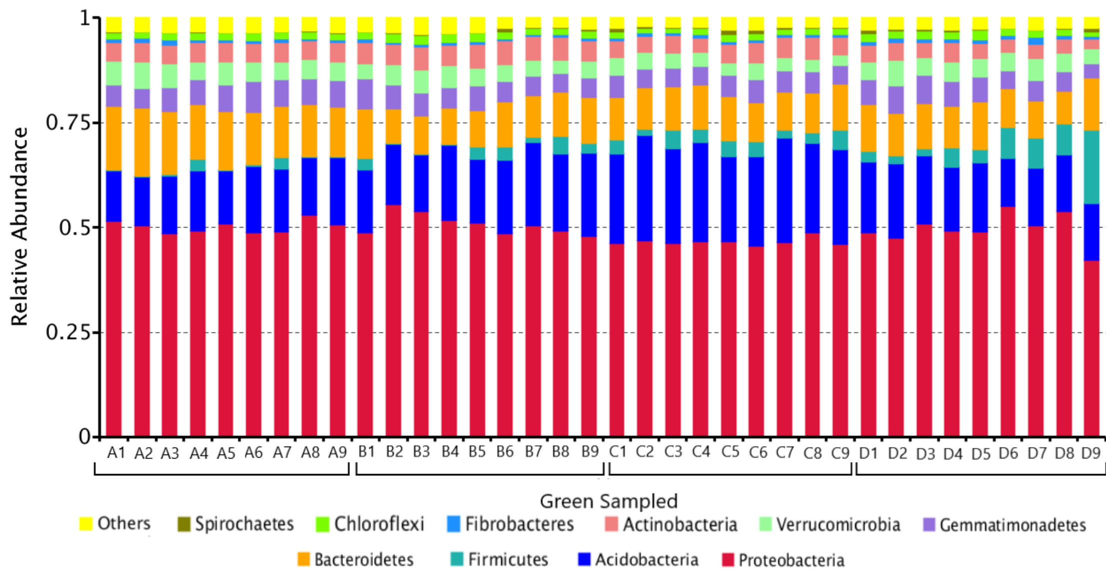


Figure 23: The phyla of the top ten bacterial species identified across all four golf courses sampled, grouped by green. The letters indicate the anonymised golf course and the numbers represent the green.

Unweighted unifrac PCoA showed grouping for courses A and C, with B overlapping both. Course D showed a huge range in bacterial community, with a wide spread of points separate to the other courses, as seen in Figure 24.

This suggests that each course had its own distinct bacterial communities with some overlap except for course D which had very different bacterial species in some greens.

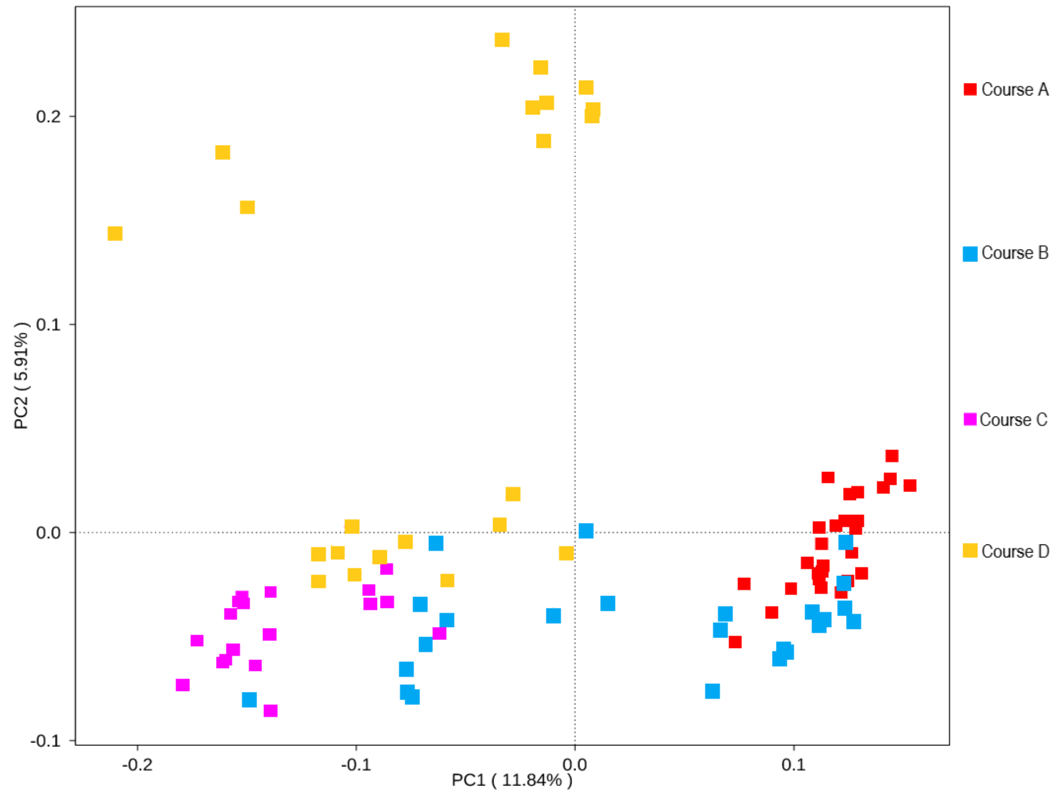


Figure 24: Unweighted Unifrac PCoA ordination plot of bacteria community for different golf courses (A-D).

The weighted unifrac PCoA still showed grouping for courses A and C, with B overlapping both as shown in Figure 25. Course D still had a different spread to the other courses. The species identified were still different to other courses but these different species were less abundant. This suggests there may be something specific to those greens causing the unique bacterial profile.

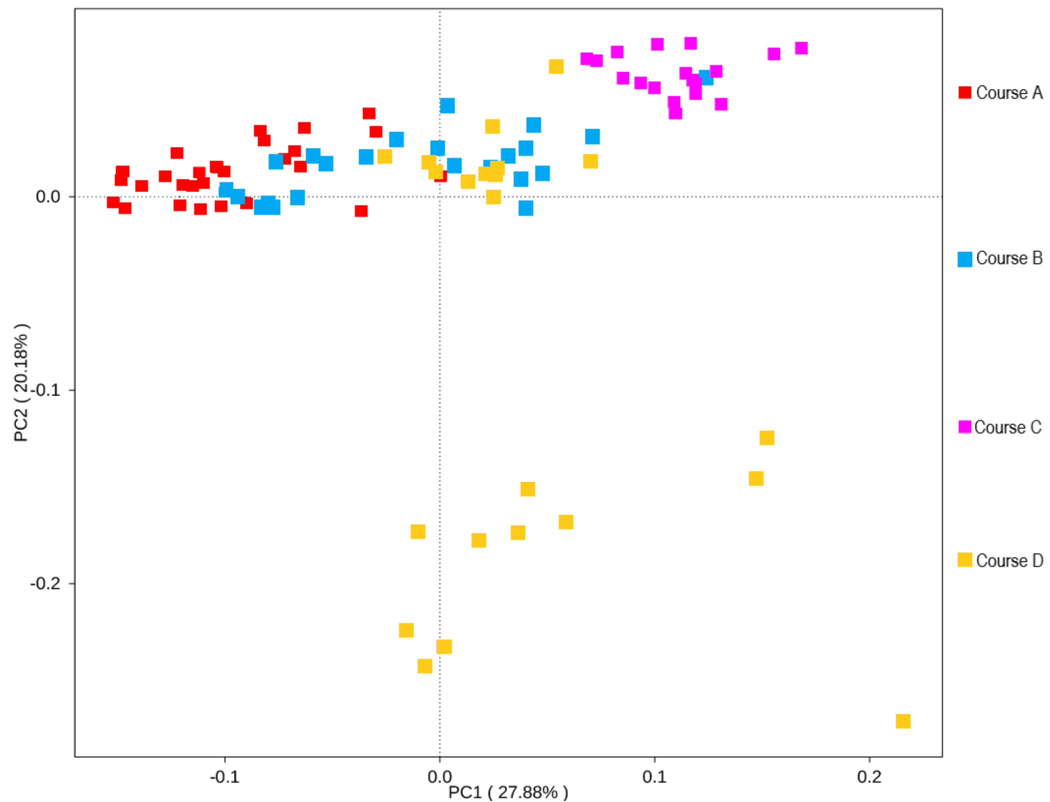


Figure 25: Weighted Unifrac PCoA ordination plot of bacteria community for different golf courses (A-D).

3.4 Discussion

3.4.1 PLFA

Each golf course was determined to have a different microbial biomass which came down to a combination of factors. Sand-based greens had a greater range of microbial biomass than soil-based greens which contradicts results by Zhang et al (2015) but confirmed the hypothesis that there would be a difference between soil types. Zhang et al (2015) found little difference between soil types of golf putting greens after they had established, despite differences early on. Alternatively, Hagley (2002) found that sand-based greens had far lower bacterial abundance than soil-based, while this trial showed sand-based had both greater and lower microbial abundance. This could be related to the

age of the course as a lot more of the sand-based greens are younger which also had a greater range of microbial biomass. Zhang et al (2015) showed that there is an initial increase in microbial population following establishment but that this evened out over time. While the Zhang et al (2015) study only lasted for one year, Bigelow et al (2002) ran a similar study over two years and also found that changes in the microbial population only occurred for the first year. There is no other published research looking into the microbial biomass of golf greens over a longer time period. It could also be that the differences between sand and soil-based greens were related to the organic matter content of the courses, with most sand-based greens having low organic matter, and so this could influence the microbial communities found there.

PCA plots described a very high proportion of variance, showing a high degree of correlation between the variables considered. There was also a bias of points sometimes forming lines which can also suggest a correlation between the variables.

Golf course location showed the greatest variation in the midlands although there was overlap between all locations. Coastal courses were found to have more bacteria and non-coastal courses had more fungi although there was overlap between the two. This could be due to differing salinity of coastal and non-coastal soils. Coastal soils show far higher salinity than other types, especially at shallower depths which is where most microbes live within the rhizosphere (Yu et al 2013).

In desert ecosystems salinity was found to have a strong influence on microbial populations, even more so than geographic distance between sampling sites

(Zhang et al 2019). Fraser et al (2018) found that alphaproteobacterial -a phylum known to be associated with golf courses- populations increased in sea grasses with salinity (Allan et al 2014). However, Fraser et al (2018) also showed some bacterial phyla significantly decreased with increasing salinity so the effects were dependent on the bacterial species present. Fungi were found to be particularly prone to salt stress (Sardinha et al 2003), with AMF spore germination particularly reduced in the presence of high salinity (Juniper & Abbott 2006). This would explain the lower fungal biomass of coastal greens compared to those inland.

There was a smaller sample size of golf courses that responded to the management survey than took part in the sampling. However, there were still nine golf courses that responded with a range of conditions. Microbial inoculants caused an increase in the number of bacteria and fungi found, as well as the total microbial biomass. Unfortunately, a lot of courses applied both bacterial and fungal inoculants and so they could not be separated for individual analysis.

Those golf courses that applied biostimulants experienced changes in microbial biomass depending on the nature of the biostimulant. Phosphites increased fungal biomass, humus increased bacteria and seaweed increased total microbial biomass. Those courses with no biostimulant application had a consistently lower microbial biomass. Phosphite applications are commonly used as a fungicide or a biostimulant. Phosphites can be oxidised to phosphate by soil fungi and bacteria, improving AMF colonisation in some crops (Gómez-Merino & Trejo-Téllez 2015) which could explain the increase in fungal biomass

observed upon application. Humus (organic matter) has already been shown to improve bacterial biomass by Zhang et al (2015), and seaweed has been shown to increase soil bacterial diversity and abundance (Wang et al 2018).

The number of active ingredients of fungicide applied per year affected microbial biomass, with the lower numbers of active ingredient showing larger microbial biomasss than those with higher number of fungicide application. There was a slight anomaly with the application of four fungicides showing greater fungal biomass than three fungicides. This is probably due to either biostimulant application as the grouping is extremely similar, or due to the timing of sampling in relation to fungicide application. This is in line with current research, as the greater number of different active ingredients used, the less likely a resistant population is to develop (Clarke et al 1997), and so a lower number of microbes would be able to survive. Further research could include considerations for species diversity using sequencing techniques.

3.4.2 Sequencing

Sequencing data was in line with PLFA results showing differences between courses and individual putting greens. For fungi there was a distinct pattern between the younger and older courses when looking at species richness but there was no pattern when taking abundance into account. This showed that the abundant fungal species identified were consistent between courses. For bacterial species richness there was clustering for different courses, but some overlapped more than others. The groupings did however stay consistent when abundance was considered. Course D had a particularly diverse bacterial

range, with some greens very distinct from other courses. When the golf course was questioned further to identify if there could be a reason for this they mentioned that some greens had been irrigated with contaminated recycled waste water five years ago due to a problem with the water recycling system, and so some of the unusual bacteria is likely to have come from then.

Glomus spp were especially prevalent out of those AMF identified. Six AMF species were identified within golf course putting greens including *F. mosseae*. This is consistent with sequencing trials in arable fields by Daniell et al (2006) who also found predominantly *Glomus spp.* Of the AMF identified, 68% could not be identified to a species level. This is consistent with previous research by Gange (1994) where 7% of spores collected from golf greens couldn't be identified. Many of the unknown species identifications were classified as "uncultured Glomus" on BLAST through sequences uploaded from a study by Hempel et al 2007. That study sequenced the soil of intensively managed grassland in Germany and so shows a consistent AMF community between an intensively managed grassland habitat and sports turf. Hempel et al (2007) also found differences in the microbial communities found in the plant roots, spores and the soil which would be an interesting further experiment for future trials to determine which of the AMF found are colonising. The average number of different AMF found per golf putting green was 25.8 which is consistent with research by Oehl et al (2003) who found that different grassland sites has 24 species on average though they found intensive management decreased the number of species found significantly.

It is common for multiple AMF species to be present in a community, but different plant species display preference for certain AMF species (Vandenkoornhuyse et al 2003). This is encouraging, as it suggests that in a mixed sward, mixed inoculums could have a positive effect for preferential host plant species and still coexist with other microbial communities. Lekberg & Waller (2016) found that there was often great variation both within plant species as well as between plant species and so environmental conditions were more important in determining the structure of the AMF community. That suggests that while plant species is important to tailoring inoculants, tailoring to management style is more important to ensure inoculants establish well. This is in alignment with the “species pool” concept in plants from Zobel (2015), who states that the primary factor in local variation of communities is the availability of species, which is affected by historical and current land use and location. This is also commonly considered in microbes (Öpik & Davison 2016), however, the community itself gives no guarantee of beneficial effects from colonisation which is far more dependent on the plant species present.

The main phylum of bacteria identified was proteobacteria (including alphaproteobacteria), making up around 50% of bacteria identified across each sampled green. This is in line with findings by Allan et al (2014) who found that alphaproteobacterial were the dominant species across golf courses. Proteobacteria are a diverse phylum, but are integral to the role of C, N and S cycling (Hortal et al 2013). *Rhizomicrobium* was identified as one of the top ten genera across all samples, showing the presence of PGPR naturally within golf putting greens. The genus *Candidatus Solibacter* was also identifed, which was originally sequenced from pastureland (Ward et al 2009). There is a lack of

current published research available for UK golf courses in order to provide a comparison. However, a golf course specific trial by Elliott et al (2008) in the USA found that the two dominant genera in bentgrass and bermudagrass species were *Pseudomonas* and *Bacillus.* While those genera were present in the UK golf putting greens sampled, they were not as prevalent as in the US courses.

3.5 Conclusions

The golf putting greens of different golf courses and within the same course were shown to vary depending on a range of factors, both for PLFA and for sequencing results. Microbial biomass determined by PLFA analysis depended on soil type, coastal or non-coastal location, age and location. Increased active ingredients used in a year reduced microbial biomass in golf greens but different biostimulants had varied effects.

Microbial biomass determined through Illumina sequencing found differences in species richness between courses but commonalities for the most abundant species identified. AMF populations were largely unidentified to a species level but were present in all golf courses sampled.

Chapter 4: What current practises change existing microbial populations?

4.1 Introduction

Pesticide applications are common in sports turf to alleviate disease and weed pressures. While sensible usage is encouraged and so usage has declined across the amenity sector in recent years (Pesticides Forum 2013), pesticide usage can influence the microbial community. Jacobsen & Hjelmsø (2014) concluded that the effect on microbial diversity greatly depended on the pesticide used, however all effects and shifts in community structures were found to last between one and three months. Effects on the microbial community were not just seen for fungicides (Yang et al 2012), but also for herbicides (Jacobsen & Hjelmsø 2014) and insecticides (Zhang et al 2009). While not all active ingredients examined in these papers are currently available through the UK market, such papers don't exist for all UK active ingredients. There are also no studies specific to a sports turf environment.

A common finding in pesticide studies is that application increases the number of pesticide degrading populations, this was found for the herbicides glyphosate, 2-4-D and MCPA (Baelum et al 2008, Lancaster et al 2009). For glyphosate it was also found that the more rounds of application, the quicker the glyphosate was incorporated into the microbial biomass, including after just four applications (Lancaster et al 2009). The rate of degradation also depends on the active ingredient observed, with a study on four fungicides finding that tebuconazole was the most persistent whereas chlorothalonil was most readily degraded. The same study found that degradation of all four pesticides was fastest in soils with a high organic matter content (Bending et al 2007). In a separate study by Muñoz-Leoz et al (2011) it was found that tebuconazole decreased both microbial biomass and soil activity as well as being slow to

break down, so can have a lasting effect on the soil microbial population. Looking specifically at AMF in a golf putting green there is no evidence that application of fungicides reduces root length colonisation (Bary et al 2005), this is thought to be because a large portion of the AMF is within the root and so protected from any active ingredients in the rhizosphere. Studies on soil fumigants reduced microbial diversity by increasing gram positive bacteria but decreasing gram negative bacteria and fungi (Jacobsen & Hjelmsø 2014). Based on current research, it was hypothesised that pesticide applications affect microbial biomass in sports turf.

There are arguments that reduced microbial diversity has little effect on soil functions as there are so many different species allowing for functional redundancy (Jacobsen & Hjelmsø 2014). This has been proven for carbon mineralisation, denitrification and nitrification (Wertz et al 2006). However, resistance to invasion by pathogenic bacteria has been shown to decline with declining species richness (van Elsas et al 2012).

When pesticides are authorised for use currently, they are assessed for physical and chemical properties, analytical methods, toxicology, residues, consumer exposure, non-dietary exposure, environmental fate and behaviour, ecotoxicology and efficacy in line with EU regulations (Health and Safety Executive 2019). Microorganisms fall under ecotoxicology and require tests on soil nitrogen transformation and soil carbon transformation to prove that nutrient cycling still occurs following pesticide application (Health and Safety Executive 2019b). However, this does not consider microbial diversity or abundance or any other roles of microorganisms within the soil and so is greatly lacking in

information. Therefore, research of this nature considering microbial diversity following pesticide application is sorely needed.

Biostimulants have already been described in section 1.3.3 as being a hugely varied group of treatments with little current research on their effects in sports turf. These trials aimed to see if there were any observable effects on soil microbial biomass following application in a golf putting green environment. It was hypothesised that biostimulants effect soil microbial biomass, especially for amino acids and humic acids which could be utilised as food by the microbes.Plant parasitic nematodes are a considerable concern for greenkeepers owing to the lack of products available for their control. Currently the most effective treatment available is the use of garlic products which have been proven to reduce nematode numbers (Flor-Peregrin et al 2016). While garlic (*Allium sativum)* has been shown to be mycorrhizal (Al-Karaki 2002), there is currently no work on the effects of garlic extracts on AMF colonisation. Interestingly- the unrelated *Alliaria petiolata*, commonly known as Garlic mustard has been shown to reduce AMF colonisation in other plants, perhaps by the toxic effects of its root extracts (Cantor et al (2011), Roberts & Anderson (2001)). Although the extent of this reduction does depend on the specific mycorrhizal species colonising the other plant (Burke 2008), garlic mustard has become a very competitive invasive species in North America following its introduction by European settlers.

An extract from garlic mustard leaves reduced the germination of AMF spores, thus reducing inoculation potential (Roberts & Anderson 2001). An extract from the whole plant can cause the same low inoculation rate as sterilised soil in tree

seedlings, showing that the phytochemistry of garlic mustard disrupts AMF colonisation (Stinson et al 2006). Vaughn & Berhow (1999) identified these phytochemicals as allyl isothiocyanate and benzyl isothiocyanate, finding them highly phytotoxic in wheat species.

Garlic has been used throughout history as an antimicrobial agent in many cultures. Garlic contains an antimicrobial chemical called allicin, shown to be antibacterial, antifungal, antiparasitic and antiviral (Ankri & Mirelman 1999). Looking at fungi specifically, allicin inhibits the germination of spores and growth of hyphae in a range of fungal species, although the specific mechanism for this is not definitive (Yamada & Azuma 1997). From this, it was hypothesised that the application of garlic extracts reduce mycorrhizal colonisation.

The aim of this chapter was to determine some of the current management practises which could affect the soil microbial communities in golf putting greens.

4.1.1 Summary of hypotheses

- Pesticide applications affect microbial biomass in sports turf.
- Biostimulants affect soil microbial biomass, especially for amino acids and humic acids which could be utilised as food by the microbes.
- The application of garlic extracts reduce mycorrhizal colonisation.

4.2 Pesticide and Growth Regulator Field Trials

4.2.1 Methods

A range of active ingredients for fungicides and herbicides were selected from those approved for use in sports turf, together with three brands of growth regulator with the same active ingredient but different adjuvants. Fungicides, Herbicides and Growth regulators were applied to the grass using a sprayer at the STRI trial site in Bingley, UK between April-October at the rates shown in Table 6. These rates were selected as the recommended rates for each product.

The trial was a Latin square layout to consider the environmental variation in the trial site and there was no border between treatments. First applications took place in April 2016 for fungicides and growth regulators, and April 2017 for herbicides. Pesticides were not applied directly to the soil as the trial aimed to replicate a golf putting green where they would be applied to the grass. The sward composition of the trial site was 60% bentgrass and 40% Poa. Untreated control plots were maintained in the same way as the treated ones with regular watering and fertilisation with 50% organic product with an analysis of 8.2.5 N.P.K. The fungicides and growth regulators were applied to four replicate plots of 2m^2. The herbicides were applied to seven replicate plots of 2m^2. Figure 26 shows the trial site.

Soil cores of 2.5cm diameter were taken in October 2016 for fungicides and growth regulators and October 2017 for herbicides, after a full season of applications had been applied. Cores were taken from the centre of plots to avoid any cross-contamination between plots. Soil cores were analysed using PLFA (see Chapter 2: General Methods, section 2.1). Normalised Difference Vegetation Index (NDVI), relative chlorophyll content (index of relative chlorophyll content) and ground hardness (Gravities, GM) were measured at the end of the trial. Measurements were taken with an NDVI meter (Trimble Greenseeker, California USA), a chlorophyll meter (FieldScout, Illinois USA) and a 0.5kg Clegg hammer (Sd Instrumentation, Bath, England). The equipment used is shown in Figure 27.

Figure 26: The trial site where assessments for fungicides, herbicides and growth regulators took place at STRI, Bingley UK.

Data from NDVI, Chlorophyll meter and Clegg hammer were analysed to find differences between all the different fungicides, the growth regulators and the herbicides using one-way ANOVAs and the means separated using a Tukey test in R 3.6.0 (R Core Team 2019). PLFA data were analysed for total microbial biomass, bacteria biomass (including actinomycetes) and fungal biomass for each treatment using a one-way ANOVA and the means separated using a Tukey test in R. The proportion of bacteria and fungi for each was analysed using a generalised linear model with quasibinomial errors in R. Normality for all data was checked using QQplots and Shapiro Wilk tests in R.

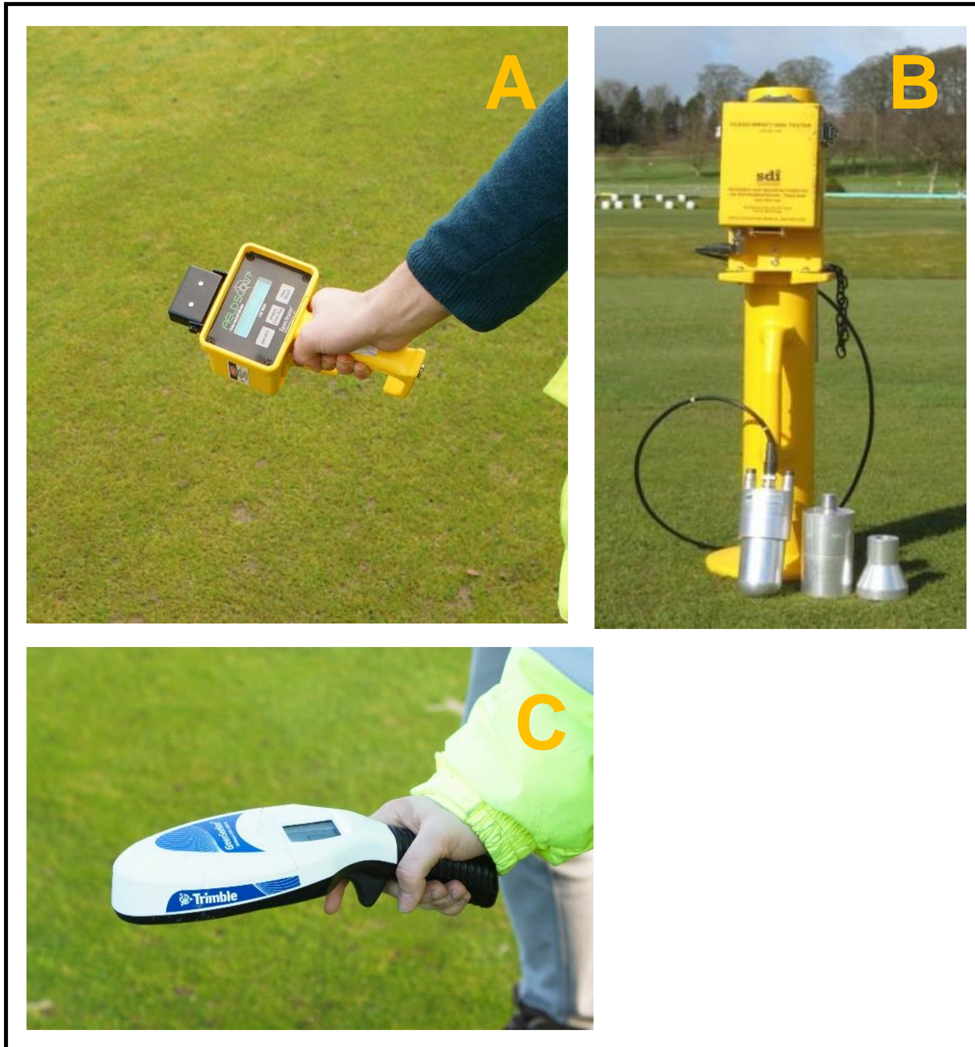


Figure 27: The equipment used to assess pesticide and biostimulant trials. A is a chlorophyll meter (FieldScout, Illinois USA) B is a 0.5kg Clegg hammer (Sd Instrumentation, Bath, England), C is an NDVI meter (Trimble Greenseeker, California USA). Credit STRI.

Type	Active ingredient	Application rate g or ml m^{-2}	Total Volume Treatment per plot/ml	Number of Applications	Time between applications
Fungicide	Trifloxystrobin & Iprodione	1.00	102.0	6	4 weeks
Fungicide	Trifloxystrobin & tebucanozole	0.10	90.2	4	8 weeks
Fungicide	Propiconazole	0.30	140.6	4	8 weeks
Fungicide	Azoxystrobin	0.25	100.5	4	8 weeks
Fungicide	Pyraclostrobin	0.13	40.3	2	12 weeks
Fungicide	Azoxystrobin & Propiconazole	0.30	120.6	4	8 weeks
Growth Regulator	Trinexapac-ethyl	0.04	100.1	4	8 weeks
Growth Regulator	Trinexapac-ethyl	0.03	80.1	1	N/A
Growth Regulator	Trinexapac-ethyl	0.04	60.1	4	8 weeks
Herbicide	Carfentrazone-ethyl, Mecoprop	0.15	100.3	1	N/A
Herbicide	Clopyralid, Florasulam, Fluroxypyr	0.20	40.4	1	N/A
Herbicide	2,4-d, Dicamba	0.35	40.7	3	4 weeks
Herbicide	2,4-d	0.33	80.7	3	4 weeks
Herbicide	Clopyralid, MCPA 2,4-d	0.30	60.6	1	N/A

Table 6: The application rates and schedule for the pesticide and growth regulator trial at STRI 2016/17.

4.2.2 Results

4.2.2.1 PLFA results

Different fungicides caused no change in the total amounts of bacteria ($F_{6,21}$= 1.333 P>0.05), fungi ($F_{6,21}$= 0.431 P>0.05) or total microbial biomass ($F_{6,21}$= 0.843 P>0.05) compared to the untreated control, as shown in Figure 28. However, different fungicide applications impacted the proportions of bacteria and fungi present in the soil ($t_{6,21}$=2.480, P<0.05) as shown in Figure 29. Bacteria were found more commonly than fungi for all treatments.

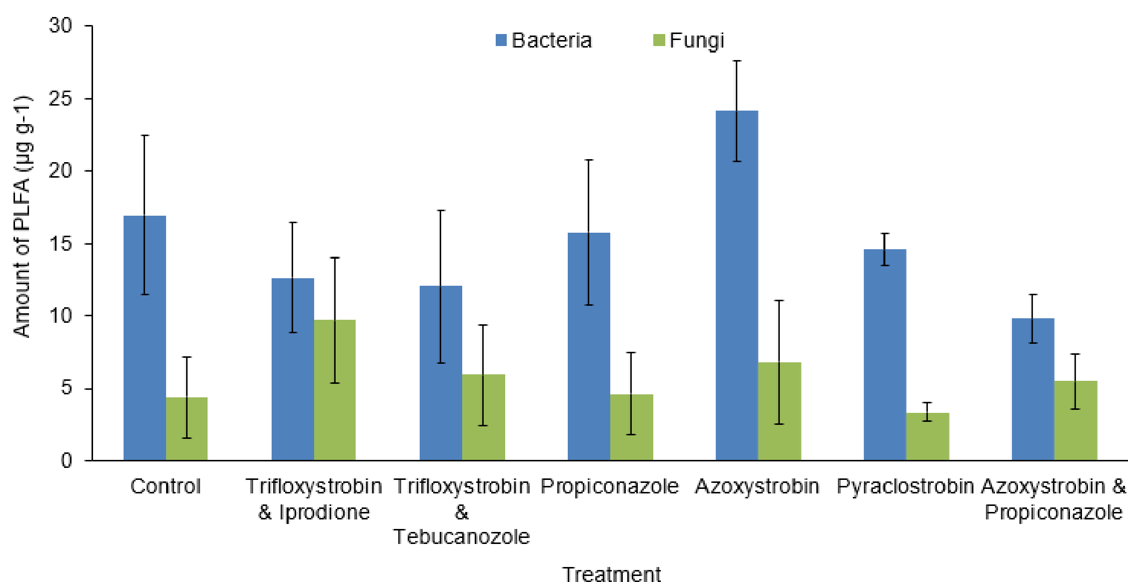


Figure 28: The mean amount of phospholipid fatty acid methyl esters found for bacteria and fungi after treatment with different active ingredients of fungicide. The error bars shown are standard error.

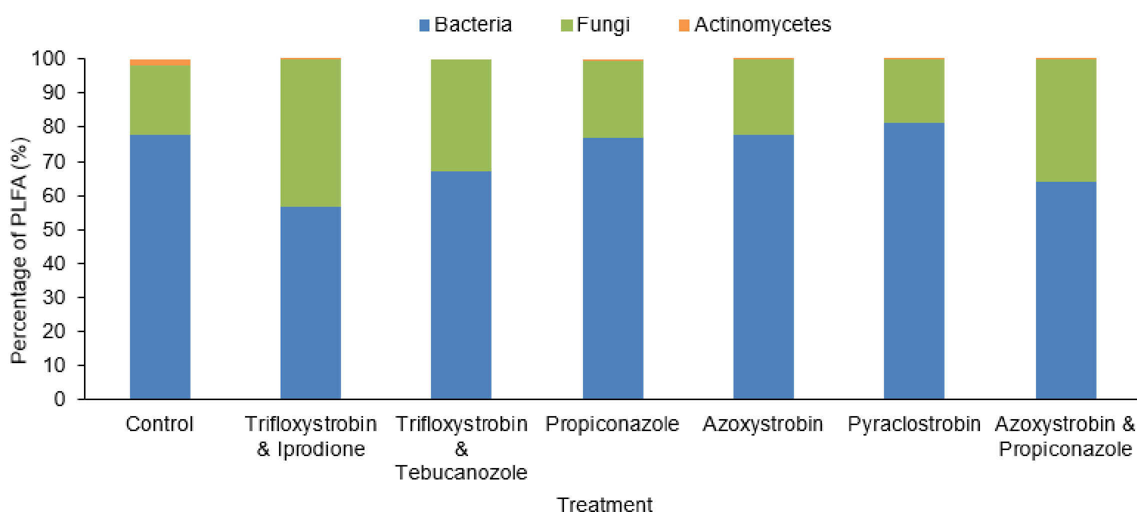


Figure 29: The proportion of phospholipid fatty acid methyl esters found for bacteria, fungi and actinomycetes after treatment with different active ingredients of fungicide.

Herbicides caused no change in the total amounts of bacteria ($F_{5,35}$= 0.399 P>0.05), fungi ($F_{5,35}$= 0.378 P>0.05) or total microbial biomass ($F_{5,35}$= 0.428 P>0.05) compared to the untreated control, as shown in Figure 30. There was also no impact on the proportions of bacteria and fungi present in the soil ($t_{5,35}$=-1.131, P>0.05) as shown in Figure 31.

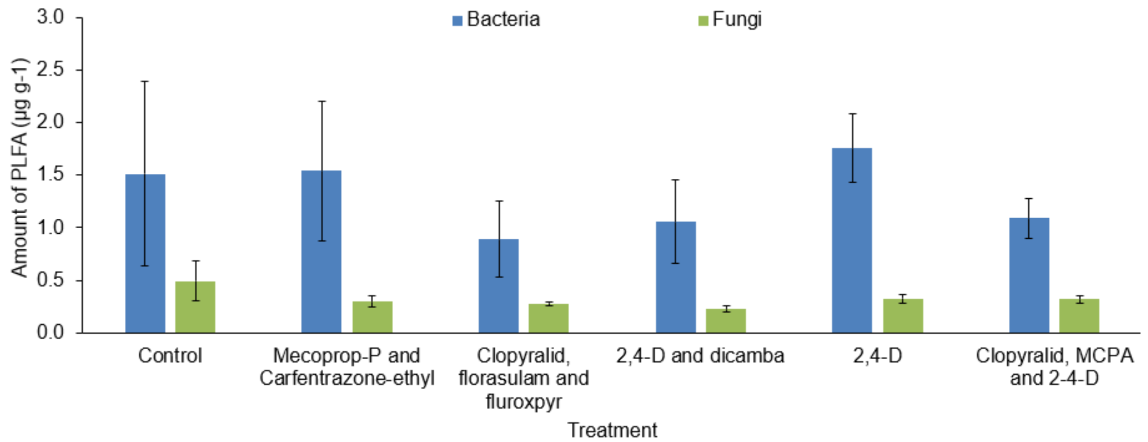


Figure 30: The mean amount of phospholipid fatty acid methyl esters found for bacteria and fungi after treatment with different active ingredients of herbicide. The error bars shown are standard error.

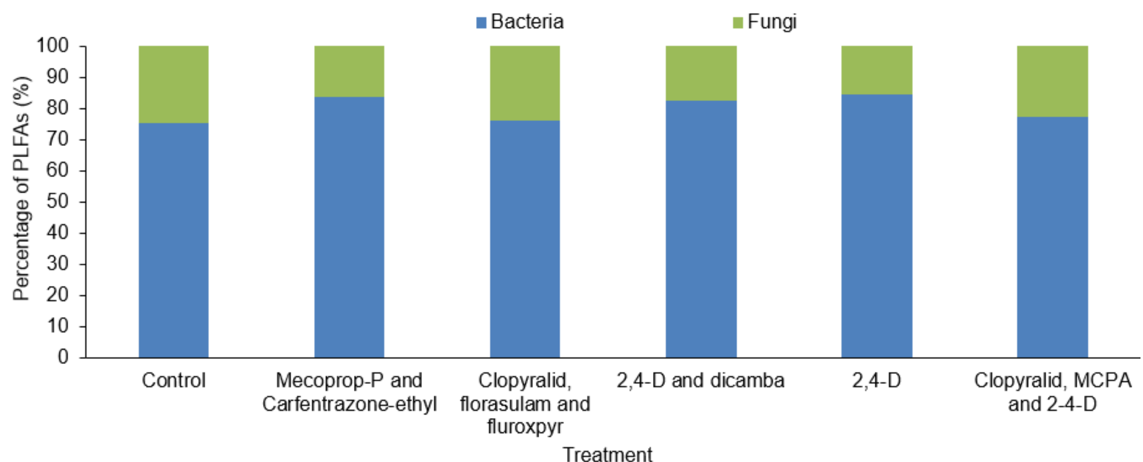


Figure 31: The proportion of phospholipid fatty acid methyl esters found for bacteria, fungi and actinomycetes after treatment with different active ingredients of herbicide.

Growth regulator products caused no change in the amounts of bacteria ($F_{3,12}$= 0.135 P>0.05), fungi ($F_{3,12}$= 0.82 P>0.05) or total microbial biomass ($F_{3,12}$= 0.47 P>0.05) compared to the untreated control, as shown in Figure 32. There was also no effect on the proportions of bacteria and fungi (P>0.05), as shown in Figure 33.

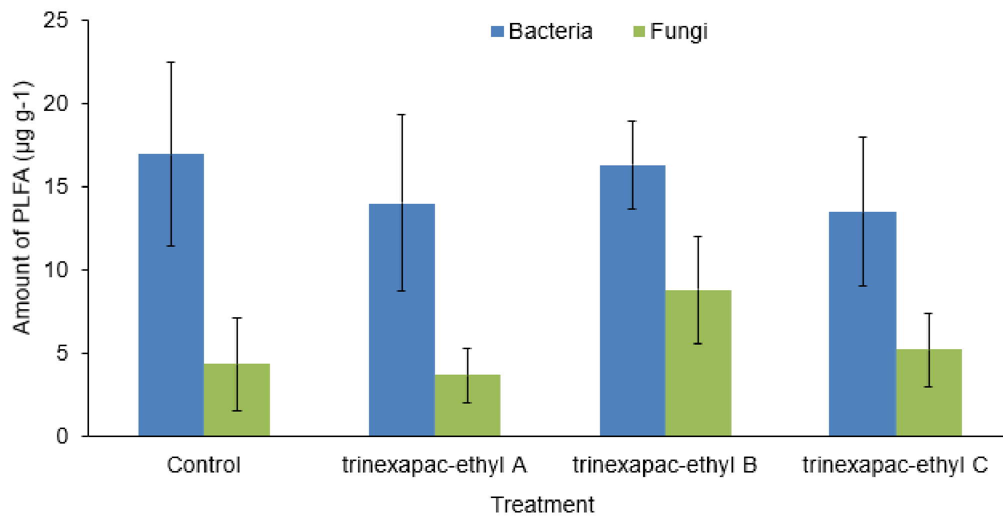


Figure 32: The mean amount of phospholipid fatty acid methyl esters found for bacteria and fungi after treatment with different growth regulator products. The error bars shown are standard error.

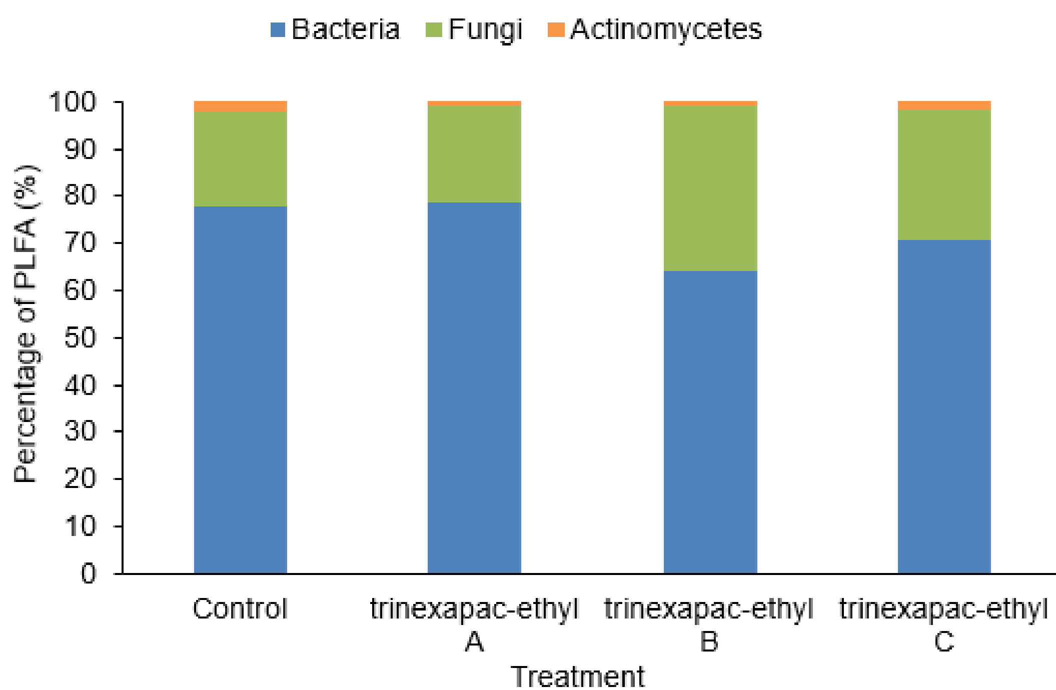


Figure 33: The proportion of phospholipid fatty acid methyl esters found for bacteria, fungi and actinomycetes after treatment with different active ingredients of growth regulator.

4.2.2.2 Fungicides

Different fungicide treatments showed no significant effect on the NDVI ($F_{6,21}$= 0.314, P>0.05) ground hardness ($F_{6,21}$=0.0833, P<0.05) or relative chlorophyll content ($F_{6,21}$= 0.057, P=0.05) for different trial plots. These graphs are shown in appendix 4, section 9.5.

4.2.2.3 Growth Regulators

Growth regulator products showed no effect on the NDVI ($F_{3,12}$=0.316, P>0.05), ground hardness ($F_{3,12}$=0.458, P>0.05), or relative chlorophyll content ($F_{3,12}$=0.646, P>0.05) of trial plots. Data not shown.

4.2.2.4 Herbicides

Different herbicides showed no significant effect on the NDVI ($F_{5,18}$= 0.499, P=0.777), ground hardness ($F_{5,18}$= 2.24, P=0.055) and relative chlorophyll content ($F_{5,18}$= 2.267, P=0.052) in different trial plots. These graphs are shown in appendix 4, section 9.5.

4.2.3 Discussion

Fungicides were found to have no impact on the total amount of bacteria and fungi within the soil. However, fungicides did have an impact on the proportions of bacteria and fungi as shown in Figure 29. In this case the proportion of fungi to bacteria became more fungal for the combination of trifloxystrobin and iprodione. It was considered that this difference between the fungicides could

be due to varying exposure to soil microbes between foliar and soil acting fungicides. However, this is unlikely as a study by Yang et al (2012) showed that four different foliar fungicides still had an impact on soil microbes in Chickpeas due to the residual accumulation of the fungicides in the rhizosphere. There did appear to be a lower biomass of microbes observed when the fungicides contained multiple active ingredients. This was concurrent with the research shown in Chapter 3 and existing research showing microbial resistance is less likely to develop when multiple active ingredients are used (Clarke et al 1997),

The reason for the lack of difference in total fungal biomass between fungicide treatments is thought to be because the microbial communities found in a simulated golf putting green may have developed resistance to pesticides commonly used in this environment. This means applying pesticides will have little effect to the microbes and they could survive pesticide applications. Even if a resistant population is not native to the habitat, multiple applications of the same active ingredient could provide enough selection pressure for such a population to develop, with Lancaster et al (2009) finding just four applications were enough to develop a population of bacteria that degraded pesticides. From this it could be hypothesised that microbial species richness is then reduced, explaining why some pesticides increased fungal biomass due to a decrease in competition. To prove this sequencing studies would need to be conducted. Reduced diversity from pesticide application has previously been shown in crops of corn and cotton by Ashworth et al (2017). However, crop plants don’t tend to have the layer of thatch seen in sports turf, which could prevent as much

pesticide entering the soil (Fresenburg 2015) and so interacting with the rhizosphere.

The lack of effect from herbicides on microbial communities when applied at the recommended rates is consistent with existing research using PLFA or plating on herbicide treated native soils (Lupwayi et al (2004), Pose-Juan et al (2017)). However, Gonod et al (2006) found that herbicide applications of 2-4-D did modify the soil community when utilising sequencing techniques. The main modification observed following application of 2,4-D was the increase in the presence of the gene which could break down 2,4-D, though this effect did disappear after 7 days. Vandenkoornhuyse et al (2003) theorised that changes in microbial community and particularly diversity could be more linked to changes in plant composition, especially pertinent for herbicides. However, as turf specific herbicides wouldn't kill the turfgrass sampled this could explain the lack of effect in golf putting greens compared to that seen in arable crops. Future research should therefore repeat this trial using Illumina sequencing to identify the diversity of the microbial community and be monitored at smaller intervals to witness this effect.

No previous published studies were found that examined the effects of plant growth regulators on soil microbial communities. This thesis found no significant impact on microbial community from the application of growth regulators, though future research should consider looking at specific species.

4.3 Biostimulant Field Trials

4.3.1 Methods

A range of commonly used biostimulants and microbial inoculants were selected from those available to sports turf. These were the products we received the most questions about while interacting with greenskeepers. Biostimulants and microbial inoculants were applied at the STRI trial site in Bingley, UK between April-October 2016 at the rates shown in Table 7. Untreated control plots were maintained in the same way as the treated ones with regular watering and fertilisation with 50% organic product with an analysis of 8.2.5 N.P.K. There were four repeat plots of $2m^2$ for each treatment in a Latin square design to account for environmental variation. The application method of each treatment is also included in Table 7.

All measurements taken and statistical analyses conducted were identical to those described above in the previous methods for pesticides (see section 4.2.1).

Biostimulant Type	Application rate g or ml m^{-2}	Total Volume Treatment per plot/ml	Number of Applications	Time between applications	Method of application
Compost Tea	13.30	626.0	12	14 days	Brewed using a compost tea brewer (water in a bucket with an air stone) for 24h before application
Soluble Seaweed	0.02	400.0	12	14 days	Powder was diluted and sprayed
Amino Acids	0.05	200.1	6	4 weeks	Liquid was diluted and sprayed
Liquid humate (humic acid l)	0.02	500.0	12	14 days	Liquid was diluted and sprayed
Powdered humate (humic acid s)	70.00	140.0	2	12 weeks	Powder was diluted and sprayed
Mycorrhizal inoculum (Plantworks)	15.00	30.0	2	12 weeks	Surface of trial plot tined and inoculum brushed into holes

Table 7: The application rate, calendar and method of application for the biostimulant trial at STRI in 2016.

4.3.2 Results

4.3.2.1 PLFA results

Different biostimulant products caused no change in the amounts of bacteria ($F_{6,21}$=0.524, P>0.05), fungi ($F_{6,21}$=0.888, P>0.05) or total microbial biomass ($F_{6,21}$=0.515, P>0.05) compared to the untreated control, as shown in Figure 34. Biostimulant applications also had no effect on the proportions of bacteria and fungi ($t_{6,21}$=1.986, P>0.05) as shown in Figure 35.

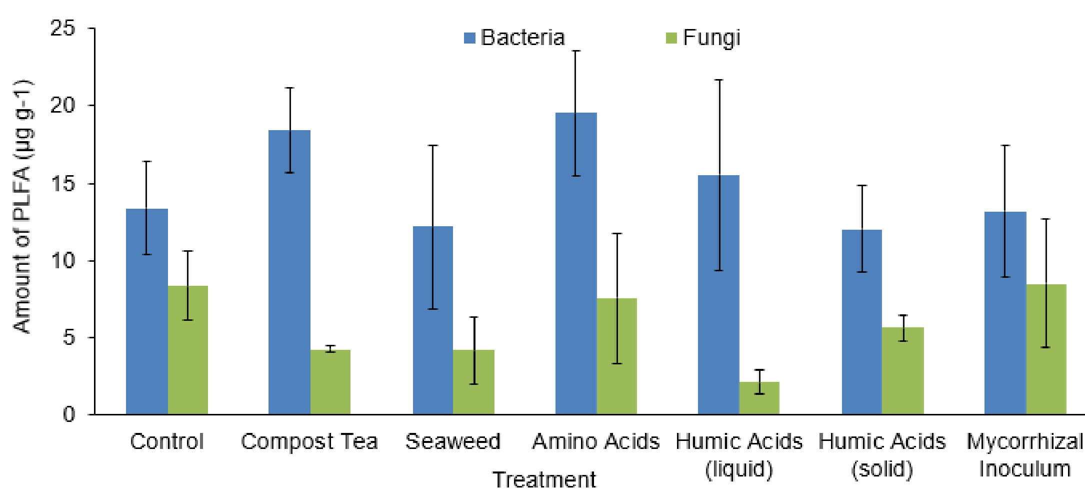


Figure 34: The mean amount of phospholipid fatty acid methyl esters found for bacteria and fungi following treatment with different biostimulants. The error bars shown are standard error.

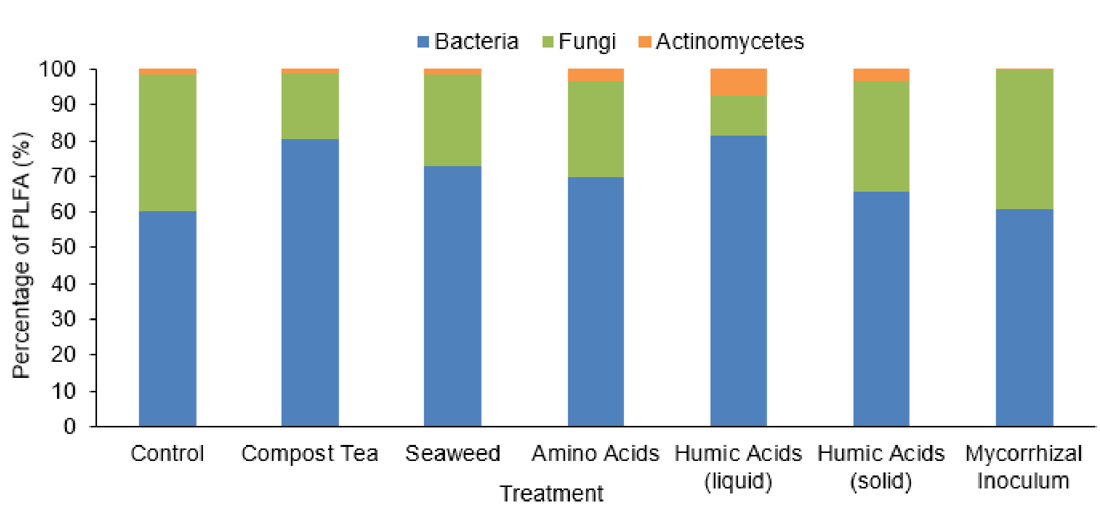


Figure 35:The proportion of phospholipid fatty acid methyl esters found for bacteria, fungi and actinomycetes after treatment with different biostimulants.

4.3.2.2 NDVI, ground hardness and chlorophyll content

NDVI readings showed no significant difference ($F_{6,21}$= 0.326, P=0.05) between different biostimulant treatments as shown in Figure 36. The overall average of NDVI readings between 0.71-0.77 suggest that there was good vegetation across the trial plot, however the large standard error shown for humic acid (solid), amino acids, humic acid (liquid) and the control suggest that there was large variation between test plots. Application of biostimulants had no effect on ground hardness ($F_{6,21}$= 0.126, P>0.05) as shown in Figure 37, or the index of relative chlorophyll for trial plots ($F_{6,21}$= 0.026, P>0.05) as shown in Figure 38.

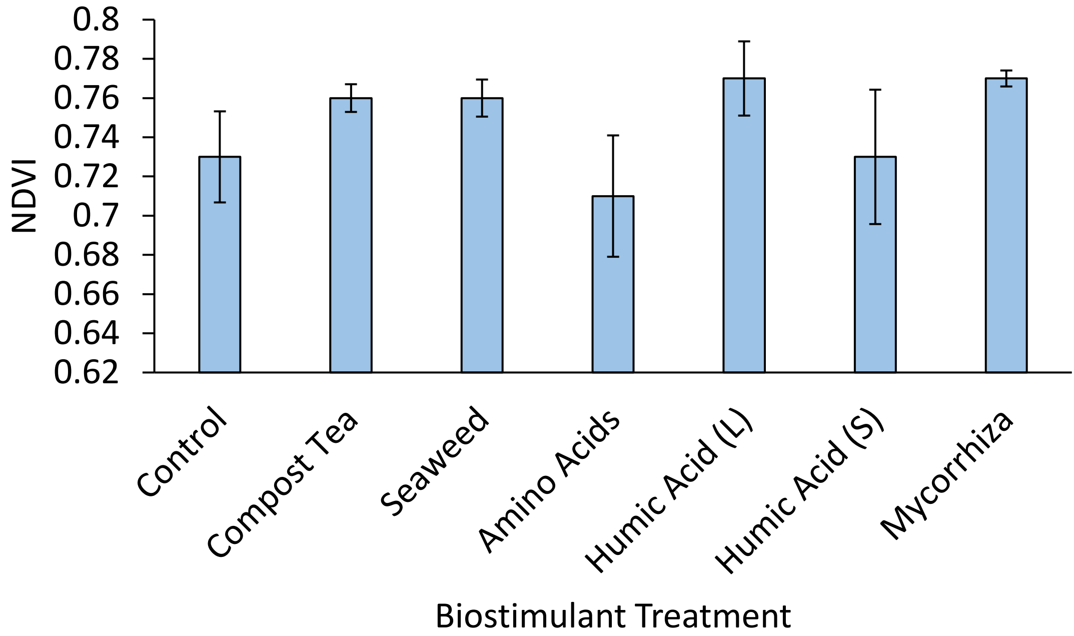


Figure 36: The impact of biostimulant treatments on the mean NDVI reading of trial plots. (L) represents liquid and (S) represents solid. The error bars shown are standard error.

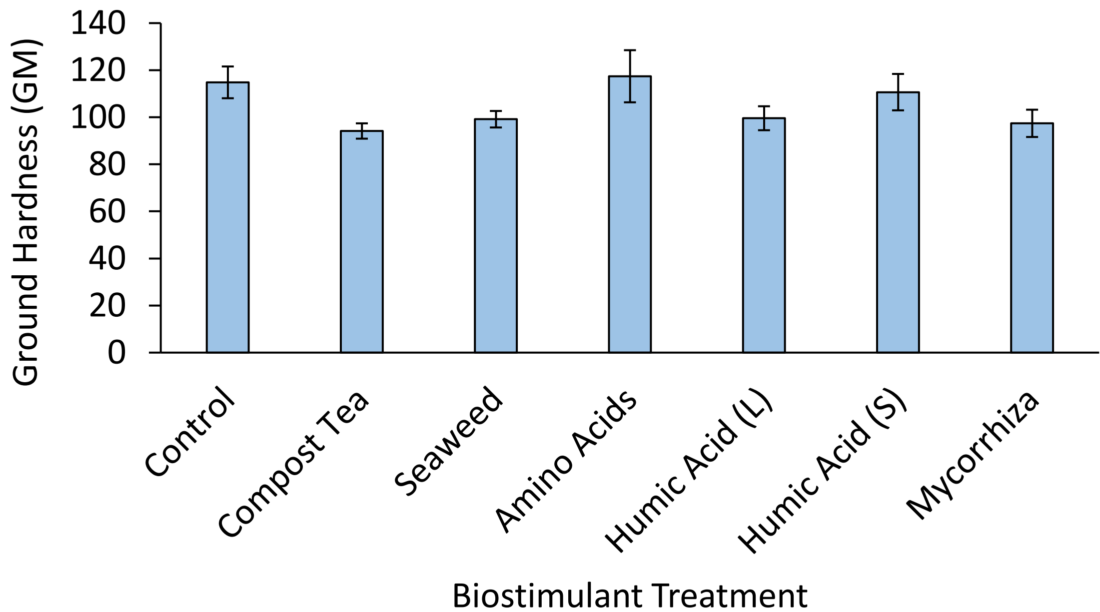


Figure 37: The impact of biostimulant treatments on the mean ground hardness of trial plots. (L) represents liquid and (S) represents solid. The error bars shown are standard error.

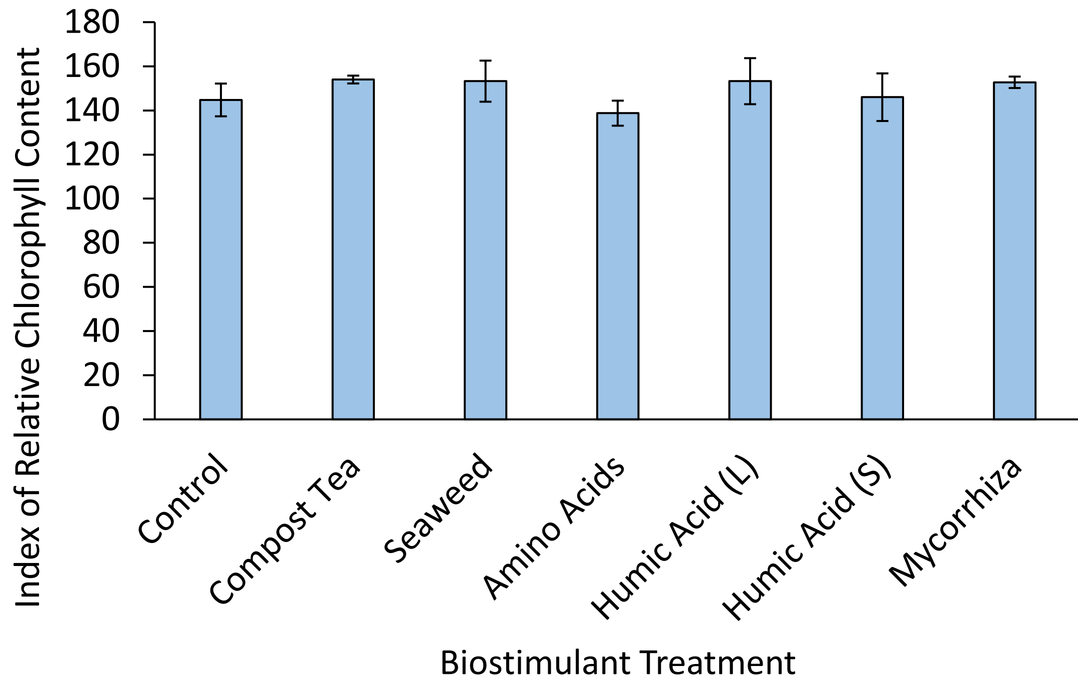


Figure 38: The impact of biostimulant treatment on the mean chlorophyll content of trial plots. (L) represents liquid and (S) represents solid. The error bars shown are standard error.

4.3.3 Discussion

While biostimulants may produce a wide range of plant beneficial effects (Rouphael & Colla 2018) they showed no effect on soil microbial biomass in this trial. This could have been due to the large variation seen between trial plots (as shown in the error bars of Figure 34). Large variation is consistent with other studies showing that PGPR and bacteria are often variable in their efficacy in the field (Gadhave et al 2016), the effects being influenced by the existing microbial populations. While previous research has shown seaweed to be a particularly effective biostimulant in increasing the populations of beneficial fungi and bacteria (Khan et al 2009) this trial showed no observable difference. This highlights one of the limitations of PLFA, which does not identify microbes

beyond whether they are bacteria or fungi, so it cannot determine if those observed are beneficial or not. Once again future research should therefore repeat this trial using Illumina sequencing to identify the diversity of the microbial community and any impact biostimulants could have on this.

Another consideration is that biostimulants are designed to aid the microbial biomass that is already present, therefore if there are few microbes already in the soil there may not be the same effects as a microbially rich soil. Therefore, the trial should be repeated again using combinations of inoculants and biostimulants to see if there is an effect with a viable microbial population.

4.4 Garlic Products

4.4.1 Methods

The experiment consisted of a two x two factorial design, with main effects of mycorrhizal and garlic extract addition and ten replicates of the four treatments. Two species of grass were sown, *Poa pratensis* and *Festuca rubra trichophylla*, at the recommended sowing rate (Appendix 2, section 9.2). Plants were grown according to the general pot set up in Chapter 2: General Methods (section 2.4), in a controlled temperature room at 20°C, with the following additions. Treatments were added after waiting for germination and five days for the grass to establish. Mycorrhiza was applied to 40 pots (20 per grass species) at a dose rate of 256kg/ha or 0.207g per pot. Pots without mycorrhizal treatment had autoclaved inoculum added at the same rate and microbial filtrate (See General Methods, 2.4.1). A commercial garlic product was applied to 40 pots at a rate of 388µl diluted in 100ml of water.

Dry weight data were analysed separately for each grass species to examine differences between treatments using a one-way ANOVA and the means separated using a Tukey test in R 3.6.0 (R Core Team 2019). Root length colonisation is proportional data so was analysed using a generalised linear model with binomial errors in R. This model was then checked for overdispersion and if found to be overdispersed it was run again but with quasibinomial errors to account for random factors. Normality for all data was checked using QQplots and Shapiro wilk tests in R.

4.4.2 Results

Garlic treatments reduced root length colonisation for *Festuca rubra trichophylla* ($t_{3,36}$=-3.867, P<0.001). Percentage root length colonisation fell from an average of 34.1% to just 6.2% which was lower than the control (6.7%) when treated with garlic. Treatment with both garlic and mycorrhiza showed no significant difference from the control ($t_{3,36}$==1.186, P>0.05). *F. r. trichophylla* also showed a significant increase in %RLC for those plants treated with mycorrhiza only against any other treatment ($t_{3,36}$=9.805, P<0.001). For *Poa pratensis* mycorrhizal treatment increased root length colonisation ($t_{3,36}$=6.756, P<0.001). The average %RLC colonisation in plants with mycorrhiza added decreased from 9.4% to 2.9% upon garlic treatment and showed no significant difference from control treatments ($t_{3,36}$=-1.004, P>0.05). These results can be seen in Figure 39.

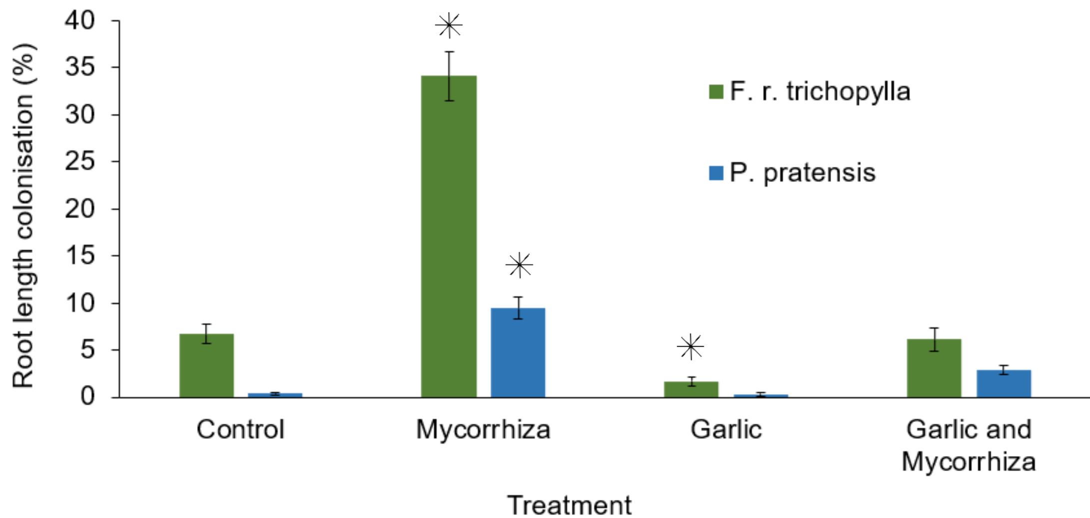


Figure 39: The mean percentage root length colonisation *for Festuca rubra trichophylla* and *Poa pratensis* upon the addition of commercial garlic treatments and mycorrhizal inoculum. The error bars shown are standard error. Starred bars were significantly different from control.

Treatments of garlic and mycorrhiza had a significant effect on dry biomass in *F. r. trichophylla* ($F_{3,36}$=9.356, P<0.001). There was a significant increase in dry biomass when both mycorrhiza and garlic were added (P<0.05), and when just garlic was added (P<0.05). Dry biomass in *P. pratensis* increased significantly between control and mycorrhiza($F_{3,36}$=7.833, P<0.01), and mycorrhiza to garlic treatment (P<0.001). This can be seen in Figure 40.

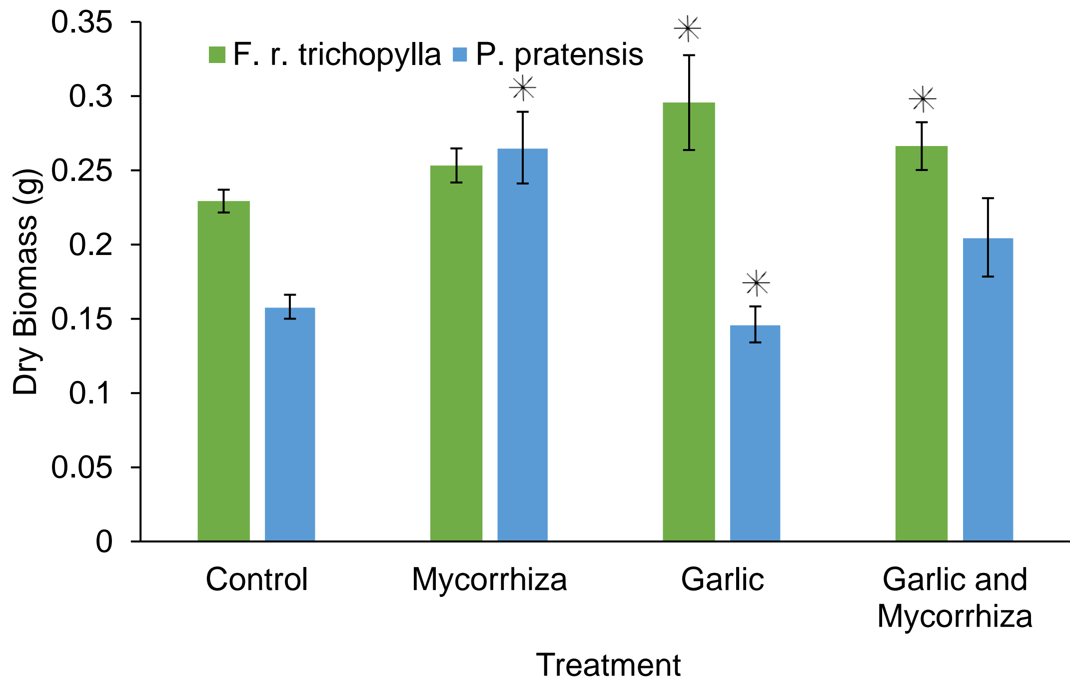


Figure 40 The mean dry biomass of *Poa pratensis* and *Festuca rubra trichophylla* upon the addition of commercial garlic treatments and mycorrhiza inoculums. The error bars shown are standard error. Starred bars were significantly different from control.

Garlic usage was also shown to have an effect in the field through the sequencing trial conducted in Chapter 3. In this trial golf course D had AMF identified to varying degrees in all greens except for green 10 where garlic had been recently applied for nematodes (data not shown). This confirms garlic treatments can reduce AMF colonisation in both pots and in the field.

4.4.3 Discussion:

Garlic treatments significantly reduced mycorrhizal colonisation in both *P. pratensis* and *F. r. trychophylla.* This upheld the hypothesis and is in line with current research on garlic mustard, which shows that plants can decrease

mycorrhizal interactions in order to reduce competition (Stinson et al 2006). There is no other research on garlic's effect on AMF colonisation, but the implications of these results are significant as garlic products are seen as an organic management technique. The reduction of AMF colonisation from garlic products suggests that those using AMF inoculums alongside garlic in their organic management schemes could be having a detrimental effect. The increase in dry biomass shown upon addition of garlic treatments in *F. r. trichophylla* suggests there may be some fertiliser quality to the extract or added to the product for this grass species, though this wasn't seen in *P. pratensis*. While garlic has been shown to be mycorrhizal with *Glomus fasciculatum* (Al-Karaki 2002), it also releases diallyl disulphide and allinin which is hydrolysed to allicin and known to be inhibitory to a wide range of microorganisms at high concentrations (Banerjee & Sakar 2003). Garlic mustard releases allyl isothiocyante which is also known to be inhibitory to bacteria and fungi (Delaquis and Sholberg 1997) and could be linked to its inhibitory effects on AMF. The fact that inhibition of AMF by garlic products was seen both in pot trials and observed in the field through Illumina sequencing shows the danger of utilising garlic products in a microbial management regime. While the phytochemicals released by garlic and garlic mustard are different, the observed effects are both inhibitory to microbes.

4.5 General discussion

Previous research with microbial inoculants has often shown disparities between field and pot trials (Gadhave et al (2016), Aamlid et al (2017)). Therefore, field trials are needed to confirm pot trial results. However, most golf

courses are wary of allowing trials on their greens due to the need to ensure greens are playable for golfers in case of any adverse effects. Those who are willing are often unsure of allowing the green to be divided into quadrats to trial different treatments. For this reason, the STRI trial sites were chosen to host field trials. The STRI replicate a golf putting green by managing the turf as if it were a golf course in terms of mowing, fertilisation, and irrigation unless specified otherwise. This allowed for comparison between treatments in a golf putting green environment. One of the limitations of the PLFA data used in these trials, is that it does not identify microbes beyond whether they are bacteria or fungi, so it cannot determine if those observed are beneficial or not. Beneficial bacteria or fungi would be those that provide a health or growth benefit to the plant and thus improve its appearance, stress tolerance and resistance to disease. Beneficial or negative effects could be measured in future trials by considering turf quality or colour, dry biomass or disease pressure alongside PLFA data.

This chapter demonstrated that current management practises can influence microbial populations though not necessarily as was expected. Pesticide and biostimulant applications both had no effect on microbial biomass except a change to the proportion of fungi to bacteria for trifloxystrobin and iprodione, rejecting the hypothesis for both trials. However, the hypothesis that garlic would reduce mycorrhizal colonisation was proven.

4.6 Conclusions

Pesticide usage made little difference to microbial PLFA biomass, though for fungicides did increase the proportion of fungi to bacteria in some cases. Biostimulants also had little effect though more research is needed to identify any effects on diversity or encouragement of beneficial species. Garlic treatments significantly reduced mycorrhizal colonisation in both *P. pratensis* and *F. r. trichophylla* and so are not compatible with a microbial management scheme.

Chapter 5: Factors affecting the application of inoculants to sports turf

5.1 Introduction

Microbial inoculants are products designed to introduce microbes to the soil. Current products on the market introduce PGPR and mycorrhiza through granular or liquid inoculants, these can be sprayed, applied as part of top dressing or as a seed coat. However, there are many factors that can affect the efficacy of an inoculant and how it is applied.

AMF populations and colonisation can be affected by multiple factors, the first of which is what microbes are already in the soil. Mycorrhiza can colonise from multiple propagules, including spores, infected root fragments or hyphae. However, not all AMF species can colonise from all types of propagule, with spores being the most effective and the efficacy of hyphae and root fragments being more species specific (Klironomos & Hart 2002). Therefore, what species and propagules are already present in the soil plays a crucial part in what will develop.

Another significant factor is the plant species, as if the mycorrhiza is not compatible with that plant it will not colonise it. Different species of AMF have different levels of specificity to plants, and molecular analysis of individual plants show they can be colonised by up to twenty different AMF species simultaneously. As well as this plants that coexist in the same location can have entirely distinct AMF species colonising them (Fitter 2005) and different extents of colonisation. In a study on the grass species *Agrostis capillaris* and *Ranunculus acris* on mountain slopes, percentage root length colonisation was 23% for *A. capillaris* but 31% for *R. acris* (Olsen 2015). In a study on turf by

Gange (1994) *A. capillaris* colonisation was also found to be up to 30% but mostly less than 10%, whereas *P. annua* experienced colonisation below 6%.

This specificity is reliant on the mechanisms of recognition between a host plant and mycorrhizal fungi, for example extensive hyphal branching occurs upon contact with a host plant root but not with a non-host plant root. This is thought to be due to the root exudates, which are either stimulatory or inhibitory depending on the fungi observed (Gadkar et al 2001). Ellouze et al (2012) found that different genotypes of chickpea produced different phytochemicals which either stimulated or inhibited the germination of AMF spores. They also found that these phytochemicals had different effects for different AMF showing a mechanism for plant host preference. There are multiple changes in plant host cells upon colonisation such as nuclear migration, condensation of chromatin and reduction or even suppression of plant immune responses that could prevent colonisation (Gadkar et al 2001). Therefore, colonisation of turf grass will be dependent on the species of fungi and grass present.

Another factor that can influence the microbial community is competition between microbes. Engelmoer et al (2014) considered the competition between *Rhizophagus irregularis* and *Glomus aggregatum* at different phosphate levels. They found that intraradical colonisation was less in mixed cultures compared to monoculture, but this was not the case for extraradical colonisation. A consistent reduction in the abundance of *G. aggregatum* in the extraradical mycelium was observed when *R. irregularis* was present, decreasing by 35% in a whole plant study and by 300% in an in-vitro system. However, *G. aggregatum* caused a reduction in the intraradical abundance of *R. irregularis*

(Engelmoer et al 2014). Spore production was also affected by the presence of competing mycorrhizal species (Bennett & Bever 2009). Although change in phosphate levels showed no effect on this competition, in a study by Engelmoer et al (2014) it has been previously shown that increasing levels of phosphate increased the inhibition of *Glomus spp.* by S*cutellospora calospora* (Engelmoer et al 2014). This change in the severity of competition is linked to the ability of the plant to reduce AMF colonisation in high phosphate through root exudates and the difference in sensitivity to this exudate of different AMF species (Pearson et al 1994). This effect could make a significant difference in golf putting greens where fertiliser input is high to make up for the removal of clippings and naturally cycling organic matter (Brown 2017), and so could increase competitive effects.

PGPR interactions are also specific to host plant and the existing microbial population of the rhizosphere. PGPR can influence root system architecture through interference with plant hormones. These changes can increase secondary metabolites and lytic enzymes, change the lignin: cellulose ratio, reduce primary root growth and increase lateral root and root hair length, which is thought to increase further PGPR colonisation (Vacheron et al 2013).

Different plant species will release different root exudates which can either be attractive or repulsive to different bacterial strains and can affect the method of colonisation (Company et al 2010). Gange & Gadhave (2018) showed that different strains of PGPR interacted differently with a brassica host plant, causing either increases or decreases in plant biomass depending on the PGPR. A previous study on *A. stolonifera* treated with four species of PGPR

that produce ACC aminase -an enzyme associated with reduced abiotic stress- also found that the grass responded differently to different PGPR, with not all of them yielding positive effects (Errickson 2018). It is therefore hypothesised that each grass species interacts differently to colonisation with different PGPR and AMF, with PGPR expected to be considerably more variable in their effect and sometimes antagonistic.

The high fertiliser application of sports turf could also influence initial colonisation, with high amounts of soluble phosphate in the soil being shown to reduce spore production (Daft & Nicolson 1969) and hyphal length (Abbott et al 1984). Menge et al (1978) showed that colonisation was more reliant on concentration of phosphate in the plant, with high plant phosphate showing reductions of root colonisation, hyphal growth and spore production. When phosphorus concentration was high in the soil, it did not make a huge difference so long as phosphate concentration was low in the root system. It is likely that in areas with consistently high phosphate concentrations the AMF populations will change to favour species tolerant of high phosphate levels (Abbott & Robson 1991). More recent research by Gosling et al (2013) confirmed that the effects of phosphate concentration on AMF colonisation are host plant specific. A significant reduction in colonisation was seen in Maize while other plants such as soybean showed no effect even at the highest concentrations of phosphate tested. However, there was a reduction in AMF diversity for both maize and soybean (Gosling et al 2013).

In a high phosphate site, Blanke et al (2005) found that nitrogen concentration affected AMF colonisation, with high plant and soil concentration of N

significantly reducing AMF colonisation. This trial suggested that when phosphate was high, the colonisation was controlled by the plants need for nitrogen which it can also receive through mycorrhizal associations. Therefore, it was hypothesised that AMF colonisation decreases for higher fertilisation rates..

There is no current recommended dose rate for AMF use in sports turf. Agricultural use is currently recommended at 4 kg ha^{-1} by Plantworks but this is a very different environment to a golf putting green. A paper by Berruti et al (2017) found that a dose rate of 10kg ha^{-1} was insufficient to cause colonisation in maize and lacked soil persistence in an arable field. However, no similar studies have been conducted on grassland or golf putting greens, so the optimal dose rate is currently unknown. It was hypothesised that the agricultural dose rate gives colonisation in turf grass.

Germination in orchids requires the aid of mycorrhiza in order to properly reproduce however there is currently very little research on the effects of mycorrhiza on the germination of other species. A study by Bryla & Koide (1990) on tomatoes showed AMF colonisation increased seed numbers due to higher fruit numbers, however on putting greens this effect would make little difference as grass is often mown too short to flower. Commercial mycorrhizal seed coats are currently sold as improving germination rates however there is little evidence to support this assertion. It was hypothesised that AMF have no effect on germination rate as they would need the root to colonise.

The aim of this chapter was to consider which management practises of sports turf could impact upon the efficacy of microbial inoculants, and how inoculants could be applied in this environment.

5.1.1 Summary of hypotheses

- Each grass species interacts differently to colonisation with different PGPR and AMF
- PGPR vary more in their effect on dry biomass and can be antagonistic.
- AMF colonisation decreases for higher fertilisation rates
- The agricultural dose rate of 4kg ha^{-1} gives colonisation in turf grass.
- AMF have no effect on germination rate as they would need the root to colonise.

5.2 Methods

5.2.1 Grass Species Mycorrhizal Consortium trial

Lolium perenne 2n, L. perenne 4n, Festuca rubra, F. r. trichophylla, F. r. commutata, Schedonorus phoenix (formerly *F. arundinacea), P. pratensis, Agrostis capillaris* and *A. stolonifera* were sown in 20cm pots at the manufacturers recommended rates shown in Appendix 2. Plants were grown according to the general pot set up (see Chapter 2: General Methods, section 2.4) in a polytunnel, with the following additions. Mycorrhizal inoculum (a consortium of 5 species, *Glomus spp A, Glomus spp B, Rhizophagus spp, Funneliformis spp A,* and *Funneliformis spp B*) provided by Plantworks was applied at a rate of 1g/pot for treated pots. There were four treated and four control replicates for each grass species.

5.2.2 Grass Species PGPR trial

The same nine grass species as section 5.2.1 were grown with six different PGPR treatments (*Azospirillum spp, Bacillus spp*, *Rhizobacterium spp, Gluconacetobacter spp, Pseudomonas spp* A, and *Pseudomonas spp* B) provided by Plantworks. Plants were grown according to the general pot set up (see Chapter 2: General Methods, section 2.4) in a polytunnel, with the following additions. No mycorrhizal inoculants were added. PGPR were added in liquid form, with 15ml of PGPR solution ($1x10^6$/ml) diluted in 1L of dechlorinated water. 20ml of each solution was added to each pot. Controls received 20ml of water. There were five treated and five control replicates for each grass species and PGPR combination. Only leaves were harvested as there was no viable way to stain roots for PGPR.

5.2.3 Mycorrhizal monocultures trial

F. r. rubra, A. capillaris and *A. stolonifera* were grown according to the general pot set up (see Chapter 2: General Methods, section 2.4) with the following alterations. Mycorrhizal inoculants of five monocultures (*Glomus spp A, Glomus spp B, Rhizophagus spp, Funneliformis spp A, Funneliformis spp B*) were applied to treated pots. There were five treated and five control replicates for each grass species and each monoculture.

5.2.4 Fertiliser trial

L. perenne (2n), F. r. trichophylla, P. pratensis and *A. stolonifera* were grown according to the general pot set up (see Chapter 2: General Methods, section 2.4) with the following alterations. There were five treated and five control replicates for each grass species and fertiliser dose rate. Fertiliser (J. Arthur

Bower's Liquid Lawn Food, NPK 14:7:7, ingredients in appendix 5 (section 9.6) was applied either at half recommended rate (20L/ha), recommended rate (40L/ha), double recommended rate (80L/ha) or not applied.

5.2.5 Mycorrhiza dose rate trial

F. r. trichophylla was grown according to the general pot set up (Chapter 2: General Methods, section 2.4) with the following alterations. Mycorrhizal inoculum (a consortium of five species *Glomus spp A, Glomus spp B, Rhizophagus spp, Funneliformis spp A, Funneliformis spp B*) was applied at the rates shown in Table 8. There were five treated and five control replicates for each dose.

Rate number	Rate in kg ha^{-1}	Rate per $9cm^2$ pot in g
1	2	0.00162
2	4	0.00324
3	8	0.00648
4	16	0.01296
5	32	0.02592
6	64	0.05184
7	128	0.10368

Table 8: The amount of inoculum applied for each rate in the dose rate trial.

5.2.6 Germination trial

This trial followed general pot set up (see Chapter 2: General Methods, section 2.4) with the following alterations. Petri dishes were filled with rootzone and five

seeds placed in each one. There were five species of grass *L. perenne 2n, F. r. rubra, P. pratensis, A. capillaris* and *A. stolonifera* used in the trial and 10 repeats of treated and control. Treated samples had 0.1g/petri dish of mycorrhizal inoculum added while control plates received the same amount of sterilised inoculum. The trial layout is shown in Figure 41 and ran for 14 days in a controlled temperature room. Petri dishes were checked daily for germination, which was defined as radical protrusion. After 14 days root and shoot length were measured for each seed using callipers. Germinating *L. perenne* seeds in a petri dish are shown in

Figure 42.

Figure 41: Trial layout for germination trial run in the controlled temperature room at Royal Holloway.

Figure 42: Germinating *L. perenne* seeds treated with mycorrhiza.

5.2.7 Statistical analysis

Differences between treatments in dry biomass were examined for each grass species with a one-way or two-way ANOVA, and the means separated using a Tukey test in R 3.6.0 (R Core Team 2019). Data was then analysed using Lorenz curves, the Gini coefficient (with a 95% confidence interval bootstrap) and Lorenz asymmetry coefficient to assess inequality between pots (see section 5.2.8: calculation of inequality).

Root length colonisation was analysed using a generalised linear model with binomial errors in R. This model was then checked for overdispersion and if

found to be overdispersed it was run again but with quasibinomial errors to consider random factors.

Differences between treatments in root or shoot length were examined for each species with a one-way ANOVA and the means separated using a Tukey test with the ‘multcomp’ package in R (R Core Team 2019).

Germination over time was analysed by plotting the germination percentage in a line graph and calculating the area under the germination progression curve (AUGPC) as described by French & Iyer-Pascuzzi (2018). These data were then analysed with a one-way ANOVA and the means separated using a Tukey HSD. Normality for all data was checked using QQplots and Shapiro-Wilk tests in R.

5.2.8 Calculation of Inequality

Inequality is a theory taken from economics to dictate the spread of wealth within a population. This concept was adapted by Jacob Weiner (Weiner & Solbrig 1984) for use in ecology to look at the equality of plant size. Inequality plots the cumulative percentage of biomass against the cumulative percentage of population to give a Lorenz curve. From this, the equality of plant biomass across a population can be measured to show if there is an inequality in plant size across a trial and whether treatments affect this (Weremijewicz & Janos (2013), Hanley & Groves (2002)). Inequality can also be quantified as a Gini coefficient developed by Corrado Gini in 1912.

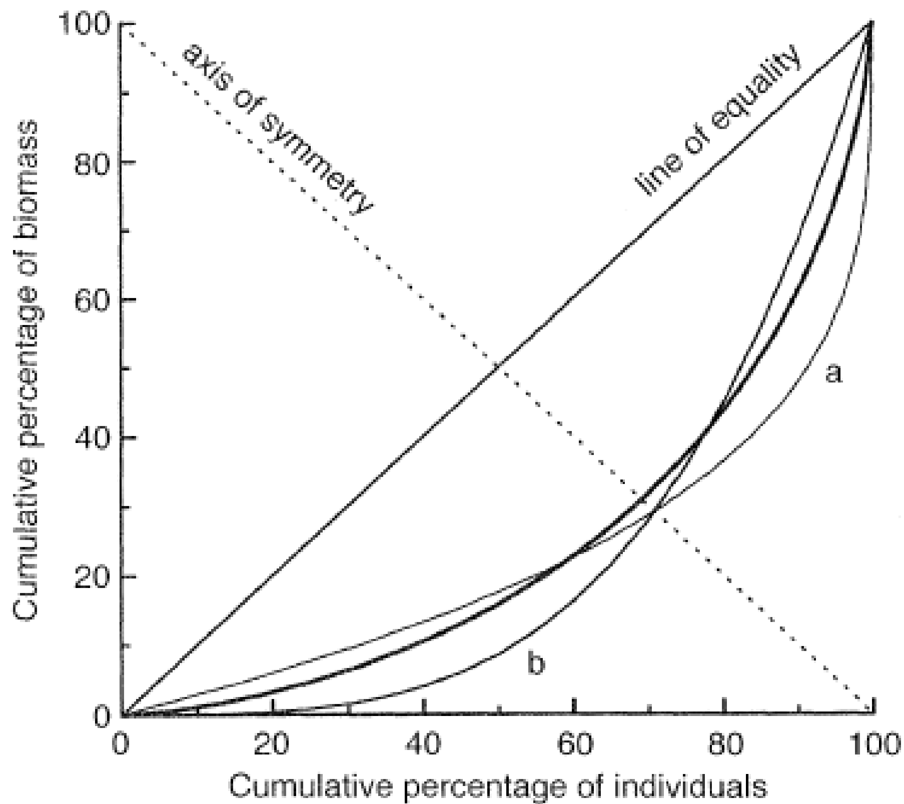


Figure 43: Taken from Damgaard & Weiner (2000), a diagram of three Lorenz curves, the bold line showing a symmetrical case and the two unbold lines showing asymmetric cases.

Figure 43 shows Lorenz curves from Damgaard & Weiner (2000), demonstrating how these curves can be symmetrical or asymmetrical. An asymmetrical line such as line a suggests a higher proportion of high biomass plants, whereas line b suggests a higher proportion of low biomass plants. The distance of the Lorenz curve from the line of equality dictates how much inequality is present between the dry biomass of different plants. The Gini coefficient measures the space between the line of equality and the Lorenz curve but doesn’t comment on the symmetry of the line, so two different Lorenz curves can give the same Gini coefficient.

Inequality was calculated using the R package *ineq* utilising dry biomass data collected from trials. Inequality was expressed as Lorenz curves, Lorenz asymmetry coefficient and the Gini coefficient. The Gini coefficient was bootstrapped to give a 95% confidence interval to allow separation between treatments. Inequality was used to determine how well different microbes were interacting with the rhizosphere by how much they increased or decreased inequality. Microbes sometimes interacted with all plants equally to reduce inequality, induced competition between plants thus increasing inequality or didn't have any effect at all (Gange & Gadhave 2018).

5.3 <u>Results</u>

5.3.1 Grass Species Mycorrhizal Consortium trial

Dry biomass increased following mycorrhizal inoculation for all species except for *A. stolonifera* which has no effect, and *Poa pratensis* which decreased ($F_{1,54}$=29.28, P<0.05) as shown in Figure 44. The extent of change in dry biomass following colonisation was dependent on the grass species ($F_{8,54}$=37.35, P=0.05), with *A. capillaris* nearly doubling in dry biomass following colonisation, while the increase was less extreme for *L. perenne (2n)*. Percentage root length colonisation increased for all grass species when treated with mycorrhizal inoculum ($Z1,_{71}$=9.875, P<0.05), shown in Figure 45.

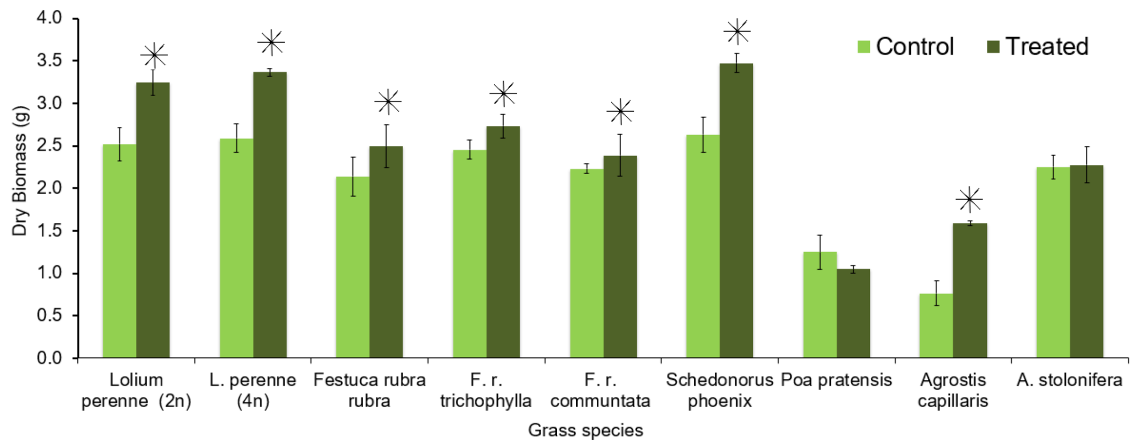


Figure 44: The change in dry biomass for different grass species when treated with a mycorrhizal inoculant. The error bars show standard error. Starred bars were significantly different from the control.

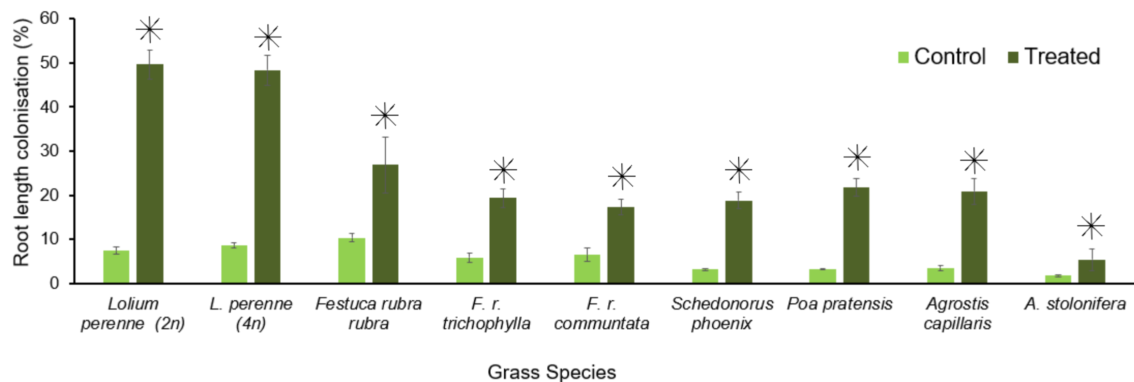


Figure 45: The change in percentage root length colonisation for different grass species when inoculated with mycorrhiza. The error bars show standard error. Starred bars were significantly different from the control.

The dry biomass of the grass in each pot was more equal between pots upon the addition of mycorrhizal inoculum in all grass species except for *Festuca rubra commutata*, which showed an increase in inequality. This can be seen in Figure 46, where *F. r. commutata* moves further from the line of equality and *A.*

capillaris moves closer to the line of equality, graphs for other species are not shown but Gini (with 95% confidence interval) and Lorenz Asymmetry Coefficients are shown in Table 9. The comparison for Gini coefficient confidence intervals demonstrated a significant decrease in inequality upon AMF colonisation for *P. pratensis, A. capillaris* and *L. perenne (4n)* and an increase in inequality for *F. r. commutata.*

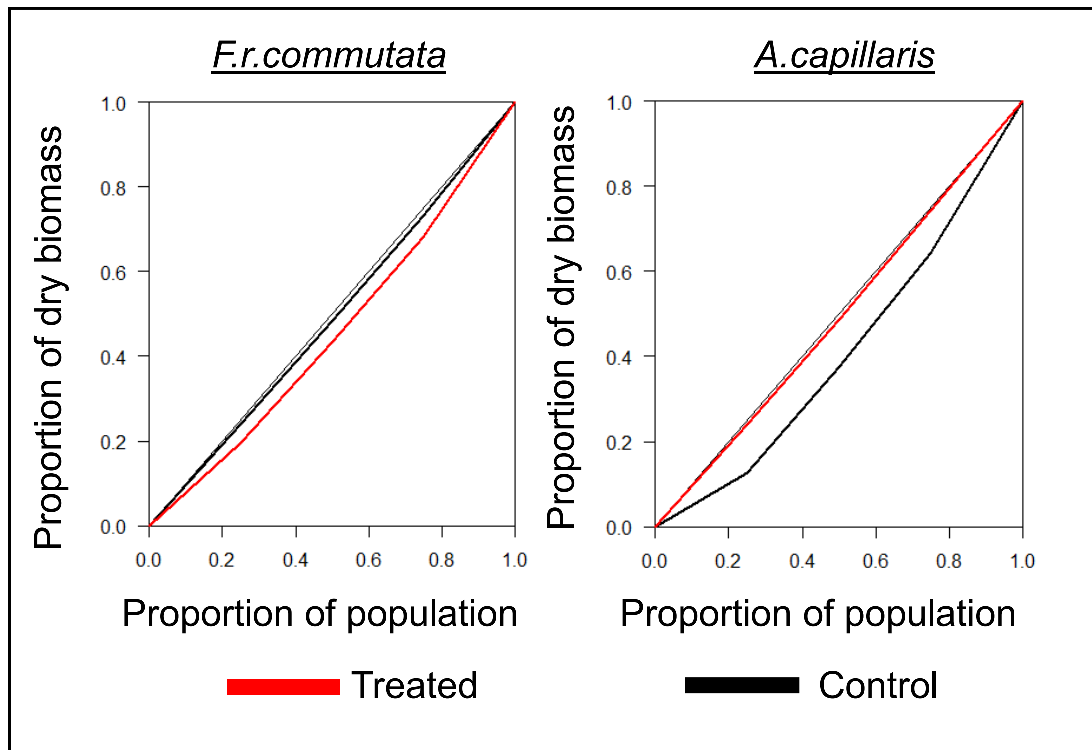


Figure 46: Lorenz curves showing the effect of mycorrhizal inoculum on the inequality of *F. r. commutata* and *A. capillaris.*

Grass Species		Gini Coefficient (95% Confidence Interval)	LAC
Lolium perenne (2n)	Treated	0.0577 (0.0351-0.108)	1.0774
	Control	0.0971 (0.0574-0.183)	1.0016
L. perenne (4n)	Treated	**0.0144** (0.0064-0.0292)	0.911
	Control	0.0758 (0.0430-0.149)	1.504
Festuca rubra	Treated	0.129 (0.0898-0.232)	1.234
	Control	0.139 (0.0894-0.255)	1.110
F. r. trichophylla	Treated	0.0627 (0.0354-0.121)	0.938
	Control	0.055 (0.0324-0.106)	1.500
F.r.commutata	Treated	**0.126** (0.0778-0.238)	1.463
	Control	0.0292 (0.0124-0.0611)	1.576
Schedonorus phoenix	Treated	0.0419 (0.0304-0.0739)	1.221
	Control	0.0953 (0.0677-0.172)	1.241
Poa pratensis	Treated	**0.0547** (0.0362-0.0997)	1.148
	Control	0.1896 (0.123-0.359)	1.392
Agrostis capillaris	Treated	**0.0209** (0.0143-0.0377)	0.869
	Control	0.236 (0.116-0.462)	0.869
A. stolonifera	Treated	0.114 (0.0704-0.219)	1.476
	Control	0.0768 (0.0389-0.151)	0.950

Table 9: The Gini Coefficient with 95% Confidence Interval and Lorenz asymmetry coefficient (LAC) for different grass species when treated with a consortium of AMF. Bold results represent treatments significantly different from their control.

5.3.2 Grass Species PGPR trial

Lolium perenne (2n) showed significant changes in dry biomass when PGPR species were added (F_{11}=4.111, P<0.05) as shown in Figure 47. *Azospirillum* species caused a significant decrease in dry biomass (P<0.05). However, the lorenz curve for *Azorospirillum* showed that inequality was reduced, meaning treated pots were a consistently smaller dry biomass than untreated pots. This is shown in Figure 48 and the Gini coefficients and Lorenz asymmetry coefficients for all grass species and PGPR trialled are shown in appendix 6 (section 9.8).

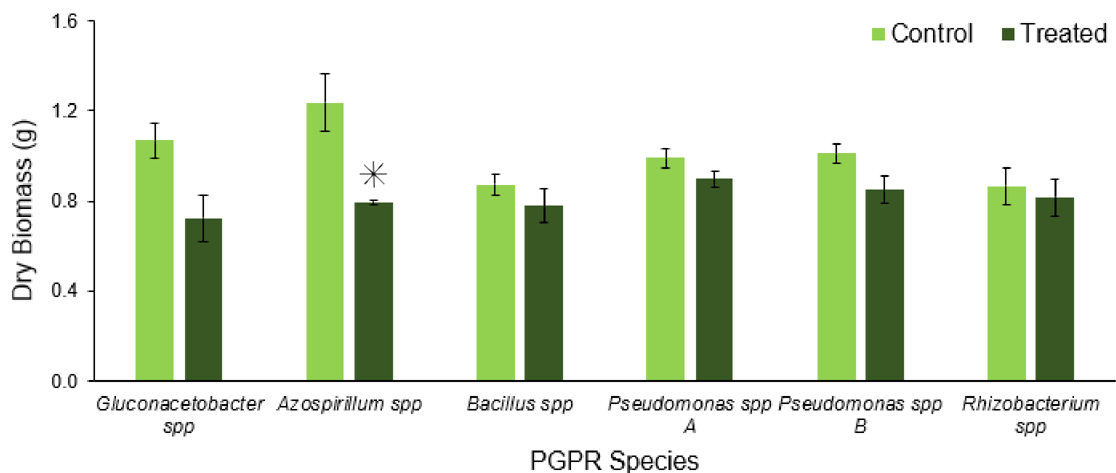


Figure 47: The changes in dry biomass when PGPR species were added to *Lolium perenne (2n).* The error bars show standard error. Starred bars were significantly different from the control.

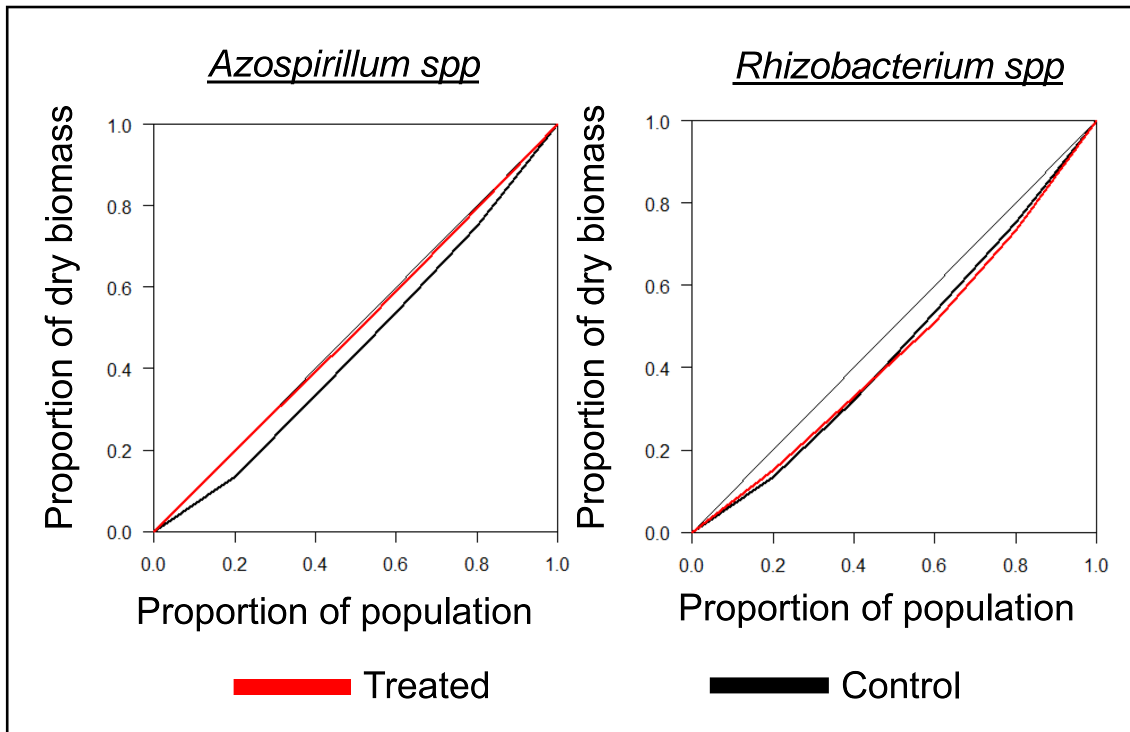


Figure 48: The Lorenz curves showing the effect of *Azospirillum spp* and *Rhizobacterium spp* inoculation to *L.perenne (2n).*

Significant increases in dry biomass were also seen for *L. perenne* (4n) (F_{11}=6.482, P<0.05, appendix 9.7) and *S. phoenix* (χ^2_{11}=34.869, P<0.05, appendix 9.7*A. capillaris* (χ^2_{11}=25.906, P<0.05) in Figure 49 showed a significant decrease upon addition of *Pseudomonas spp* A and *A. stolonifera* increased when inoculated with *Rhizobacterium spp* (χ^2_{11}=20.417, P<0.05) shown in Figure 50.

No significant changes in dry biomass were shown upon the addition of PGPR treatments for *P. pratensis* (χ^2_{11}=14.188, P>0.05, appendix 9.7), *F. r. trichophylla* (χ^2_{11}=16.499, P>0.05, appendix 9.7), *F. r. rubra* (F_{11}=0.075, P>0.05, appendix 9.7) and *F. r. commutata* (F_{11}=1.356, P>0.225, appendix 9.7).

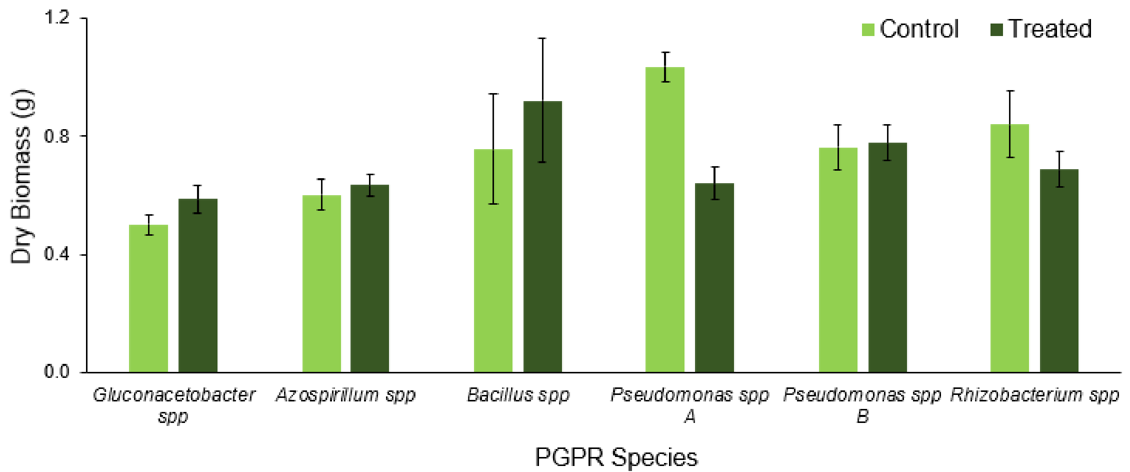


Figure 49:The changes in dry biomass when PGPR species are added to *Agrostis capillaris.* The error bars show standard error.

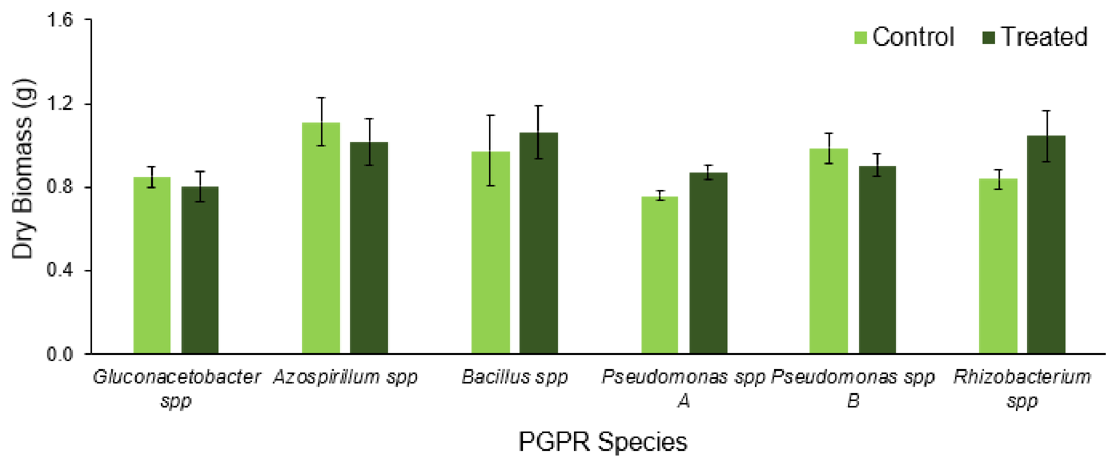


Figure 50: The changes in dry biomass when PGPR species are added to *Agrostis stolonifera*. The error bars show standard error.

Despite the increase in dry biomass shown when *A.stolonifera* was treated with *Rhizobacterium spp* in Figure 50, there was actually an increase in the inequality between treated pots (shown in Figure 51). The treated line on the lorenz curve bulges at the top end of the proportion of biomass axis (Lorenz asymmetry coefficient of 1.424), showing a disproportionate amount of larger biomass pots which account for the increase in dry biomass overall. However,

not all PGPR treatments caused a change in equality as shown for *Pseudomonas species B.*

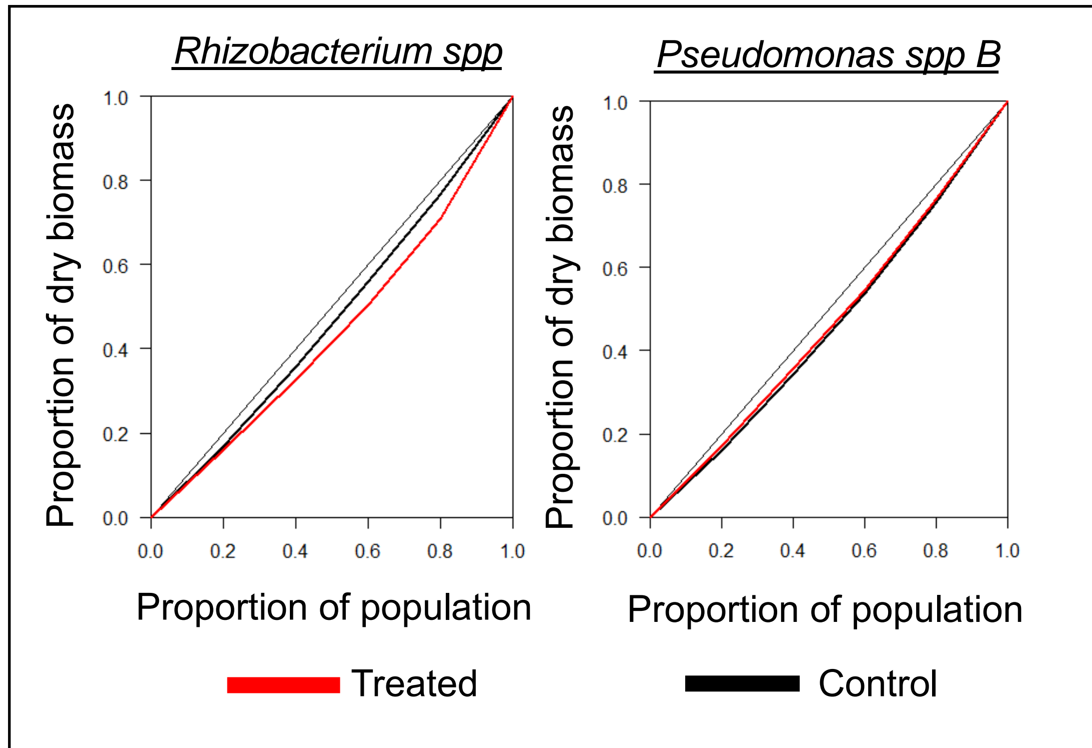


Figure 51: Lorenz curves showing the effect of treatment with *Rhizobacterium spp* and *Pseudomonas spp 2* on *A. stolonifera.*

In appendix 6 (section 9.8) significant decreases in inequality were shown in *L. perenne (2n)* treated with *Azospirillum spp,* and *S. phoenix* treated with *Gluconacetobacter spp.* Significant increases in inequality were observed for *L. perenne (4n)* treated with *Rhizobacterium spp*, and *S. phoenix* treated with *Rhizobacterium spp*.

5.3.3 Mycorrhizal monocultures trial

Overall, the addition of mycorrhizal inoculants caused a significant increase in the dry biomass of *F. r. rubra* ($F_{1,44}$=8.480, P<0.05) compared to control

treatments. The extent of change in dry biomass depended on the AMF species applied ($F_{4,44}$=2.902, P<0.05), with *Funneliformis A* ($F_{1,8}$=12.09, P<0.05) and *Glomus spp B* ($F_{1,8}$=12.4, P<0.05) both showing significant increases while other AMF monocultures showed little change.

A. capillaris experienced significant increases in dry biomass for *Rhizophagus spp* ($F_{1,8}$=9.737, P<0.05) and *Funneliformis spp B* ($F_{1,8}$=7.558, P<0.05). Other species of AMF did not significantly affect the dry biomass of *A. capillaris.* For *A. stolonifera* mycorrhizal inoculants increased dry biomass ($F_{1,43}$=4.112, P<0.05), particularly *Funeliformis spp B* ($F_{1,8}$=19.46, P<0.05). These results are shown in Figure 52.

For all grass species and monocultures, treatment with a mycorrhizal inoculant significantly increased percentage root length colonisation. The statistics for these results are summarised in Table 10, and the data visualised in Figure 53.

	Glomus spp A	*Rhizophagus spp*	*Funneliformis spp A*	*Glomus spp B*	*Funneliformis spp B*
Festuca rubra	$Z_{1,8}$=7.297 P<0.05	$Z_{1,8}$=4.253 P<0.05	$Z_{1,8}$=7.557 P<0.05	$Z_{1,8}$=7.898 P<0.05	$Z_{1,8}$=7.253 P<0.05
Agrostis capillaris	$Z_{1,8}$=4.889 P<0.05	$Z_{1,8}$=4.200 P<0.05	$Z_{1,8}$=5.643 P<0.05	$Z_{1,8}$=3.779 P<0.05	$Z_{1,8}$=4.511 P<0.05
Agrostis stolonifera	$Z_{1,8}$=4.262 P<0.05	$Z_{1,8}$=3.435 P<0.05	$Z_{1,8}$=3.696 P<0.05	$Z_{1,8}$=3.773 P<0.05	$Z_{1,8}$=3.114 P<0.05

Table 10: GLM outputs for the change in percentage root length colonisation for different grass species upon the addition of different mycorrhizal monocultures.

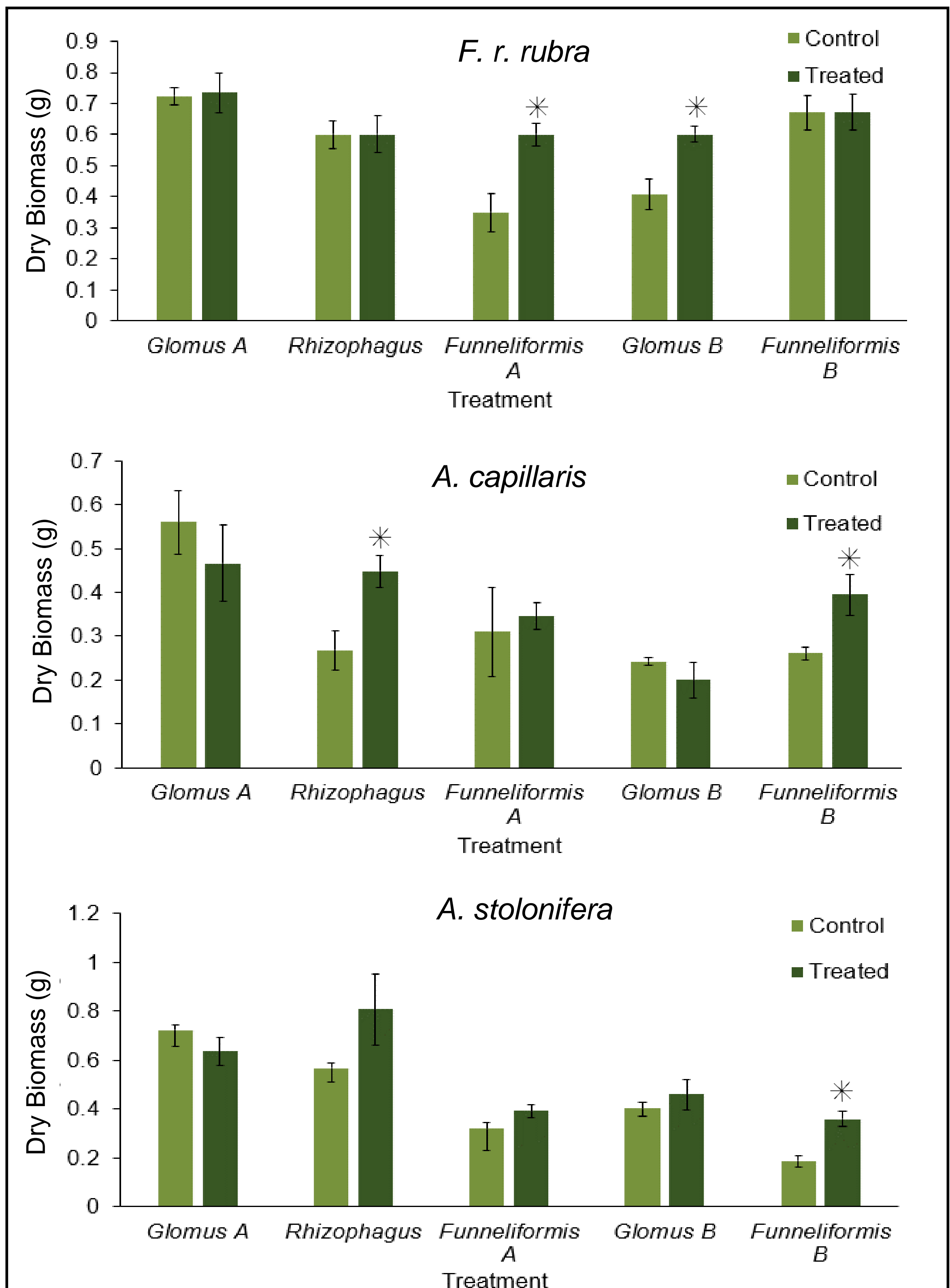


Figure 52: The change in dry biomass following inoculation with different mycorrhizal monocultures in *F. r. rubra, A. capillaris* and *A. stolonifera*. The error bars show standard error. Starred bars were significantly different from the control.

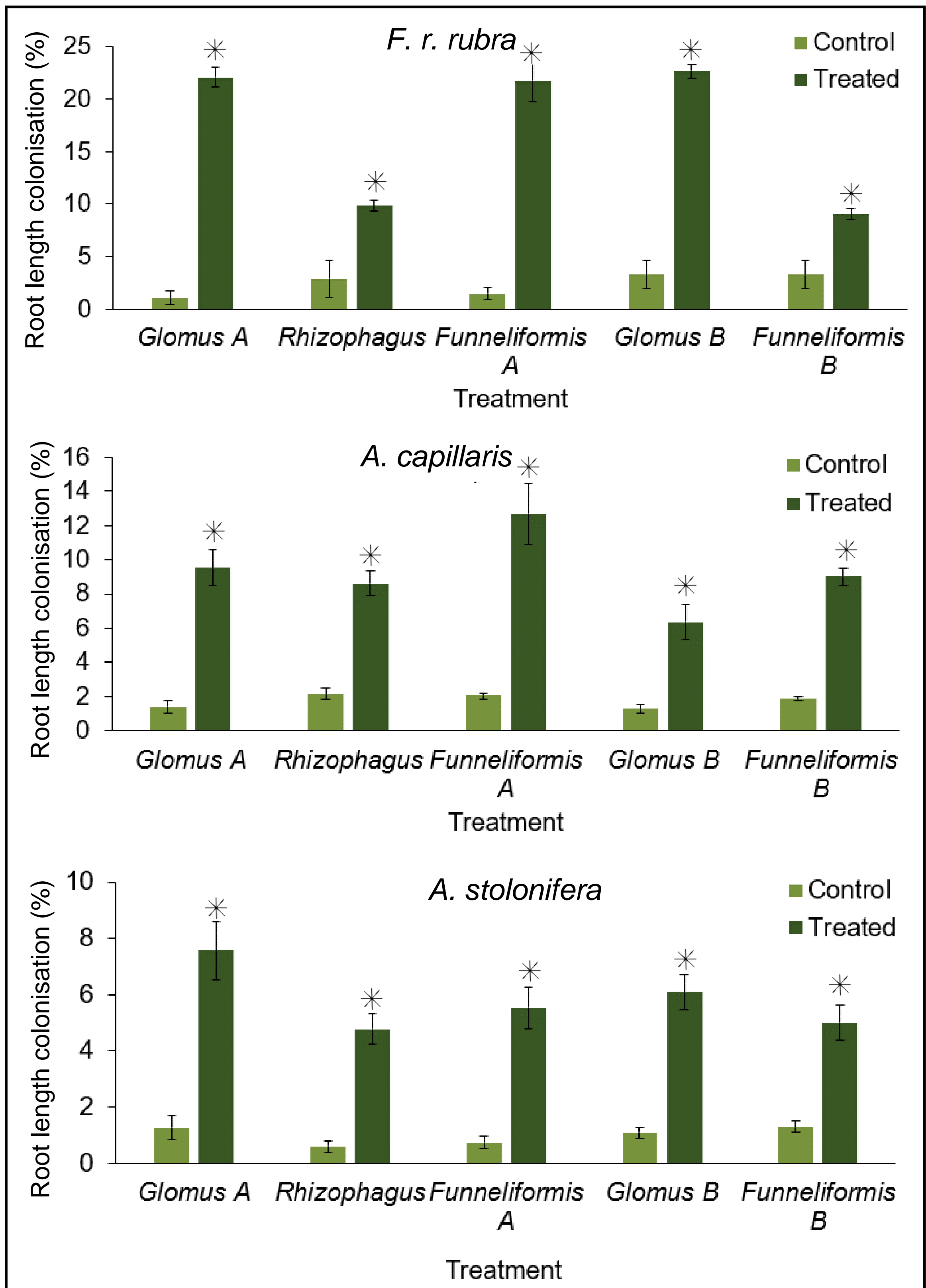
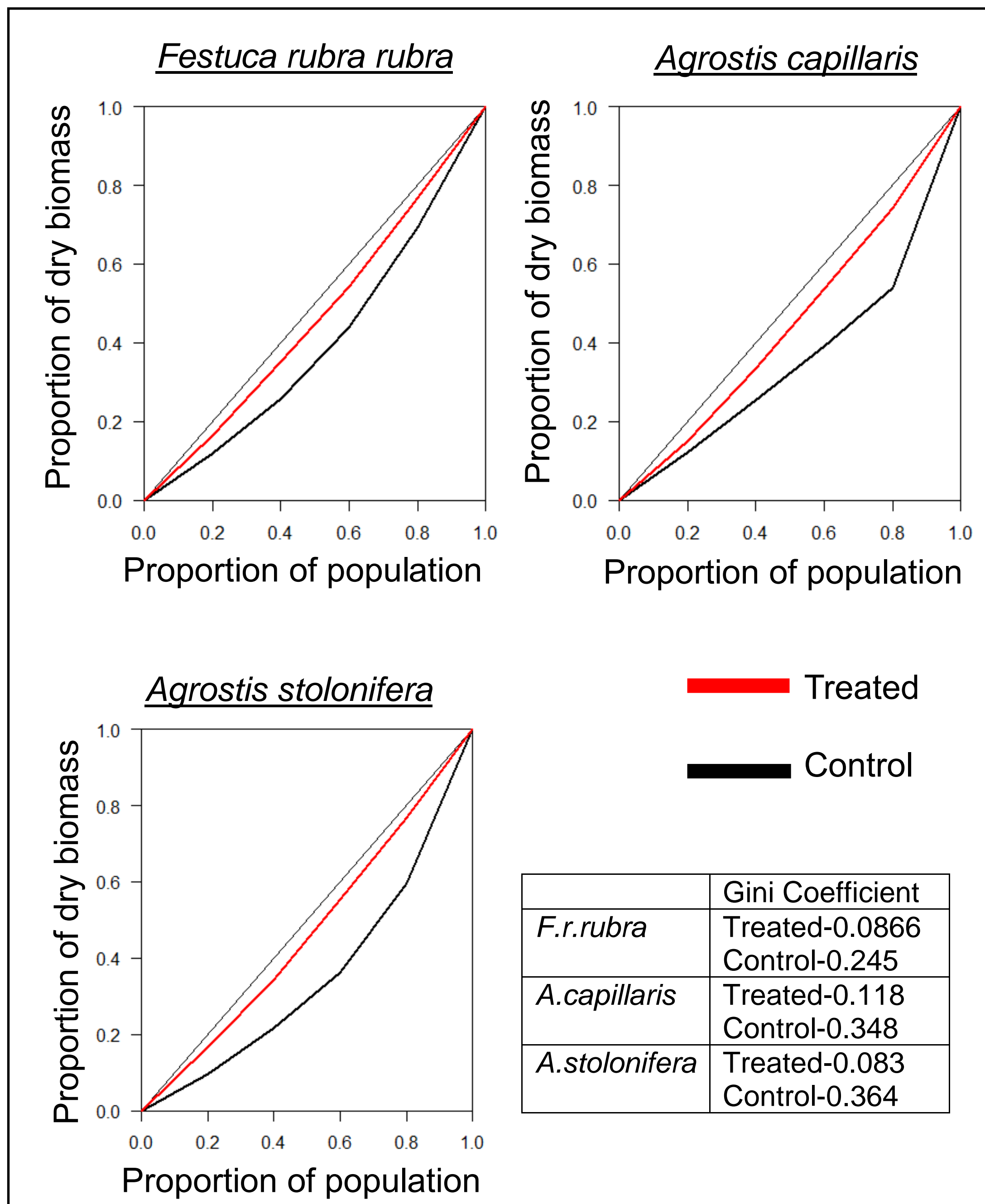


Figure 53: The change in percentage root length colonisation following inoculation with different mycorrhizal monoculture in *F. r. rubra, A. capillaris* and *A. stolonifera.* The error bars show standard error. Starred bars were significantly different from the control

As the error bars in Figure 52 show, there were a lot of changes in variability between different treatments depending on the species, the statistics for these are summarised in appendix 7 (section 9.9). The only AMF monoculture that consistently reduced inequality for all grass species tested was *Funneliformis spp A*, as shown in Figure 54.



	Gini Coefficient
F.r.rubra	Treated-0.0866 Control-0.245
A.capillaris	Treated-0.118 Control-0.348
A.stolonifera	Treated-0.083 Control-0.364

Figure 54: The changes in the inequality of dry biomass per pot for different grass species when inoculated with *Funneliformis spp A.* The table shows Gini coefficients quantifying the difference from the line of equality.

5.3.4 Fertiliser trial

Application of mycorrhizal inoculant significantly increased the dry biomass of treated plots ($F_{1,24}$=6.358, P<0.05) in *A. stolonifera*. Higher fertiliser rates also increased dry biomass ($F_{3,24}$=6.782, P<0.05). These data are shown in Figure 55. Root length colonisation increased with application of the mycorrhizal inoculant ($Z_{1,27}$=7.604, P<0.05) but decreased with high rates of fertilisation ($Z_{3,27}$=4.545, P<0.05), as shown in Figure 59.

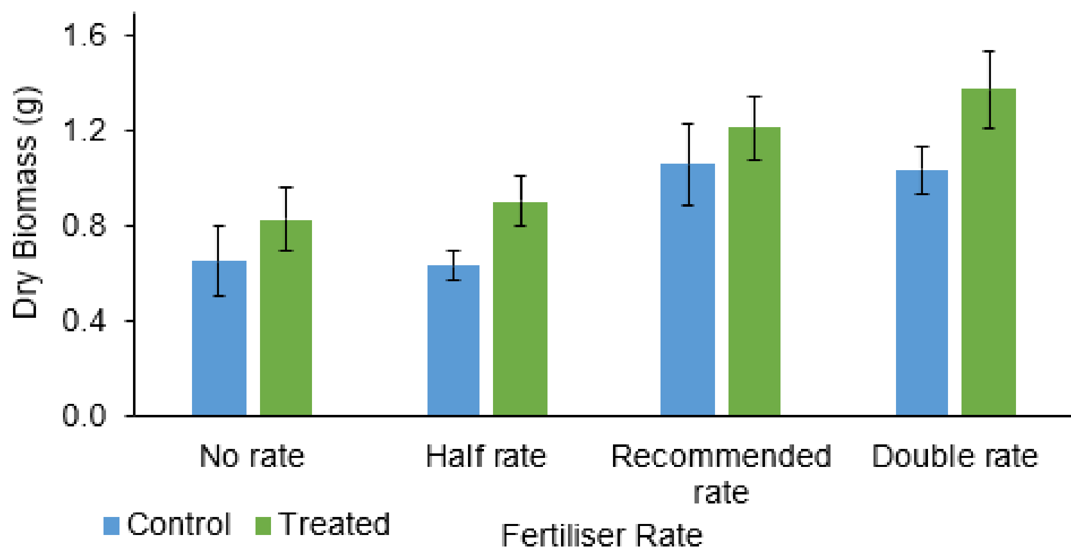


Figure 55: The change in dry biomass in *A. stolonifera* from treatment with mycorrhizal inoculant and different fertiliser rates. Error bars show standard error.

Application of mycorrhizal inoculant significantly increased the dry biomass of treated *P. pratensis* ($F_{1,24}$=14.532, P<0.05) for all fertiliser rates. Higher fertiliser rate also increased dry biomass ($F_{3,24}$=5.474, P<0.05). These data are shown in Figure 56. Root length colonisation increased with application of the mycorrhizal inoculant ($Z_{1,27}$=9.062, P<0.05). The fertiliser rate also decreased percentage root length colonisation ($Z_{3,27}$=3.663, P<0.05), as shown in Figure 59.

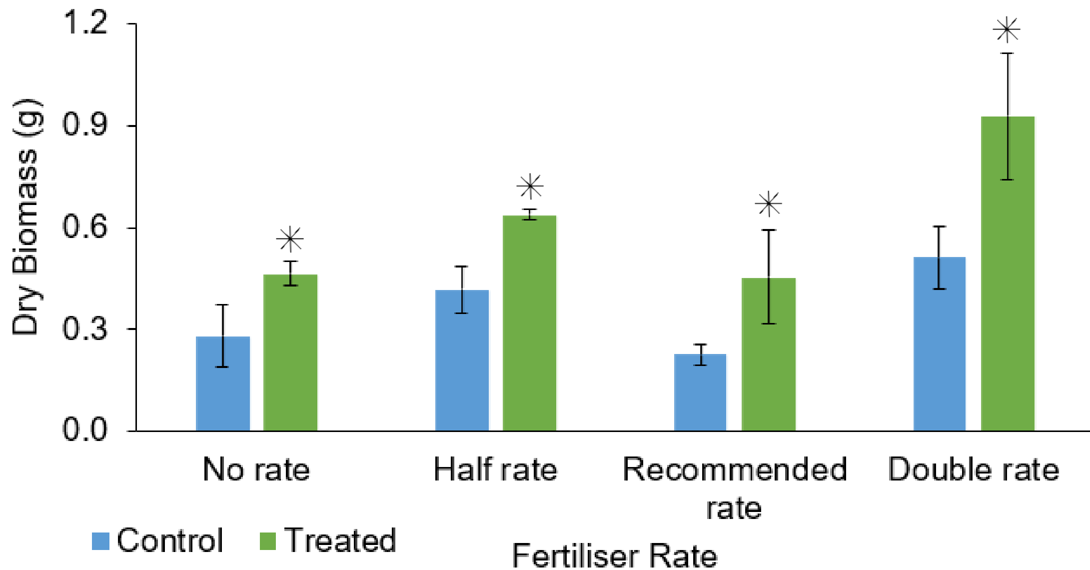


Figure 56: The change in dry biomass in *P. pratensis* from treatment with mycorrhizal inoculant and different fertiliser rates. Error bars show standard error. Starred bars showed significant difference from the control.

In *F. r. trichophylla* application of mycorrhizal inoculant had no significant effect on dry biomass for recommended and double rates of fertiliser($F_{1,24}$=2.141, P>0.05), but increased dry biomass at half rate fertilisation ($F_{1,5}$=8.564, P<0.05) and no fertilisation ($F_{1,6}$=2.869, P>0.05) . Increasing fertiliser rate increased dry biomass ($F_{3,24}$=3.479, P<0.05). These data are shown in Figure 57. Root length colonisation increased with application of the mycorrhizal inoculant ($t_{1,27}$=2.888, P<0.05) however, fertiliser rate had no effect on the root length colonisation ($Z_{3,27}$=0.316, P>0.05) as shown in Figure 59.

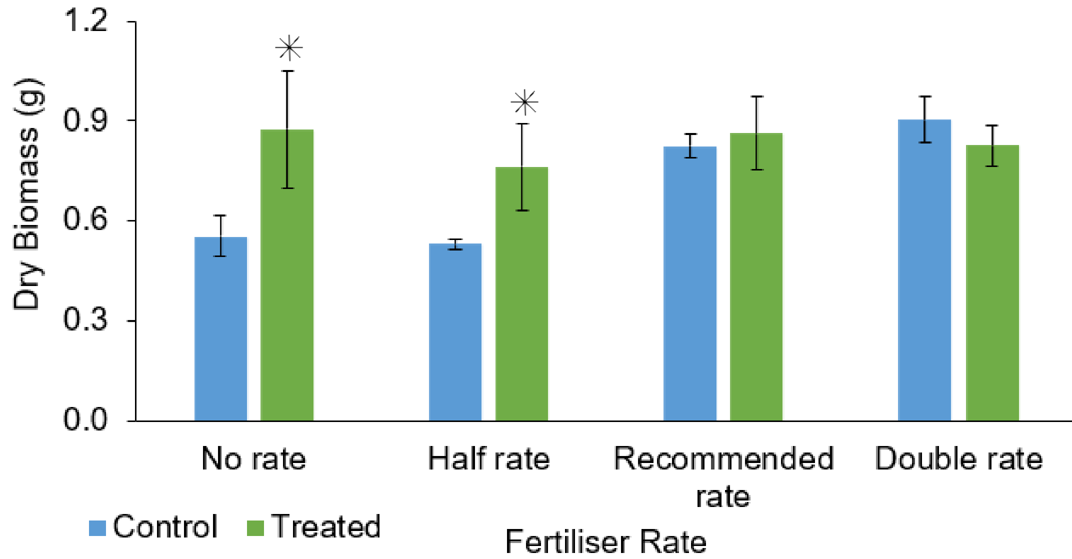


Figure 57: The change in dry biomass in *Festuca rubra trichophylla* from treatment with mycorrhizal inoculant and different fertiliser rates. Error bars show standard error. Starred bars show significant difference from the control.

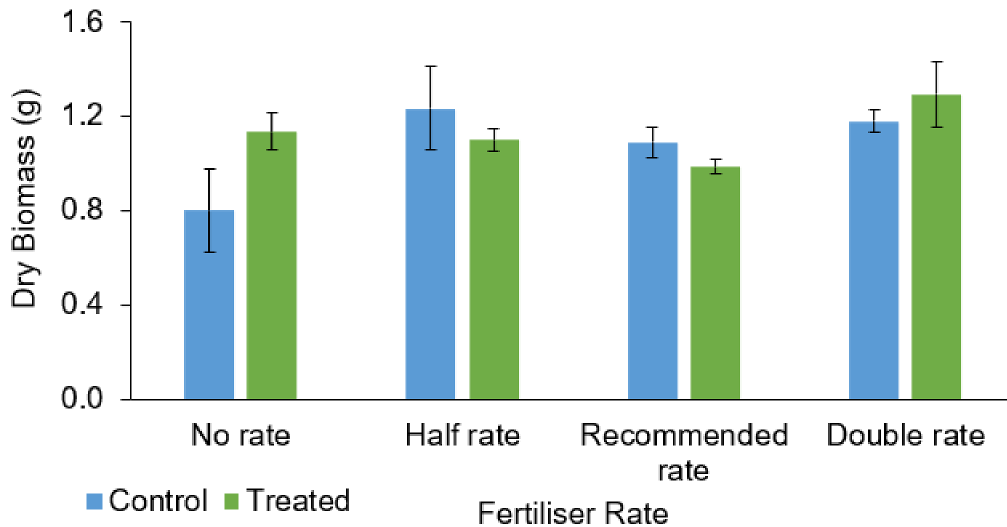


Figure 58: The change in dry biomass in *Lolium perenne (2n)* from treatment with mycorrhizal inoculant and different fertiliser rates. Error bars show standard error.

In *L. perenne (2n)* mycorrhizal inoculant had no significant effect on dry biomass ($F_{1,24}$=0.10, P>0.05) but fertiliser increased it ($F_{3,24}$=3.109, P<0.05).

These data are shown in Figure 58. Percentage root length colonisation increased with the application of the mycorrhizal inoculant ($Z_{1,27}$=11.507, P<0.05) while it decreased with increasing fertiliser rate ($Z_{3,27}$=4.368, P>0.05), as shown in Figure 59.

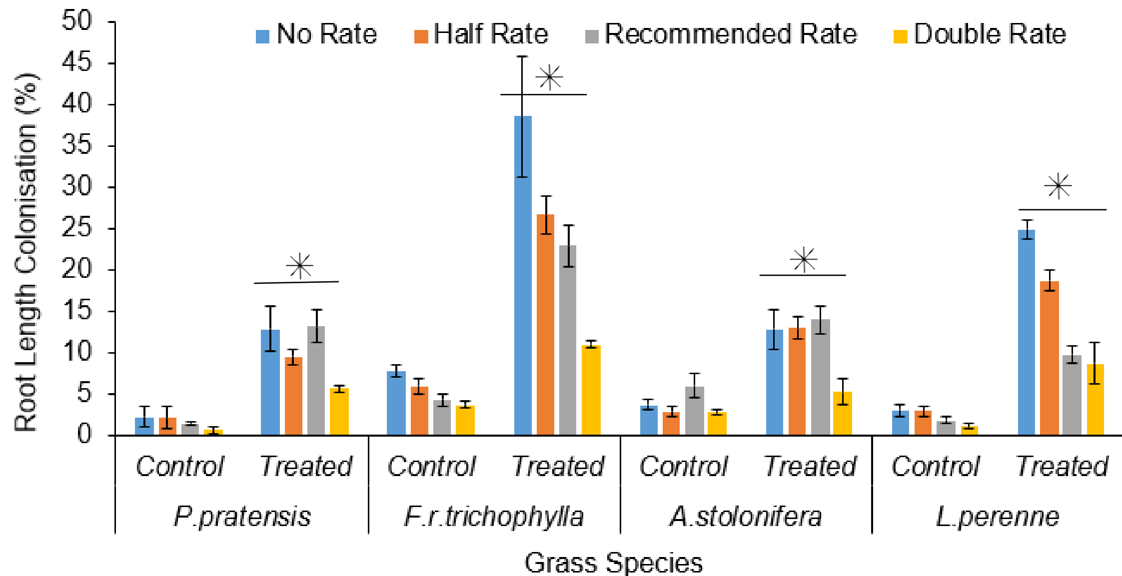


Figure 59: The change in % root length colonisation for pots treated with mycorrhizal inoculant and different fertiliser rates. Error bars show standard error. Starred bars show significant difference from the control.

5.3.5 Dose rate trial

Treatment with mycorrhizal inoculant significantly increased percentage root length colonisation for all dose rates ($Z_{1,62}$=10.358, P<0.05). Average root length colonisation increased as dose rate increased (shown in Figure 60) except for a slight decrease at 16Kgha^{-1} ($Z_{6,62}$=4.096, P<0.05).

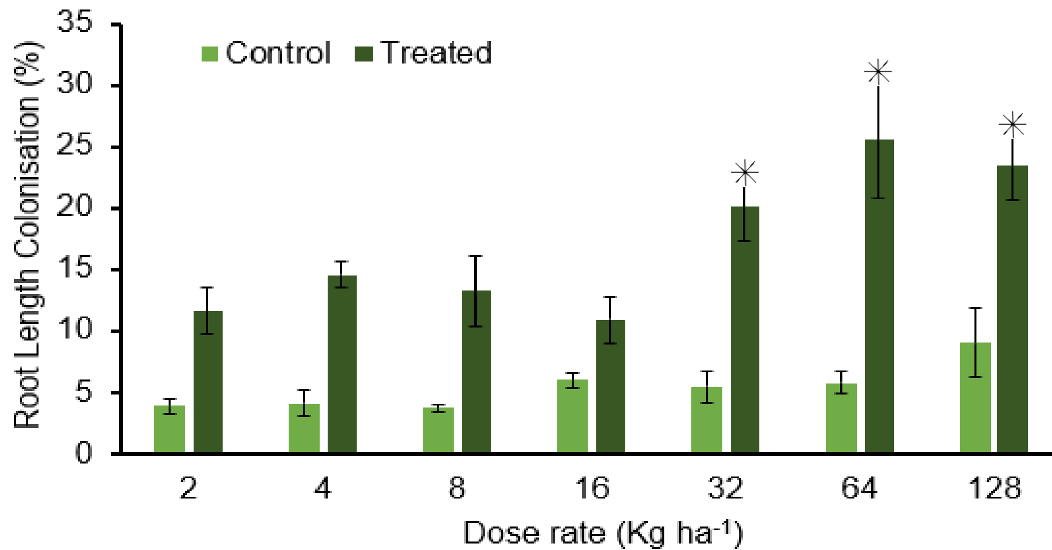


Figure 60: The change in % root length colonisation in *F. r. trichophylla* for different dose rates of AMF inoculant. The error bars show standard error.

Different doses of mycorrhizal inoculant did not significantly affect the dry biomass of *F. r. trichophylla* ($F_{6,56}$=1.204, P>0.05), except for 32kg/ha ($F_{1,8}$=5.787, P<0.05) as shown in Figure 61. A rate of 64kg ha^{-1} also caused an increase though this was not found to be significant ($F_{1,8}$=3.034, P=0.12).

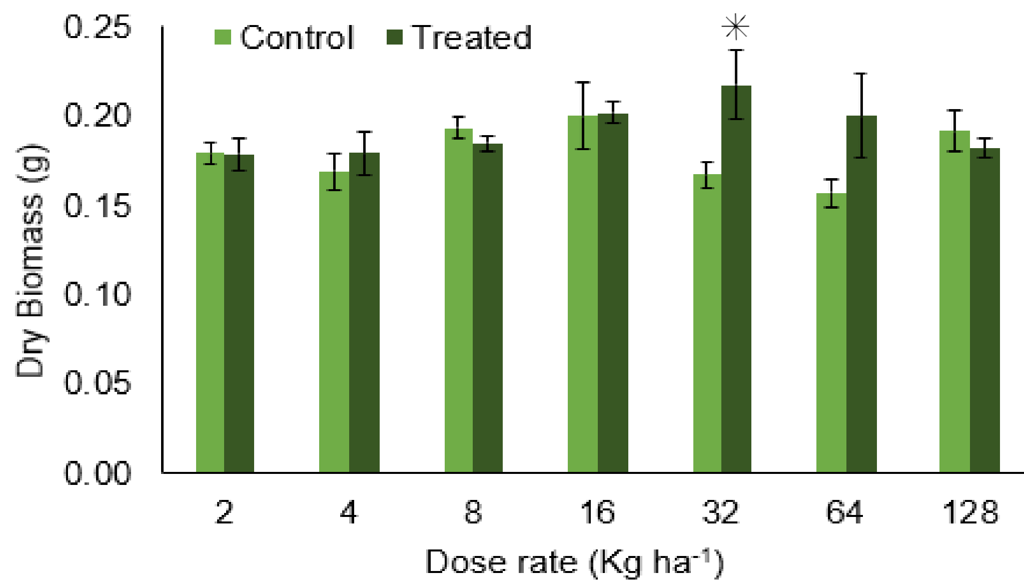


Figure 61: The dry biomass of *F. r. trichophylla* for different dose rates of mycorrhizal inoculant. The error bars show standard error.

Grass Species	Treatment	Final Germination/%
Lolium perenne (2n)	Mycorrhiza	100
	Control	100
Poa pratensis	Mycorrhiza	94
	Control	94
Festuca rubra rubra	Mycorrhiza	94
	Control	90
Agrostis Capillaris	Mycorrhiza	92
	Control	90
Agrostis stolonifera	Mycorrhiza	92
	Control	100

Table 11: The final germination percentage for different grass species when treated with mycorrhizal inoculant.

5.3.6 Germination trial

Final percentage germination (shown in Table 11) showed no significant difference between mycorrhizal and control for *L. perenne* ($t_{2,19}=0$, $P>0.05$), *P. pratensis* ($t_{2,19}=4.693$, $P>0.05$), *F. r. rubra* ($t_{2,19}=4.693$, $P>0.05$), *A. capillaris* ($t_{2,19}=0.354$, $P>0.05$) or *A. stolonifera* ($t_{2,19}=0.006$, $P>0.05$).

The area under the germination progression curve was calculated using the graphs plotted in Figure 62. While a significant difference in germination was shown between grass species ($F_{4,90}=62.333$, $P<0.05$), there was no significant difference between mycorrhizal and control ($F_{1,90}=0.635$, $P>0.05$).

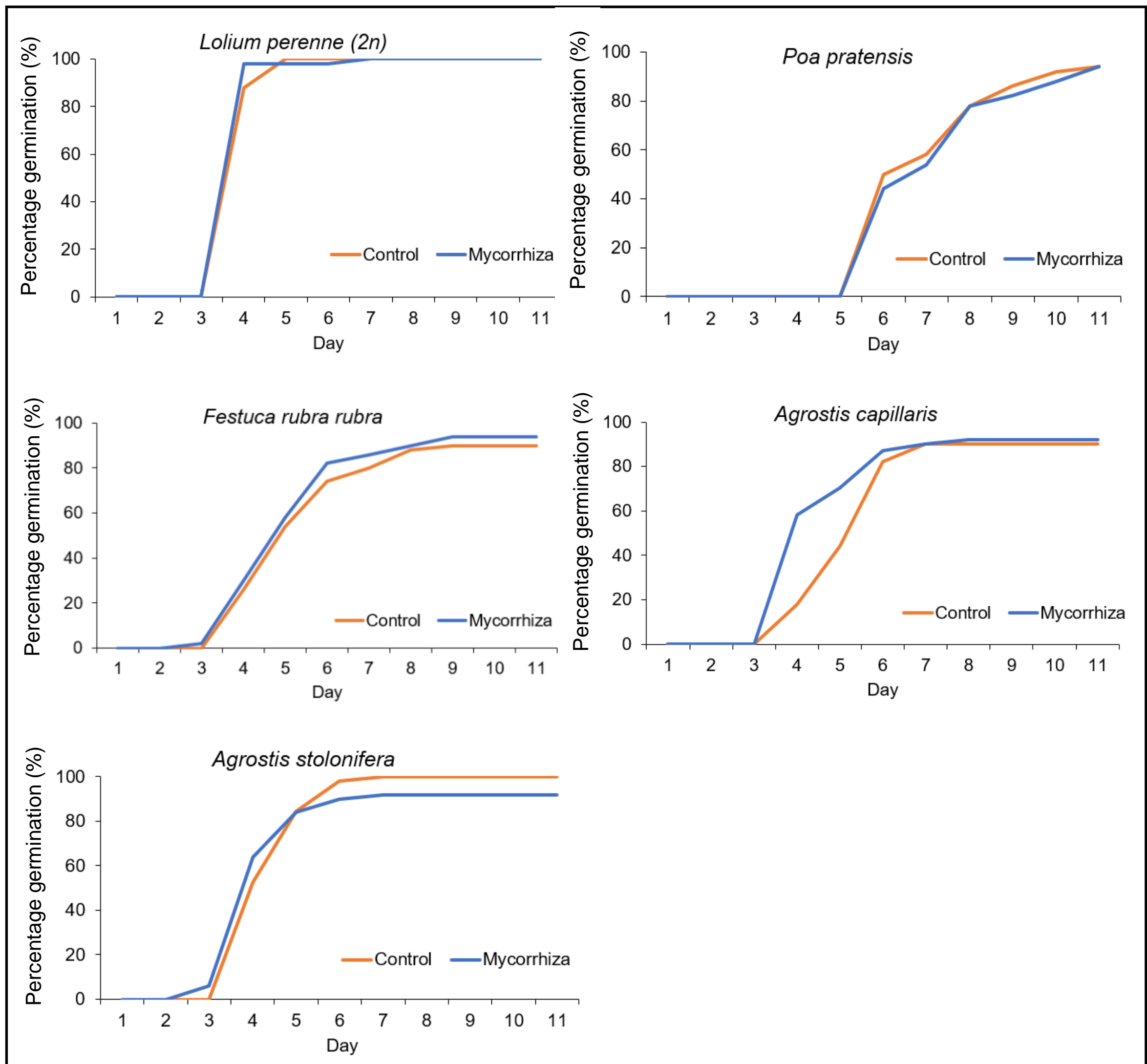


Figure 62: The germination over time for different grass species treated with mycorrhizal inoculant.

Shoot length after 14 days decreased upon the addition of mycorrhizal inoculant for *L. perenne* ($F_{1,96}$=8.806, P<0.05) and *A. stolonifera* ($F_{1,101}$=13.92, P<0.05). *A. capillaris* increased in shoot length ($F_{1,107}$=5.64, P<0.05) and *P. pratensis* ($F_{1,88}$=0.326, P>0.05) and *F. r. rubra* experienced no change ($F_{1,89}$=2.72, P>0.05). There was also a significant difference in shoot length between grass species ($F_{4,479}$=498.173, P<0.05) and the grass species affected the extent to

which mycorrhiza changed shoot length ($F_{4,479}$=5.879, P<0.05). These data were visualised in Figure 63.

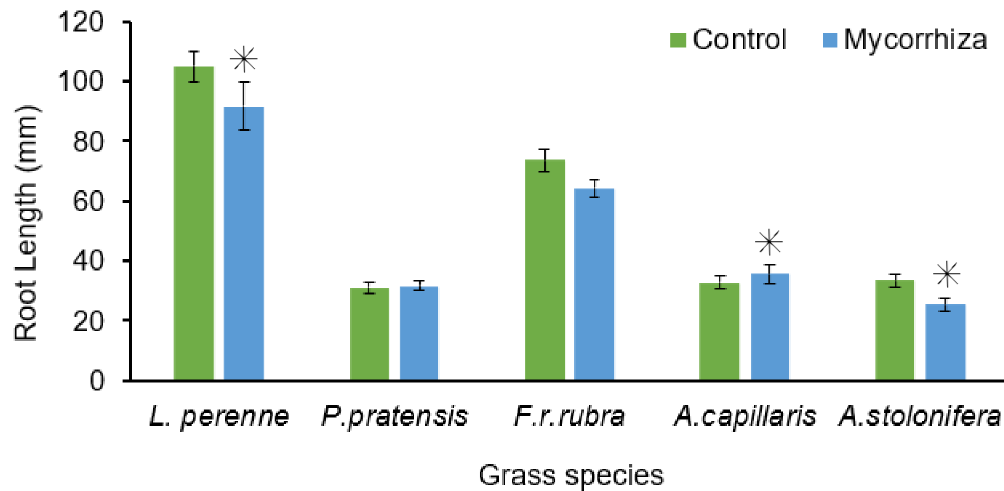


Figure 63: The difference in average shoot length (mm) for different grass species following colonisation with a mycorrhizal inoculant. Error bars show standard error.

Root length after 14 days decreased upon the addition of mycorrhiza for *F. r. rubra* ($F_{1,89}$=4.51, P<0.05) and *A. stolonifera* ($F_{1,101}$=7.11, P>0.05) as shown in Figure 64. *L. perenne* experienced a decrease that was close to significance ($F_{1,96}$=3.868, P=0.052) but *A. capillaris* ($F_{1,105}$=1.53, P>0.05) and *P. pratensis* ($F_{1,88}$=0.043, P>0.05) showed no change. Root length changed for different grass species ($F_{4,479}$=113.708, P<0.05) and the grass species affected the extent to which mycorrhiza changed root length ($F_{4,479}$=2.894, P<0.05).

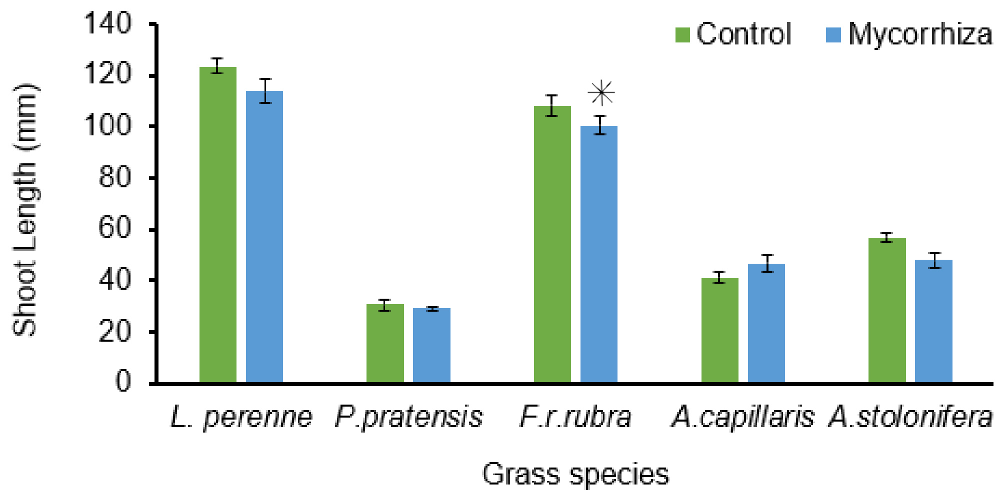


Figure 64: The difference in root length (mm) for different grass species following colonisation with a mycorrhizal inoculant. Error bars show standard error.

5.4 Discussion

5.4.1 Grass species mycorrhizal consortium and mycorrhizal monocultures trial

The results of grass colonisation with a mycorrhizal consortium are concurrent with existing research, in that AMF will colonise different grass species to different extents (Gustafon & Casper 2006, Gadhave et al 2016). This same result was then found with monocultures of different species of AMF, showing that both grass species and AMF species make a difference to colonisation. Gange (1994) had previously shown that different grass species experienced different percentages of colonisation in a golf putting green, but this trial expanded upon this to consider a wider range of turfgrasses.

Host plant preference for AMF species has been previously shown in the grasses *A. capillaris, P. pratensis* and *F. rubra* in semi-natural pastureland (Vandenkoornhuyse et al 2003), and interestingly this was shown for different AMF combinations too and not just single species. This host plant preference was demonstrated in the AMF monocultures trial as beneficial growth effects were only seen for certain species of AMF and these beneficial species differed between the grasses trialled. For example, *Glomus spp B* produced an increase in average dry biomass for *F. r. rubra,* and no effect for *A. stolonifera* and *A. capillaris.* The only AMF found to be consistently compatible with all three grass species was *Funneliformis spp A* which reduced inequality consistently, however an increase in dry biomass was only seen for *F. r. rubra.* This demonstrates how useful measures of equality can be alongside measurements of dry biomass.

Not all AMF monocultures increased dry biomass, unlike all treatments with the mycorrhizal consortium. This is concurrent with findings by Gustafon & Casper (2006) when treating *Andropogon gerardii* seedings with nine different AMF species. They found that certain AMF, such as *G. etunicatum,* do not cause an increase in dry biomass unless paired with other AMF, in their case *G. intraradices.* They also found that effects on dry biomass were greater with the two AMF combined than individually, suggesting plants gain an advantage from colonisation of more than one AMF species (Gustafon & Casper 2006). However, it was also demonstrated in these trials that while *A. stolonifera* experienced no change in dry biomass following inoculation with a consortium, it did show positive changes in dry biomass for *Funeliformis spp B.* This suggests that not all plants prefer a consortium of AMF. Öpik et al (2006) found

that the average AMF taxa per plant was 8.3 in grasslands however this did vary between species. Future research should therefore consider trialling each of the different mycorrhiza monocultures in varying combinations to see which collaborate and whether any antagonism occurs.

5.4.2 Grass PGPR trial

PGPR showed very variable effects on dry biomass for five of the grass species tested, with both increases and decreases recorded. The inequality results for PGPR were also found to be variable depending on the grass species and PGPR observed. This upheld the hypothesis and agrees with recent research by Gadhave et al (2016), who used similar data to argue for the necessity of inoculants tailored to the plant and existing soil community. There were also more negative effects on dry biomass seen for PGPR than for AMF suggesting that PGPR are less reliable as a commercial inoculant. Antagonistic effect of PGPR on different plant species has been showed before by Gange & Gadhave (2018), and Errickson (2018). These negative effects could be caused by antagonism of the plant hormone or enzyme production by PGPR, as they are known to interact with this to improve their own colonisation (Vacheron et al 2013). However, these trials are limited in that they explore the growth effect of PGPR, which are far less well known than their induction of biotic and abiotic tolerance. Therefore, the other benefits of PGPR could still make them worthwhile if they are appropriately tailored.

5.4.3 Fertiliser trial

Greater phosphate uptake has been linked directly to better plant growth, changes in leaf morphology and carbon allocation. These effects are a by-product of the increased phosphorus uptake and similar effects can be seen by increased phosphate fertilisation (Grimoldi et al 2005). Plant growth effects are dependent on the mycorrhizal species observed and the time after inoculation, with a study between *Funneliformis mosseae* (an AMF, formerly known as *Glomus mosseae*) and *Hebeloma leucosarx* (an ectomycorrhizal fungus) showing a greater increase in root length for plants inoculated with *G. mosseae*, but equal long-term beneficial effects in shoot length and dry weight between the two fungi (Van der Heijden 2001).

In this trial higher fertiliser rates always increased dry biomass for all four plant species. However, the effects of AMF inoculants were slightly less consistent. *A. stolonifera* and *P. pratensis* had significantly higher dry biomass following colonisation with an AMF inoculant for all fertiliser treatments. This was particularly interesting as *P. pratensis* did not increase in dry biomass upon colonisation in the mycorrhizal consortium trial. This suggests that the extent of *P. pratensis* colonisation is linked to the available nutrients. *F. r. trichophylla* showed no change in dry biomass for recommended or double rates of fertilisation but did increase at half rate or no rate when colonised with an AMF inoculant. In fact, the dry biomass of no rate and half rate fertilisation when colonised with AMF was comparable to the dry biomass at the recommended and double rates. These results are promising as they suggest that AMF inoculants could be effective even in high fertilisation, contradictory to what was

hypothesised. This has been shown previously by Treseder & Allen (2002) who postulate that the initial nutrient status of the ecosystem and the specific AMF species determine how much AMF are influenced by high P. Therefore, the AMF in the consortium could be more compatible with high phosphate environments than those used in other research.

L. perenne showed no difference in dry biomass between grass inoculated with AMF and control pots. This could suggest that the only benefit of AMF in *L. perenne* are at extremely low fertiliser rates as even at half rate the grass experienced no increase in dry biomass from AMF inoculation. This may be because the grass can already gain everything it needs from the fertiliser and so the nutrient benefits of AMF are no longer beneficial.

The application of an AMF inoculant increased percentage root length colonisation for all grass species. However, it decreased with increasing fertiliser rate for *L, perenne, A. stolonifera* and *P. pratensis* as hypothesised. Vandenkoornhuyse et al (2003) showed that biocide usage had less of an effect on fungal diversity than applications of nitrogen or lime, showing that fertilisation can have a detrimental effect on fungi. Similarly, N and phosphate were found to have inhibitory effects on different AMF species with *Glomales spp* severely inhibited by phosphate and *Diversisporales spp* by N (Camenzind et al 2002).

5.4.4 Dose rate trial

As dose rate increased the percentage root length colonisation increased except for 16 Kg ha^{-1}. This slight decrease is thought to be due to the very small

amount of inoculant needed for each pot which meant the lower dose rates only used dust rather than granules. This dust may have a higher spore concentration than the granules which could account for the decrease when doses switched from dust to granules.

Dry biomass increased for the doses 32kg ha^{-1} and 64kg ha^{-1} but showed a slight decrease for 128kg ha^{-1} . In the past it has been suggested that the introduction of excess AMF propagules for certain species can cause a decrease in shoot biomass in *Medicago sativa* (Janoušková et al 2013). This is thought to be due to induced competition between AMF propagules over a certain density which in turn reduced plant benefit (Janoušková et al 2013). This has also been found in Soybean by Niwa et al (2018), so potentially the same effect is being observed here in grass as 128kg ha^{-1} is an excess dose compared to the recommended 4kg ha^{-1} for agriculture. Based on this, the recommended dose rate for use of AMF inoculants in golf putting greens is between 32-64 kg ha^{-1}.

5.4.5 Germination trial

AMF did not cause a change in overall percentage germination or in germination time as modelled by the AUGPC. There is very little current research on the impact of AMF on germination success but a paper by Allison (2002) showed that germination success is largely linked to the biomass of the parent plant. So, the seeds of mycorrhizal parents who experience increased growth have better germination success, and those whose parent plants are inhibited by AMF have lower biomass and so lower germination success. As the

seeds used in this trial came from a supplier the size of the parent plant is not known and therefore an effect could have occurred. For this reason, future research should repeat this trial under multiple generations to see if parent biomass does effect germination success. Donath and Eckstein (2010) found that seed size and ground cover had a significant impact on germination success, with larger seeds being more successful but only with adequate ground cover; potentially due to predation. As grass seeds are sown on the surface of the soil the smaller seed size would therefore be an advantage. However, as the research in this thesis was conducted in a control temperature room with no chance of predation, these effects were not observed.

Shoot length decreased for *L. perenne* and *A. stolonifera,* stayed the same for *P. pratensis* and *F. r. rubra* and increased for *A. capillaris* following AMF colonisation. The decrease in shoot length correlates with existing research suggesting AMF colonisation can cause early growth depressions in soybeans (Bethlenfalvay et al 1982) and wheat (Li et al 2005) which are often overcome later in development. This growth depression is thought to be due to demand for carbon from the AMF as well as a reduction in the direct phosphate uptake pathway in favour of the AMF pathway, which isn't fully established upon colonisation (Jacott et al 2017). As this trial took place for two weeks, colonisation may have caused a growth depression without the chance for growth to balance out after a longer time period.

Root length decreased for three of the grass species trialled when inoculated with AMF. The reason for the decrease in root length could be due to the reduced carbon availability in the roots causing them to grow more slowly. This

contradicts existing research in citrus plants. Wu et al (2010) found that AMF inoculation increased root length and diameter, but that this effect was different between AMF species. However, Wu et al (2010) measured roots five months after germination which may have allowed for the lower growth from reduced C content to balance out. There was no current published research on the effect of AMF inoculation on root length in grass.

5.5 Conclusions

Different species of AMF both as monocultures and a consortium, and different PGPR have variable effects depending on the grass species colonised. Different fertiliser rates had different impacts on dry biomass of the grass species trialled but higher rates of fertiliser consistently reduced % root length colonisation across all species. AMF inoculation could allow for a reduction in fertiliser usage in some grasses, especially *P. pratensis.* AMF colonisation can occur at dose rates as low as 2kg ha^{-1}, and colonisation generally increased as the dose of inoculant increased. However, this was limited and there is the possibility of “overdosing”. Germination time and percentage showed no impact from colonisation with AMF, but root and shoot length were either increased or decreased depending on the grass species observed.

Chapter 6: Benefits gained from using microbial inoculants in sports turf

6.1 Introduction

In previous chapters the current microbial populations of sports turf and the more generic benefits presented by AMF and PGPR were discussed. This chapter covers the potential benefits specific to a golf putting green environment that can be gained using microbial inoculants.

6.1.1 Mycorrhiza and thatch

Thatch is the layer of organic matter that forms from dead grass and new shoots above the soil surface. Thatch can be decomposed quickly in low input pitches and is useful to a degree for ball control. However, the high fertilisation of golf courses mean that the grass grows quickly and cannot always be broken down at an appropriate rate by the soil microbes available (Munshaw 2014). Thatch can be increased by soil acidity and grass species being grown that take longer to break down, as well as low earth worm populations (Munshaw 2014). Thatch is an issue for turf as it can harbour diseases and pests, prolongs high humidity, causes shallow roots and affect the playability of the green. However, keeping a certain amount of thatch can stop leaching of pesticides into the soil (Fresenburg 2015).

Thatch can be easily managed through mechanical dethatching to loosen the organic matter or cut through the layer to the soil below (Munshaw 2014). There are microbial products on the market currently claiming to use microbes to reduce thatch, and growing interest in such products (Frost 2006) although there is little published evidence to support or dismiss their use. Microbes have been shown to increase turf quality in bermudagrass, which could be linked to the effects of *Bacillus spp.* and actinomycetes on organic matter in the soil.

Actinomycetes were particularly important in lignocellulose degradation which could be responsible for the decrease in organic matter (Zhang et al 2015). AMF of the Glomales order have been shown to acquire nitrogen from organic material and increase the rate of decomposition of *L. perenne* leaves in the soil (Hodge et al 2001). However, this has not been shown in a turfgrass environment where thatch build up is on the surface of the soil. Based upon current research it was hypothesised that mycorrhiza have no effect on thatch decomposition at the surface but increase decomposition of organic matter in the soil.

6.1.2 Microbial inoculants and disease

There is increasing evidence of certain microbes aiding in turfgrass disease suppression, summarised in Table 12. One such example is the use of *Bacillus amyloliquefaciens* in reducing the incidence and severity of grey leaf spot (*Magnaporthe oryzae)* in perennial ryegrass (Rahman et al 2015). The bacteria aid the plant's immune response through accumulation of hydrogen peroxide, increased peroxidase deposition on uninfected leaves and callose deposition, which helps to strengthen cell walls and limit pathogen invasion and spread (Rahman et al 2015). *B. amyloliquefaciens* can also produce antifungal peptides that inhibit hyphal growth in the vascular tissues and around stomata. *B. amyloliquefaciens* is of industrial interest as it is seed-transmitted and so would be easy to apply in the field (White & Chen 2014).

Dollar Spot, caused by *Sclerotinia homeocarpa*, is a common fungal disease in turf grasses at temperatures over 10°C. A reduction in the severity and occurrence of dollar spot has been observed in soils with higher microbial

populations. Bentgrass grown in sterilised compost showed greater disease incidence and severity than non-sterilised composts (Workman & Waltz Jr 2012). Leaf spot, caused by *Drechslera poae,* can be inhibited by *Bacillus lentimorbus* in perennial ryegrass, with significant effects comparable to the fungicide propiconazole on disease severity and incidence. The same study found antagonistic effects against the anthracnose-causing organism *Collectotrichum cereale* (formerly *Collectotrichum graminicola)* (Viji & Uddin 2001). Take-all disease, caused by *Gaeumannomyces graminis var. tritici* can be inhibited by strains of *Bacillus subtilis* and *Bacillus cereus* in wheat, a close comparison to turf grass. The strains, *B. subtilis* B908 and *B. cereus* A47 were effective at reducing take-all, while another strain of *B. subtilis*, B931 was more effective than other treatments at reducing rhizoctonia root rot (Ryder et al 1999). This demonstrates the importance of bacterial strain on disease suppression, as two different strains of the same bacterial species have effects on different turf grass diseases. Such strain specificity implies that to be used effectively, the bacteria must be tailored to the diseases faced (Gange & Hagley 2004) making the identification of existing microbes in the soil even more important.

Disease suppression can also be achieved using fungi. *M. nivale* was antagonised by *Streptomyces* bacteria and the fungus *Gliocladium catenulatum* (Espevig et al 2014). *Streptomyces* releases antifungal antibiotics (nigericin) when grown in the soil or grass rhizospheres (Trejo-Estrada et al 1998). *G. catenulatum* was parasitic on the pathogenic fungal hyphae and thus reduced the efficacy of the pathogen by reducing its surface area and access to the roots (McQuilken et al 2001).

As shown in Table 12, much of the current disease suppression trials in turf grass have been completed using *L. perenne.* Considering the specific nature of microbial interactions this could mean results differ for other grass species. The effects of suppression against *M. nivale* shown in agar were repeated, and while promising results were shown in vitro, in field trials using *Festuca rubra* the significant reduction was small (-1%) using either of the *Streptomyces spp.* or *G. catenulatum* (Aamlid et al 2017). There was also no significant reduction found in greens with *Poa annua* or *Agrostis capillaris* (Aamlid et al 2017). These trials were conducted in Norway and so temperature could have had an effect, but the necessity for more research in a variety of host organisms is obvious.

Disease	Causative organism	Microbe offering suppression	System	*Reference*
Grey leaf spot	*Magnaporthe oryzae*	*Bacillus amyloliquefaciens*	*Lolium perenne*	Rahman et al (2015)
Anthracnose	*Collectotrichum graminicola*	*Bacillus lentimorbus*	*Lolium perenne*	Viji & Uddin (2001)
Take-all patch	*Gaeumannomyces graminis* var. *tritici*	*Bacillus subtilis* and *Bacillus cereus*	Triticum aestivum *L. cv. Spea*	Ryder et al (1999)
Rhizoctonia root rot		*B. subtilis,* B931	Triticum aestivum *L. cv. Spea*	Ryder et al (1999)
Dollar Spot	*Sclerotinia homeocarpa*	*Bacillus lentimorbus*	*Lolium perenne*	Workman & Waltz Jr (2012)
Microdochium patch	*Microdochium nivale*	*Streptomyces spp. Gliocladium catenulatum*	Grown on Agar plates	Espevig et al (2014)
Leaf spot	*Drechslera poae*	*Bacillus lentimorbus*	*Lolium perenne*	Viji & Uddin (2001)

Table 12: A summary of the suppression effects shown with microbes to diseases.

Mycorrhizal fungi have been associated with the reduction of certain diseases and disease symptoms in grasses, summarised in Table 13. These effects are dependent on the AMF species, soil conditions and whether colonisation is established before disease pressure (Azcón-Aguilar & Barea 1996). A meta-analysis by Borowicz (2001) concluded that AMF reduced pathogen growth in 50% of studies included in the analysis. It was found that the effects are highly variable but generally have a positive effect on disease tolerance, and that plants in general benefit more from AMF when under some kind of disease stress (Borowicz 2001).

Disease	Causative Organism	Microbe offering suppression	System where suppression was shown	Reference
Take All	*Gerumannomyces graminis*	*Glomus fasciculatus*	Wheat *(Triticum aestivum)*	Graham & Menge (1982)
Microdochium patch	*Microdochium nivale*	Mixed AMF	Golf putting green	Gange & Case (2003)
Fusarium oxysporum	*Fusarium oxysporum*	*Glomus spp*	*Vulpia ciliata spp*	Newsham et al (1995)

Table 13: A summary of the suppression effects shown with AMF and diseases.

While a lot of studies have been completed in other hosts, there are few looking specifically at grasses in a golf putting green system. One turfgrass specific trial found a significant negative correlation between *M. nivale* incidence and mycorrhizal colonisation, suggesting that *M. nivale* incidence can be reduced by AMF (Gange & Case 2003). This would have huge implications in the golf industry as *M. nivale* is the most prevalent turf grass disease in the UK and so needs to be explored further.

The mechanisms through which AMF improve disease suppression are through microbial interactions, improvement of plant nutrient uptake, competition, improved root structure and plant immune response increase (Xavier & Boyetchko 2004). Mycorrhiza can change the qualities of root exudates and therefore the microbial population of the rhizosphere both in diversity and quantity. Microbial interactions are important as they can antagonise pathogenic microbes through the mechanism of competition or increase populations of beneficial microbes and their access to the plant (Huang et al 2003).

Mycorrhiza are particularly important in rhizosphere chemical changes, as not only do they alter root exudates, but can release chemicals that attract beneficial bacteria meaning chemical composition of the soil will differ significantly to that of non-mycorrhizal soil (Jones et al 2004). The improvements in nutrient uptake and root structure improve plant health and can compensate for root damage and loss (Borowicz 2001). If the plant is healthy it is more likely to be able to defend against disease, however mycorrhizal colonisation causes modulation of plant defences during its establishment. This modulation causes the accumulation of phenolic

compounds and JA-defence responses not only in the roots but throughout the plant, priming the plant for an attack and making the immune response quicker and more effective (Jung et al 2012, Huang et al 2003). This is called induced systemic resistance and can also take place through inoculation with PGPR (Benduzi et al 2012). Mycorrhizas also take up sites for colonisation or infection in the roots, and so reduce the room for other fungi to access the roots (Azcón-Aguilar & Barea 1996). Based upon the positive effects shown in other grass species, it was hypothesised that *M, nivale* incidence reduces when AMF and PGPR are applied to the trial site.

6.1.3 Mycorrhiza, waterlogging and drought tolerance

Weather extremes can be a serious issue to turf grasses, depending on season and course location. Specific turfgrass can be genetically engineered to cope with extreme conditions. A particularly successful strain is a drought tolerant *Festuca rubra commutata* seed that requires just 10% of the water needed by most other species (Clevenger 2015). Drought puts turf under high stress, leaving it more susceptible to disease and wear.

Dry patch is a condition in sports turf where some areas of a golf green become water repellent. This is through the release of water repellent chemicals by certain fungi (for example, the fairy ring mushroom, *Marasmius* oreades) in the soil and causes severe drought conditions (York 1993) . Current practises rely on the use of wetting agents to help spread water throughout the soil preventing both drought and waterlogging, and ease symptoms like dry patch.

AMF can aid water uptake in plants independently of nutrient uptake through extension of the root absorptive-surface area using extraradical hyphae. The importance of the hyphae aside from the better root absorption from higher phosphate levels was shown by the removal of the extraradical hyphae and the observation of a serious drop in transpiration rate (Hardie 1985). The effect of AMF on water uptake has been linked to the different species of plants studied, with citrus plants often showing no effects, but lettuce, rose, soybean and wheat all showing alterations to transpiration with AMF inoculation (Augé 2001). AMF have also been shown to aid drought resistance through the mechanism of drought avoidance, whereby the plant experiences less of the effects of drought such as necrosis and wilting and will wilt at a lower soil moisture content than un-colonised plants (Augé 2001). Species of AMF differ in their ability to aid drought resistance, with different *Glomus* spp showing different levels of soil depletion though all were greater than that of un-inoculated plants. There is also a direct correlation between the amount of mycelium produced and the extent of water depletion of the soil, suggesting this is what causes the species variation in water uptake (Marulanda et al 2003).

Waterlogging tolerance of different grass species can range from days to weeks (Wang & Jiang 2007). Waterlogging reduces canopy cover by 30% but does not affect shoot dry matter in hybrid *Cynodon dactylon* (hybrid bermudagrass) and common *Eremochloa ophiuroides* (centipede grass). However, root dry matter was concentrated near the surface for *E. ophiuroides* and was reduced by 40% in *C. dactylon* under mimicked conditions of waterlogging (Wherley et al 2007). The reduction in root growth caused by waterlogging is because of reduced oxygen availability, which is exacerbated in high temperatures due to increased

oxygen demand. Reduced oxygen availability increases anaerobic respiration in the grass, shown by increased root alcohol dehydrogenase and lactate dehydrogenase activity (Wang & Jiang 2007).

By 2025 it is expected that over half of the human population will be living in countries under water stress, something we have known for 20 years already (Arnell 1999). With climate change, rainfall is shifting to become less frequent in some areas but heavier in others, leading to more extreme water conditions. Therefore finding a solution to drought and waterlogging stress is a necessity for the turfgrass industry worldwide, as limited water supplies are likely to prioritise drinking water and food production. The R&A has outlined water sustainability as a key priority for its Golf Course 2030 initiative (R&A, 2019), so the use of microbes to reduce drought stress could be essential in future management schemes. Based upon current evidence, it was hypothesised that grasses treated with AMF respond better to drought conditions than untreated.

6.1.4 Mycorrhiza and *P. annua*

The effect of AMF on weeds is highly dependent upon whether the weed itself is mycorrhizal. While mycorrhizal weeds can experience benefits like desirable mycorrhizal plants, non-mycorrhizal plants can experience antagonism from AMF colonisation. This antagonism can reduce germination, survival and growth rates. The antagonistic effects on non-mycorrhizal species vary considerably in short term (3 month) experiments and so depends entirely on the species present (Vatovec et al 2005). AMF can deter parasitic weeds by altering root exudates upon colonisation. This reduced the levels of chemicals

like strigolactones released, usually used as a trigger for parasitic weed seeds to germinate (Jung et al 2012).

P. annua is the most important weed in golf putting greens, due to its ability to overtake an entire sward and its susceptibility to drought and disease. The reason *P. annua* is such a competitive weed is that despite low mowing heights it is still capable of producing 168,000 seeds per m^2 at peak season compared to just 4,900 for other fine grasses like *A. stolonifera* (Lush 1988). A study by Gange (1998) found there was less *P. annua* abundance with higher AMF colonisation, even in a golf course with a sward of over 50% *P. annua*. This reduction of *P. annua* abundance was originally thought to be because of the presence of more mycorrhizal grass species, such as bentgrass, outcompeting *P. annua* with the help of the mycorrhiza. However even without other grass species *P. annua* may still be reduced (Gange 1998). Current theories suggest the amount of carbon provided to the AMF compared to the improved phosphate and N uptake are unbalanced in *P. annua*, whereby the grass is losing more carbon that it is gaining in phosphate and nitrogen. This in turn reduces plant growth and allows *P. annua* to be outcompeted by other fine grasses (Gange 1999). From this it was hypothesised that *P. annua* dry biomass is reduced following colonisation with different species of AMF.

6.1.5 Summary of hypotheses

- Mycorrhiza have no effect on thatch decomposition at the surface but increase decomposition of organic matter in the soil.
- *M, nivale* incidence is reduced when AMF and PGPR were applied to the trial site.

- Grasses treated with AMF respond better to drought conditions than untreated.
- *P. annua* dry biomass is reduced following colonisation with different species of AMF

6.2 Methods

6.2.1 Thatch trial

Four treatments were applied to 4 replicate plots of 2m^2 turf in a factorial design, and maintained as a golf putting green at STRI Bingley, UK. The trial took place in one area prone to thatch and one area more typical of a normal golf course, with an existing sward of 60% *Festuca spp* and 40% *Poa* spp. The sward was not changed to ensure the grass system was well established and so would produce thatch as expected. The treatments were 15gm^{-2} of a consortium of five AMF species (*Glomus spp A, Glomus spp B, Rhizophagus spp, Funneliformis spp A, Funneliformis spp B*), a consortium of PGPR (*Bacillus spp B*, *Gluconacetobacter spp, Pseudomonas spp* A), both consortiums of PGPR and Mycorrhiza and a non-treated control of nothing. PGPR were added as 15ml of PGPR solution (1x10^6 cfu/ml) diluted in 5L of dechlorinated water. The treatments were applied once in April 2018.

6.2.1.1 Tea bag index

Litter bags are commonly used in studies to measure decomposition in the soil. Litter bags have been used before in turf grass to consider the impact of temperature and nitrogen addition (Henry & Moise 2015) but not to look at the effect of microbial inoculants. The Tea Bag Index was developed by Keuskamp et al (2013) in order to create a standardised method across biomes and ecosystems which can measure decomposition. The method utilises two tea bags with contrasting decomposability to give an insight into the rate and stabilisation factor of the soil.

Figure 65: A hole changer used in golf courses to swap holes on a green.

The method does face some of the same disadvantages as other litter bag studies in that there is potential to exclude certain microbes which may not enter the litterbag. The placement of the tea bags can also greatly affect the decomposition observed and so it is vital to ensure placement is consistent between plots (Akesson 2017). It is noted in the method that while the Tea Bag Index does give data on decomposition rates and stabilization factors it is not as precise as conventional litter bags (Keuskamo et al 2013). However this method

was used because it is cost effective and easily comparable if greenskeepers or others wanted to replicate the experiment.

Liptons tea bags (one rooibos and one green tea) were buried 15cm below the surface on each repeat plot, following the method laid out in Keauskamp et al (2013), but using a hole changer (see Figure 65). After three months tea bags were dug up, dried and weighed.

Recovered tea bags were dried at 30℃ for two days to remove any water in the tea bags. Tea bags were weighed, compared to unburied weights and analysed using the equation by Keauskamp et al (2013) to calculate S (proportion of labile fraction remaining) and K (rate of decomposition) values.

6.2.1.2 Loss on ignition data

After three months a 2.5cm diameter soil core was taken from the centre of each plot for analysis by loss on ignition. Loss on ignition data was collected for the different depths of the soil cores, 0-10mm, 10-20mm, 20-30mm and 30-40mm. Different portions of the soil cores were dried overnight in an oven at 105°C, passed through a sieve, weighed and placed in beakers. Samples were then ignited at 360°C for two hours and reweighed to calculate organic matter content.

6.2.2 Disease trial

Eight treatments and a control were applied to four replicate plots each of $2m^2$ turf maintained as a golf putting green at STRI Bingley, UK in 2018/19.

Treatments included microbial inoculants, iron products, both preventative and curative fungicides for comparison, and manual dew removal. Enriched biochar was included as a comparison product that is currently gaining in popularity in the industry. Enriched biochar is added to turf to improve soil quality and aid disease suppression. The biochar is a biostimulant that is combined with AMF, *Trichoderma*, seaweed and worm casts and so provides a different biological treatment to the microbial inoculants.

The plots had a sward of 60% *Festuca spp* and 40% *Poa* spp. The treatments for the 2018/19 trial are shown in Table 14. All treatments are commercially available and were applied at the manufacturer's recommended rate in October 2018 except for the curative fungicide which was applied when disease started showing. PGPR were added in liquid form, with 15ml of PGPR solution ($1x10^{13}$cfu/ml) diluted in 5L of dechlorinated water. Mycorrhizal inoculum was added to the soil by making drainage holes in the turf and brushing inoculum to fill the holes. Disease assessments took place every two weeks and gave a percentage based upon disease severity and cover of the plot. An example of a disease covered plot is shown in Figure 66. A preliminary disease trial was conducted in winter 2017/18 with the same format but five treatments and a control at the same application rates, and monthly disease assessments.

Treatment	Application rate/ g or ml m^{-2}	Time treated
Preventative fungicide (azoxystrobin)	0.03	Start of trial
Curative fungicide (propiconazole)	0.05	First sign of disease
Iron treatment	2.00	Start of trial
Enriched Biochar	500	Start of trial
Mycorrhizal inoculant (Plantworks)	15.0	Start of trial
PGPR (Plantworks)	1.88	Start of trial
Mycorrhiza and PGPR (Plantworks)	15 and 1.88	Start of trial

Table 14: The application rates for different treatments in the disease trial.

Figure 66: *Microdochium nivale* on the disease trial plot at STRI Bingley.

6.2.3 Turf Quality and Colour trial

This trial was set up and maintained as in the thatch study (6.2.1). Turf quality and turf colour assessments were conducted every 2 weeks. Assessment was on a scale of 1-10 following the methodologies used by STRI (no date A & no date B).. Turf quality considers sward density, live grass cover, uniformity and any disease pressure. The scale for turf colour is based on desirability, with 1 being brown to 10 being dark green. All assessments were completed by the same person to avoid subjectivity.

6.2.4 Drought trial

F. r. rubra, A. capillaris, *A. stolonifera* and *P. annua* were grown according to the general pot set up (see Chapter 2: General Methods, section 2.4) with the following alterations. Watering was the same as general set up until grass had germinated and had grown to 2.5cm tall. Following this the grass was watered according to the three watering regimes laid out in Table 15. There were five treated and five control replicates for each grass species and water treatment.

Watering regime	**Method**
Drought	Watered whenever the grass started to wilt.
Normal	Watered as needed every two days.
Waterlogged	Pots were kept in grow bag trays filled with water. Water was topped up daily.

Table 15: The watering regimes used for the drought trial.

6.2.5 *Poa Annua* trial

P. annua was sown according to the general pot set up (see Chapter 2: General Methods, section 2.4) with the following alterations. Mycorrhizal inoculants of five monocultures (*Glomus spp A, Glomus spp B, Rhizophagus spp, Funneliformis spp A, Funneliformis spp B*) and a consortium of the five species were applied to treated pots. There were five treated and five control replicates for treatment.

6.2.6 Statistical Analysis

Differences between treatments in dry biomass were examined with a one-way or two-way ANOVA and the means separated using a Tukey test in R 3.6.0 (R Core Team 2019). Root length colonisation and percentage organic matter lost on ignition data were analysed using generalised linear models with binomial errors in R. This model was then checked for overdispersion and if found to be overdispersed it was repeated with quasibinomial errors to consider random factors.

Disease percentage data was plotted on a line graph to find the area under the disease progression curve (AUDPC) before analysis of this area through a one-way ANOVA. The means were separated using a Tukey test with the 'multcomp' package. Plant health and colour were analysed by adding up a total value for each plot over the assessment period and then analysing these data with a one-way ANOVA. The means were separated using Tukey HSD.

Dry weight data for *P. annua* was further analysed using Lorenz curves, the Gini coefficient and Asymmetry coefficients to assess for inequality (see section: 5.2.8).

6.3 Results

6.3.1 Thatch trial

6.3.1.1 Loss on ignition data

Different microbial treatments had no effect on the percentage organic matter of cores taken from a naturally thatch-prone trial plot ($t_{9,48}$=-0.173, P>0.05), as shown in

Figure 67. There was no difference in the organic matter between depths of soil except for 10-20mm which had a lower amount of organic matter ($t_{3,48}$=-2.067, P<0.05).

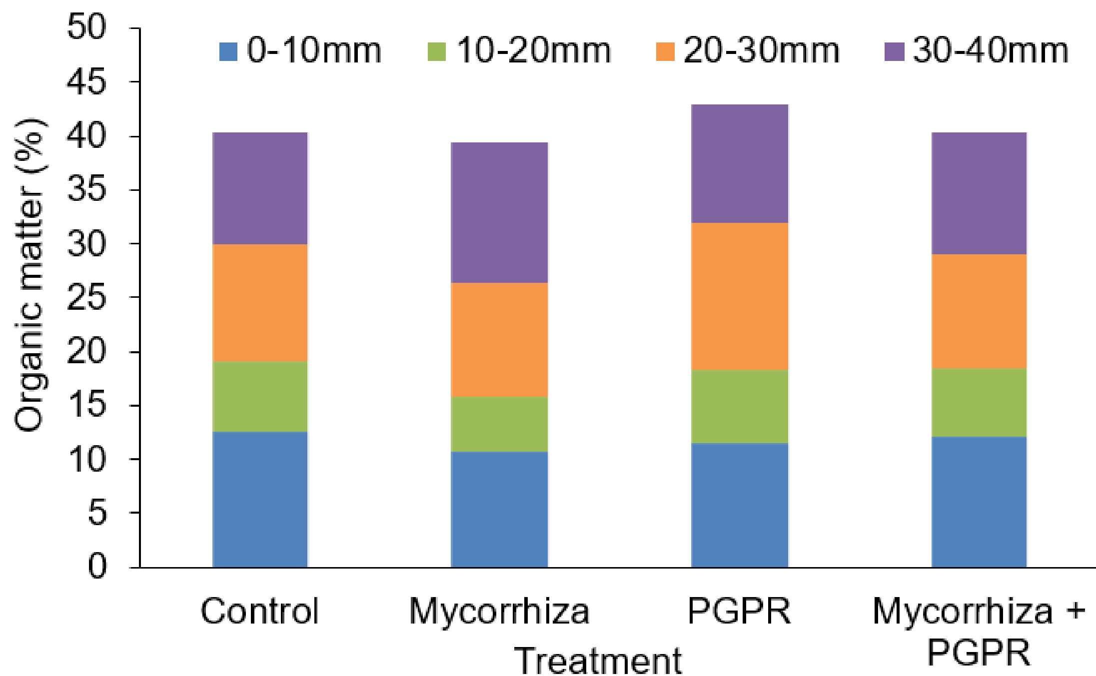


Figure 67: The average percentage organic matter at different depths of cores taken from a naturally thatch-prone trial plot with different treatments.

For the non-thatch-prone trial plot (Figure 68) there was a significant increase in the organic matter for the combined treatment of Mycorrhiza + PGPR ($T_{3,48}$=3.216, P<0.05). There was also a significant difference between the organic matter of all depths of the soil core (10-20mm ($T_{3,48}$=-4.144, P<0.05),

20-30mm ($T_{3,48}$=-5.007, P<0.05) and 30-40mm ($T_{3,48}$=-4.821, P<0.05), decreasing the further from the surface they were.

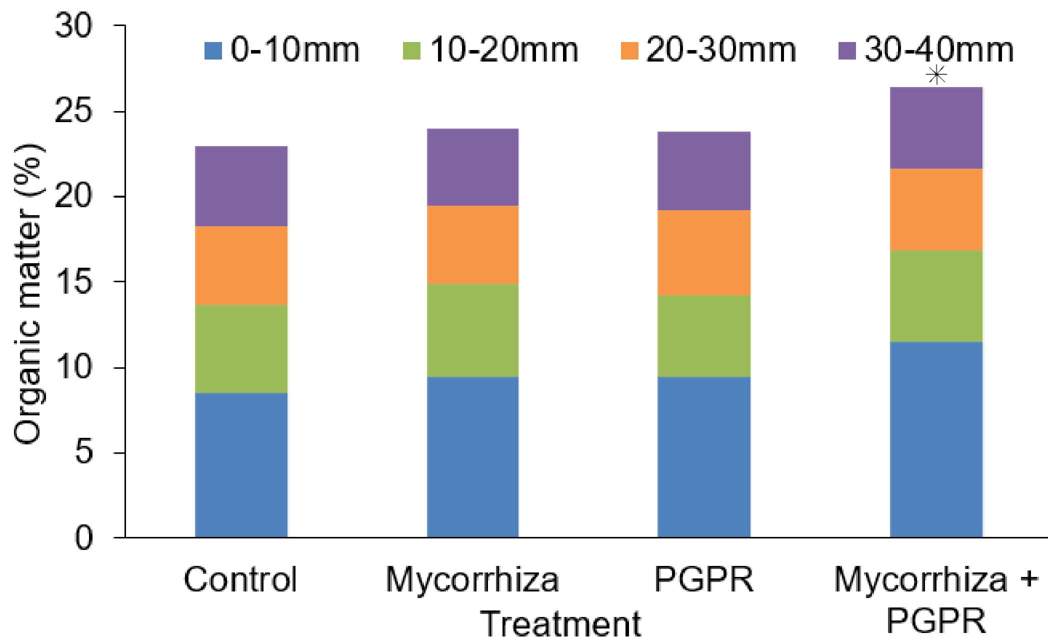


Figure 68: The average percentage organic matter at different depths of cores taken from a trial plot not prone to thatch with different treatments.

6.3.1.2 Tea bag index

There was no difference in decomposition rate (K value) between microbial treatments in either the thatch prone ($F_{3,12}$=0.185, P>0.05) or non-thatch prone ($F_{3,12}$=0.4454, P>0.05) trial plots. This is shown in Figure 69.

The proportion of the labile fraction remaining (S) is interpreted as the lower the value the more complete the decomposition of the organic matter. The S values showed no change and so the completeness of decomposition is not affected by microbial treatment with mycorrhiza or PGPR in either thatch prone ($F_{3,12}$=0.544, P>0.05) or non-thatch prone ($F_{3,12}$=0.8203, P>0.05) trial plots as shown in Figure 70.

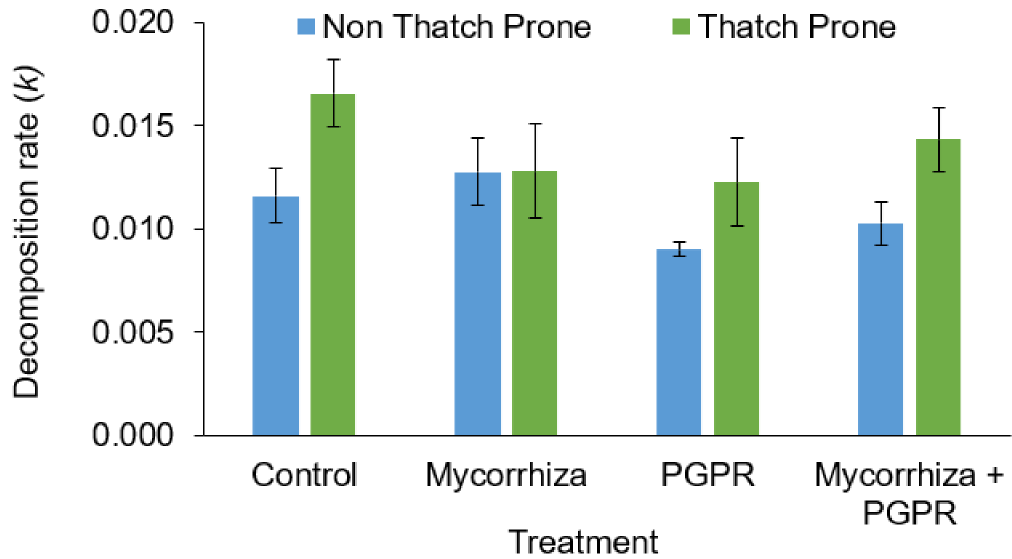


Figure 69: The rate of organic matter decomposition for trial plots in thatch prone or non-thatch-prone areas with different microbial treatments. Error bars show standard error.

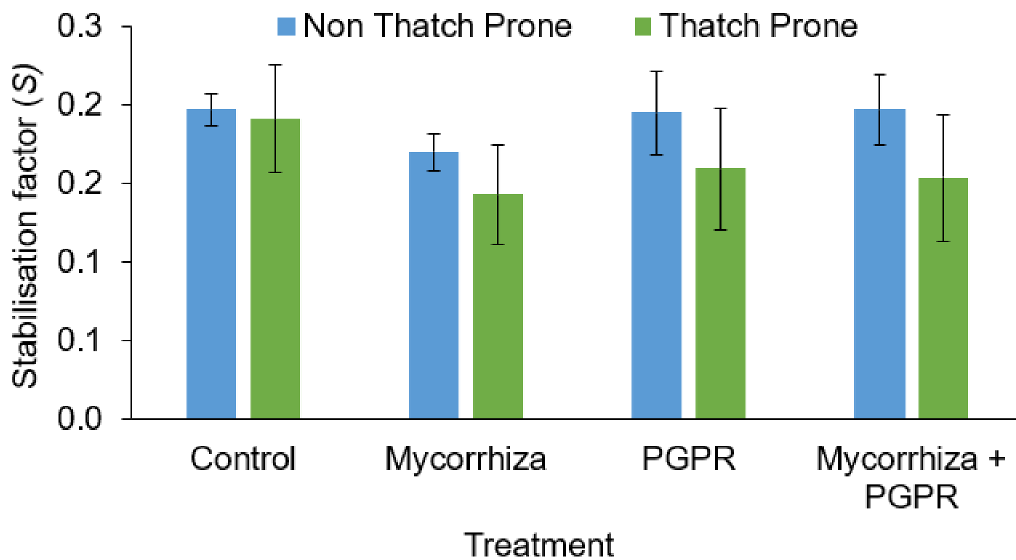


Figure 70: The proportion of the labile fraction remaining (S) shown for trial plots in thatch prone or non-thatch-prone areas with different microbial treatments. Error bars show standard error.

6.3.2 Disease trial

For the 2017/18 disease trial the percentage disease cover over time was plotted in
Figure *71*. Different treatments had no effect on disease cover over time

($F_{5,18}$=0.369, P<0.05) as calculated using AUDPC.

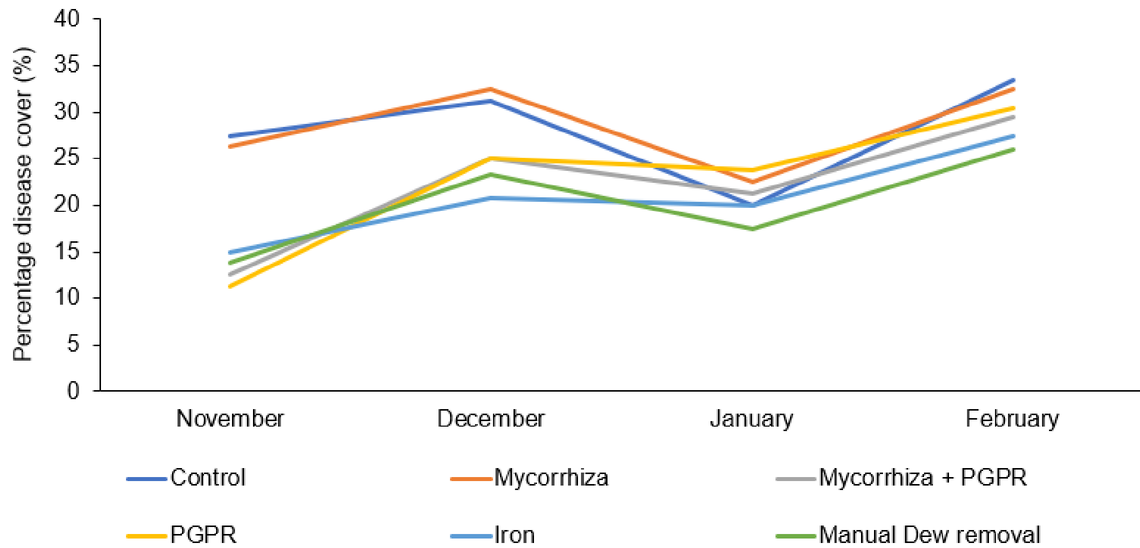


Figure 71: The disease progression curve of *Microdochium nivale* for different treatment plots over time, 2017/18.

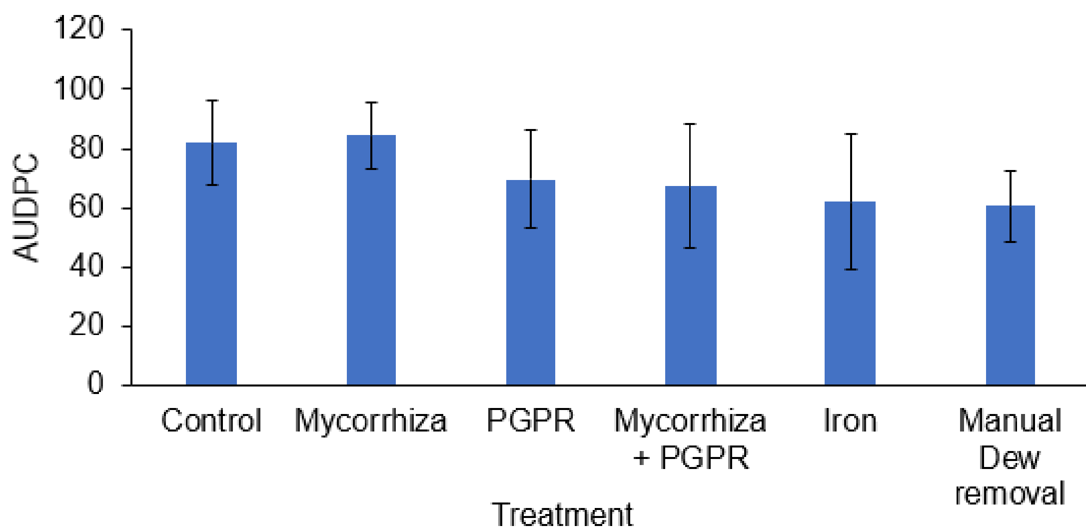


Figure 72: Area under disease progression curve (AUDPC) for different treatments in the 2017/18 disease trial. Error bars show standard error.

The AUDPC graph for 2017/18 (Figure 72) showed differences in size between error bars and so the trial was repeated for 2018/19 with more treatments. The disease progression curves for 2018/19 are shown in Figure 73.

The preventative fungicide caused a significant reduction in percentage disease cover on 02/11/2018 ($t_{8,27}$=-3.976, P<0.05), 16/11/2018 ($t_{8,27}$=-2.66, P<0.05) and 28/11/2018 ($t_{8,27}$=-2.299, P<0.05). Treatment with mycorrhiza caused an increase in disease cover on the 16/11/18 ($t_{8,27}$=2.593, P<0.05) and the 28/11/18 ($t_{8,27}$=3.025, P<0.05), and treatment with Mycorrhiza + PGPR showed an increase in disease cover on 16/11/2018 ($t_{8,27}$=2.515, P<0.05).

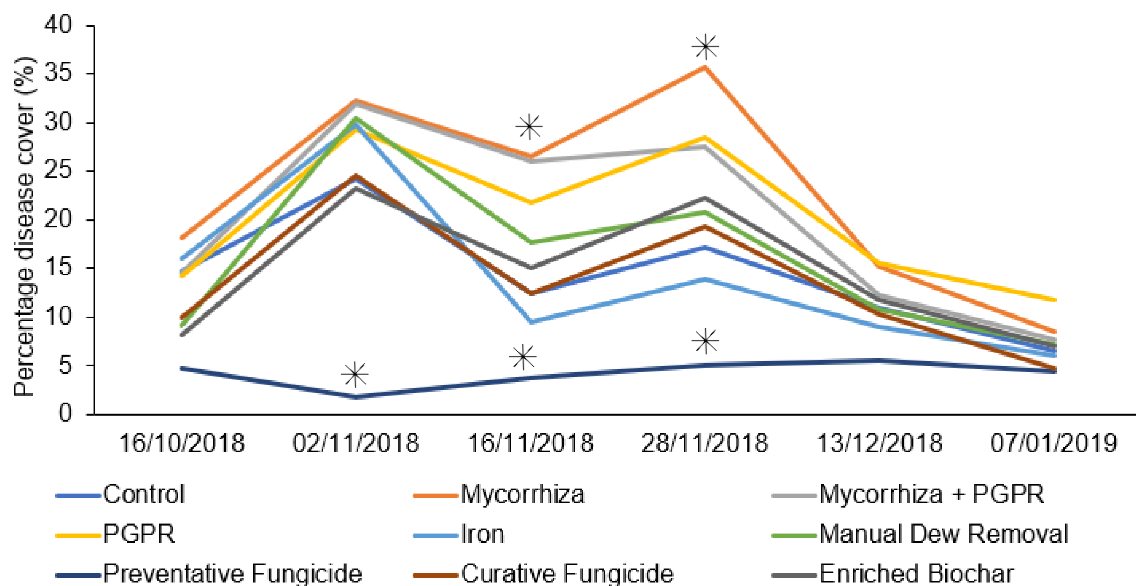


Figure 73: The disease progression curve of *Microdochium nivale* for different treatment plots over time, 2018/2019. Starred points show significant difference from the control for that assessment date.

The AUDPC analysis showed a difference in percentage disease cover between treatments over the trial period ($F_{8,27}$=5.381, P<0.05) as shown in

Figure 74. Preventative fungicide had lower disease incidence than other treatments while AMF addition increased disease incidence relative to the control.

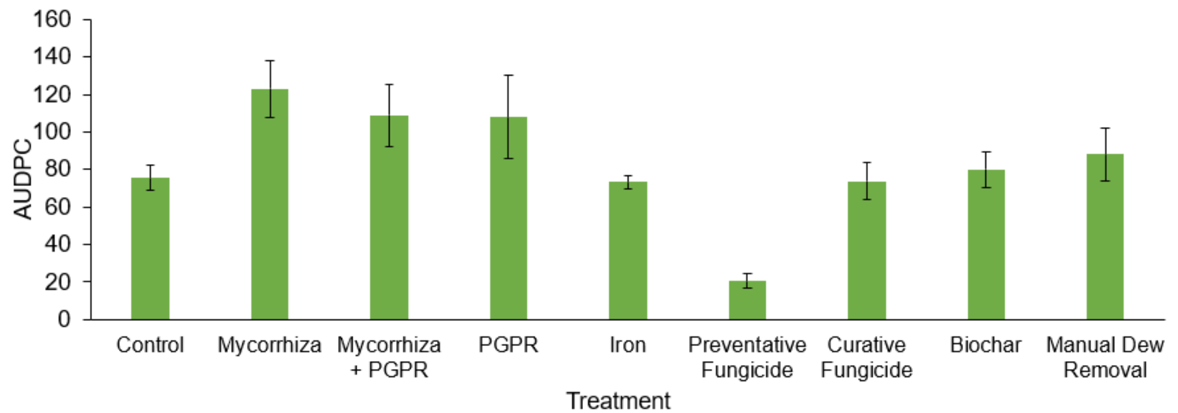


Figure 74: Area under disease progression curve (AUDPC) for different treatments in the 2018/19 disease trial. Error bars show standard error.

6.3.3 Turf Quality and Colour trial

Turf quality was consistently better for thatch prone areas (shown in Figure 75) however turf colour was consistently worse (Figure 76). There was no difference between treatments in turf quality or colour for thatch prone or non-thatch-prone areas as summarised in Table 15.

	Turf Quality	Turf Colour
Thatch Prone	$F_{3,12}=0.583$, $P>0.05$	$F_{3,12}=0.234$, $P>0.05$
Non-thatch-prone	$F_{3,12}=0.452$, $P>0.05$	$F_{3,12}=1.148$, $P>0.05$

Table 16: The statistical results for turf quality and turf colour assessments following microbial treatments of thatch prone and non-thatch-prone trial plots.

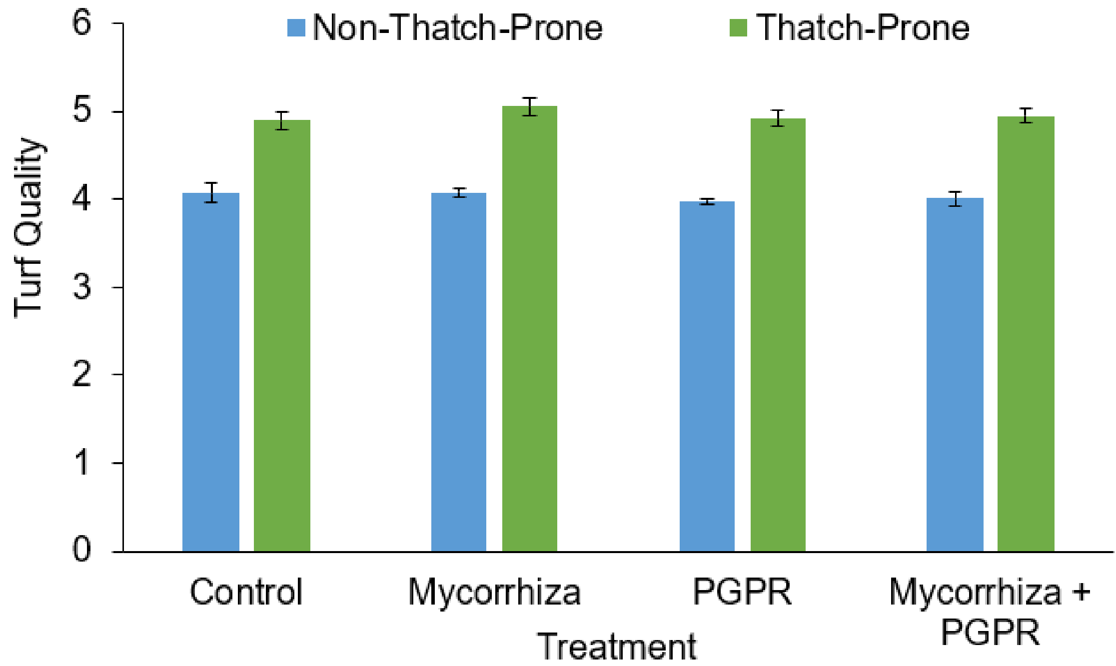


Figure 75: The turf quality of thatch prone and non-thatch prone trial plots treated with different microbial inoculants. Error bars show standard error.

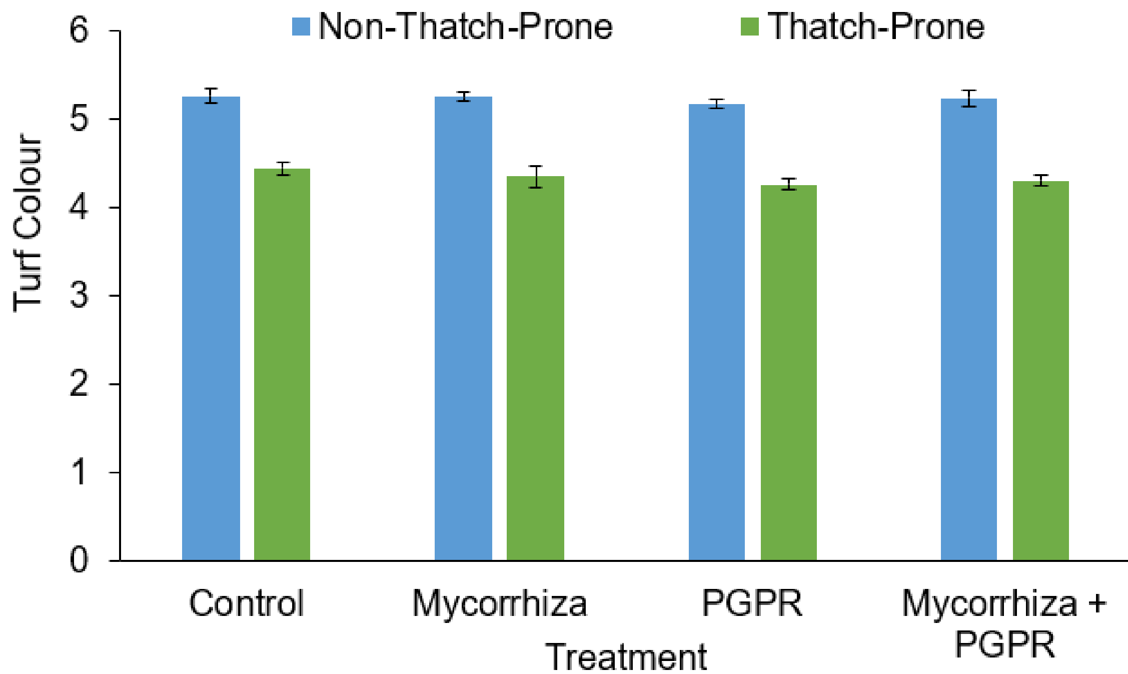


Figure 76: The turf colour of thatch prone and non-thatch prone trial plots treated with different microbial inoculants. Error bars show standard error.

6.3.4 Drought trial

Dry biomass increased following mycorrhizal colonisation in all watering conditions for *A. stolonifera* ($F_{1,24}$=11.092, P<0.05), *A. capillaris* ($F_{1,24}$=4,662, P<0.05), and *F. r. rubra* ($F_{1,24}$=14.805, P<0.05) (Figure 77). Dry biomass also increased consistently with water application across all species, so wetter conditions caused higher biomass (*A. stolonifera* ($F_{2,24}$=36.480, P<0.05), *A. capillaris* ($F_{2,24}$=22.847 , P<0.05), *F. r. rubra* ($F_{2,24}$=51.306, P<0.05) and *P. annua* ($F_{2,24}$=16.558, P<0.05).

In *P. annua,* treatment with mycorrhiza affected how much watering regime impacted dry biomass, significantly reducing loss of dry biomass in drought conditions ($F_{2,24}$=5.223, P<0.05). However, under normal watering conditions *P. annua* experienced a decrease in dry biomass consistent with other trial results. All dry biomass data are shown in Figure 77.

Addition of mycorrhiza increased percentage root length colonisation for all grass species and watering regimes except *A. capillaris* when waterlogged. These results are summarised in Table 17 and displayed in Figure 78

	Watering Regime		
Grass Species	Waterlogged	Normal	Drought
P. annua	$Z_{1,8}$=2.392, P<0.05	$Z_{1,8}$=5.017, P<0.05	$Z_{1,8}$=4.561, P<0.05
F. r. rubra	$Z_{1,8}$=2.413, P<0.05	$Z_{1,8}$=6.152, P<0.05	$Z_{1,8}$=5.171, P<0.05
A. capillaris	$Z_{1,8}$=0.226, P>0.05	$Z_{1,8}$=5.087, P<0.05	$Z_{1,8}$=4.340, P<0.05
A. stolonifera	$Z_{1,8}$=3.597, P<0.05	$Z_{1,8}$=4.989, P<0.05	$Z_{1,8}$=3.192, P<0.05

Table 17: The statistical results for change in percentage root length colonisation following addition of mycorrhizal inoculants in different watering regimes.

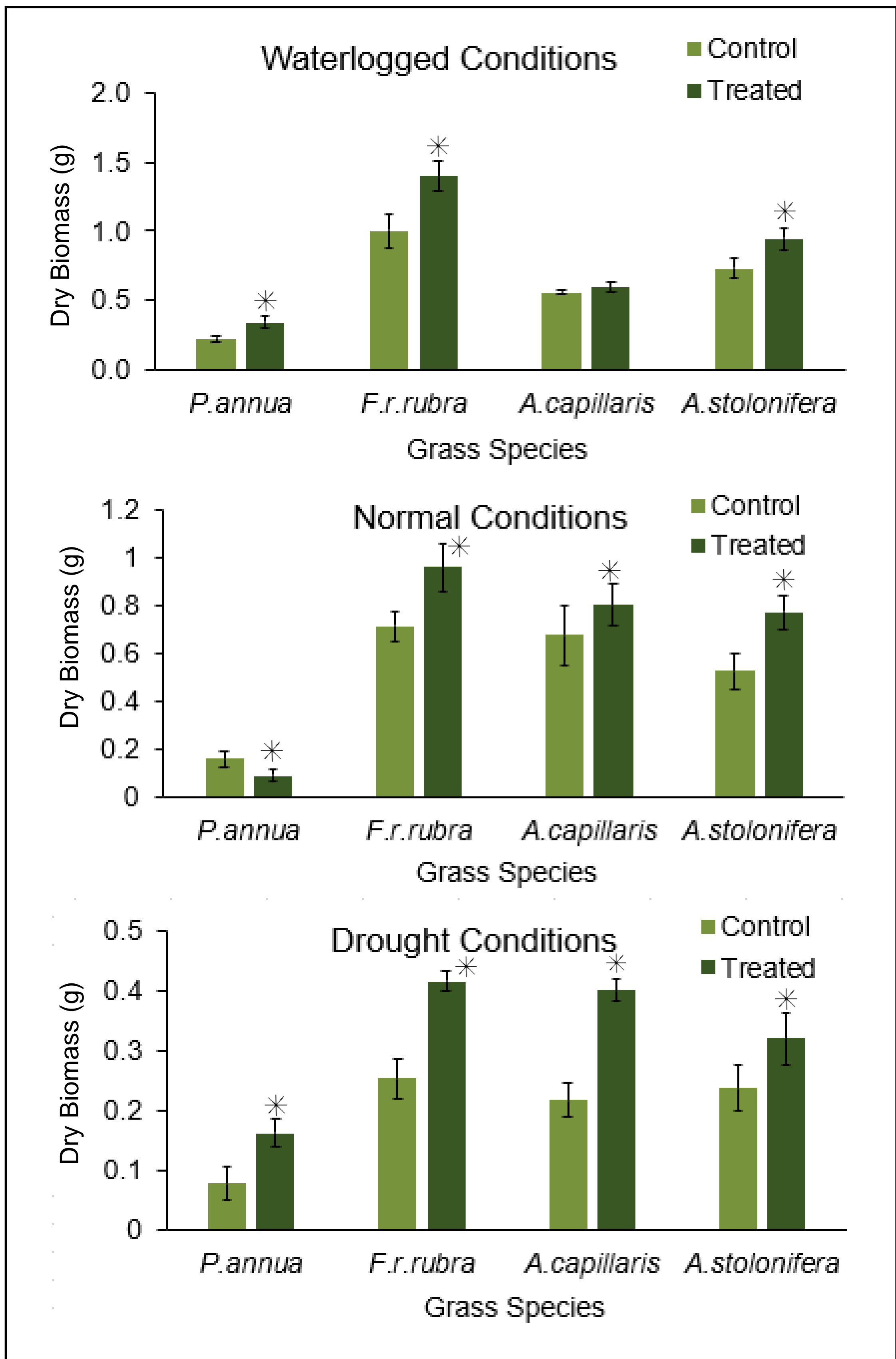


Figure 77: The difference in dry biomass for turf grass treated with mycorrhiza or control under different watering regimes. Error bars show standard error. Starred bars show significant difference from the control.

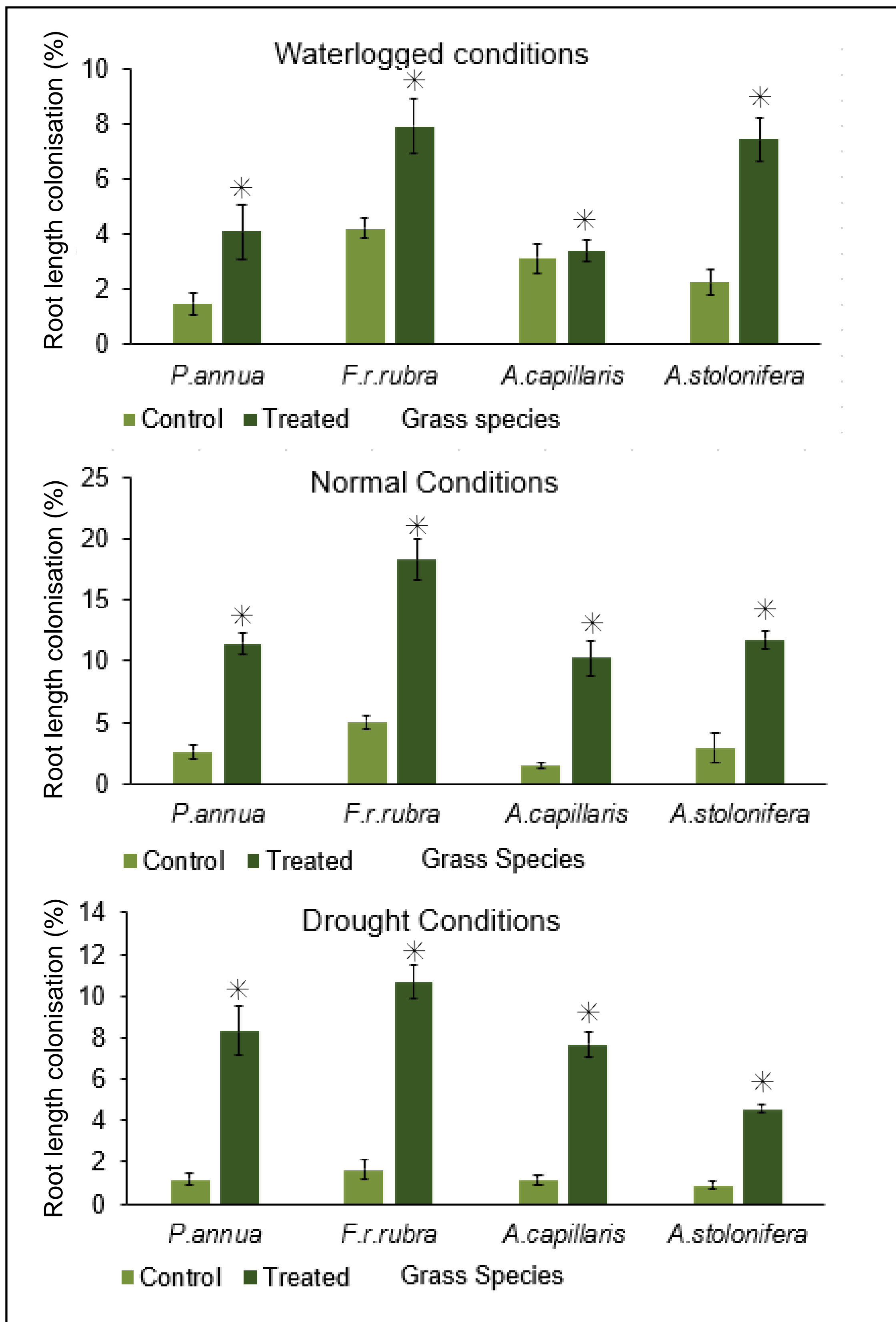


Figure 78: The difference in percentage root length colonisation for turf grass treated with mycorrhiza or control under different watering regimes. Error bars show standard error. Starred bars were significantly different from the control.

6.3.5 *Poa Annua* trial

The dry biomass of *P. annua* (shown in Figure 79) decreased following colonisation with all mycorrhizal inoculants tested ($F_{6,28}=3.487$, $P<0.05$), especially for *Glomus spp A* ($P<0.05$) and the consortium of species ($P<0.05$). Percentage root length colonisation increased consistently for each AMF species added ($Z_{6,28}=13.401$, $P<0.05$), as shown in Figure 80.

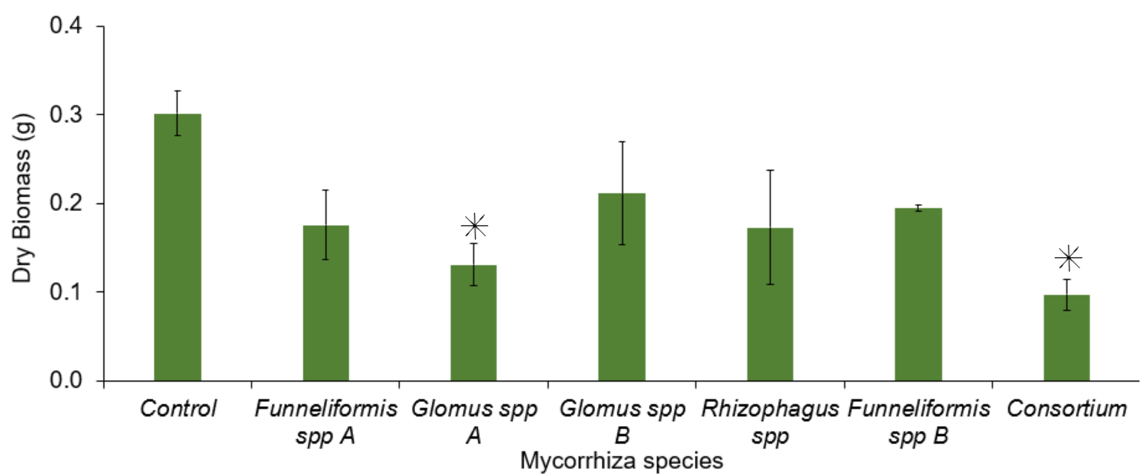


Figure 79: The dry biomass of *Poa annua* when treated with different monocultures of mycorrhiza. The error bars show standard error. Starred bars were significantly different from the control.

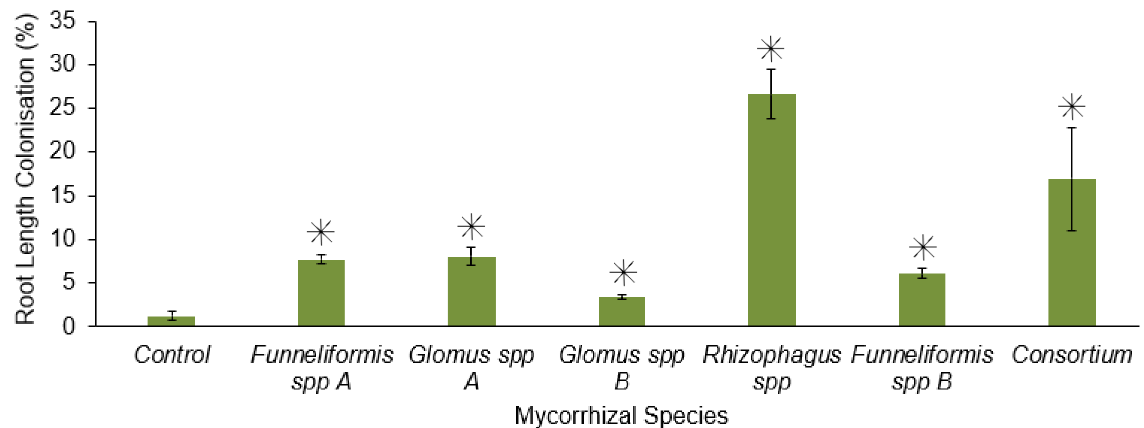


Figure 80: The percentage root length colonisation in *P. annua* following addition of mycorrhizal inoculant. Error bars show standard error. Starred bars were significantly different from the control.

Inequality results for *P. annua* (Table 18) showed thet all AMF species increased inequality and caused greater variation in plant size except for *Funneliformis spp B. Glomus spp A* caused a significant decrease in dry biomass and also had a significant increase in inequality shifting towards smaller plants (indicated by coefficient values less than unity). The Lorenz asymmetry coefficients showed that most AMF caused a shift to smaller plants (shown by values less than one), except for *Funneliformis spp* A and B and the consortium of AMF.

	Gini Coefficient (95% confidence interval)	Lorenz Asymmetry Coefficient
Control	0.102 (0.0740-0.170)	1.257
Glomus spp A	**0.371** (0.229-0.629)	0.904
Rhizophagus spp	0.170 (0.109-0.294)	0.958
Funneliformis spp A	**0.397** (0.219-0.714)	1.172
Glomus spp B	**0.382** (0.201-0.679)	0.931
Funneliformis spp B	**0.0211** (0.0161-0.0345)	1.267
Consortium	0.218 (0.159-0.370)	1.263

Table 18: The Gini Coefficient with 95% Confidence Interval and Lorenz asymmetry coefficient (LAC) for *P. annua* when treated with different AMF. Bold results show significant differences from control.

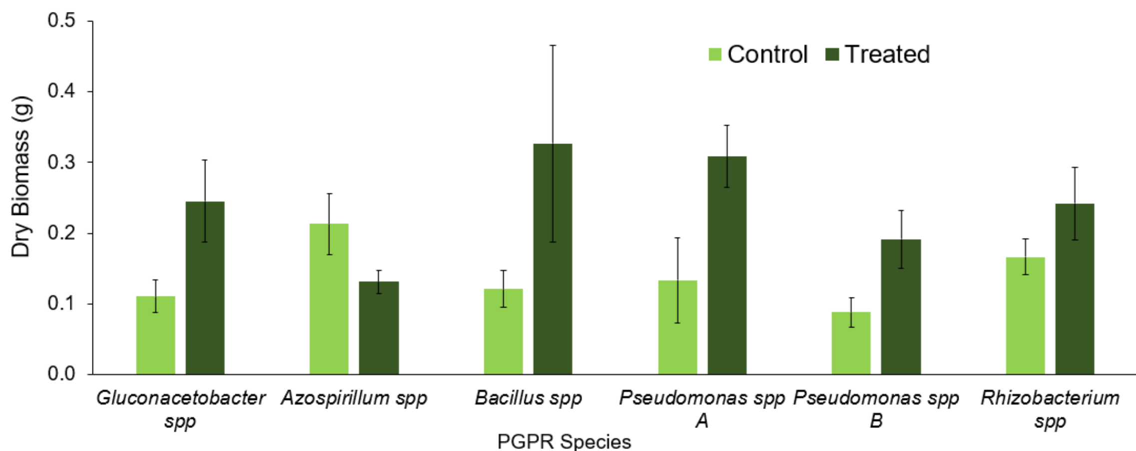


Figure 81: Dry biomass of *Poa annua* when treated with different monocultures of PGPR. Error bars show standard error.

Dry biomass of *P. annua* changed significantly between treatments of different PGPR monocultures (χ^2_{11}=22.656, P<0.05) with most species increasing biomass, except for *Azospirillum spp* which decreased it (shown in Figure 81).

PGPR had a varied effect on the inequality of *P. annua* as shown by the results in Table 19. In particular, *Bacillus spp*, caused an increase in inequality by encouraging larger plants and R*hizobacterium* by causing a shift to smaller plants (indicated by coefficient values less than unity). *Pseudomonas spp A* was the only PGPR to significantly reduce the inequality on *P. annua* and make plant size more consistent between treated pots.

PGPR Species		Gini Coefficient (95% confidence interval)	LAC
Gluconacetobacter spp	Control	0.286 (0.136-0.530)	1.143
	Treated	0.324 (0.156-0.596)	1.103
Azospirillum spp	Control	0.278 (0.150-0.496)	0.962
	Treated	0.168 (0.108-0.291)	0.998
Bacillus spp	Control	0.287 (0.197-0.493)	1.216
	Treated	0.497 (0.336-0.921)	1.331
Pseudomonas spp A	Control	0.550 (0.394-0.964)	1.307
	Treated	**0.194** (0.0949-0.358)	1.134
Pseudomonas spp B	Control	0.309 (0.219-0.516)	1.141
	Treated	0.287 (0.0849-0.568)	0.829
Rhizobacterium spp	Control	0.199 (0.122-0.361)	1.399
	Treated	0.273 (0.038-0.569)	0.761

Table 19: The Gini Coefficient with 95% Confidence Interval and Lorenz asymmetry coefficient (LAC) for *P. annua* when treated with different PGPR. Bold results show significant differences from control.

6.4 Discussion

6.4.1 Thatch trial

Thatch was assessed using loss on ignition data to quantify the thatch content of soil cores and the tea bag index to consider the rates and completely of decomposition in the plots. AMF colonisation could not be determined as not enough root fragments could be recovered from the soil cores for analysis owing to the soil type being quite dense and the fragile roots of the grass.

6.4.1.1 Loss on ignition data

Microbial treatments mostly had no effect on the organic matter content of soil cores in thatch-prone or non-thatch-prone areas, agreeing with the hypothesis in 6.1.1. An exception was the increase in the organic matter of non-thatch prone plots treated with a combination of AMF and PGPR. This could have been due to disruption of the existing microbial community responsible for organic matter cycling due to competition with the added species, causing them to be less effective (Gadhave et al 2016). The reason for the lack of effect for other treatments could have been because thatch is mostly at the surface of the soil from the deposition of dead leaves whereas PGPR and AMF are situated in the rhizosphere. This means that AMF and PGPR are unlikely to influence surface organic matter as it is not near them. There is also a possibility - especially for thatch-prone sites- that microbes are not the limiting factor in nutrient cycling and so other factors prevent thatch breakdown, such as anaerobic conditions. Zhang et al (2015) found a correlation between organic matter and bacteria as organic matter provided more food for certain bacteria species but found no relationship for fungi. This implies that while greater thatch

presence could encourage the establishment of bacteria, there is unlikely to be an interaction with AMF.

6.4.1.2 Tea bag index

There was no difference found in thatch-prone or non-thatch-prone areas for decomposition rate or completeness using the tea bag index, rejecting the hypothesis in **Error! Reference source not found.**. This is not wholly s urprising as there is only one paper recording AMF increasing the rate of decomposition (Hodge et al 2001) and while this did use *L. perenne* leaves in a litter bag, they were not in a golf putting green environment. Golf putting greens have already been shown in Chapter 3 to have very specific microbial communities. This could have prevented the effective break down of the tea bag litter or meant that any AMF effective at decomposing organic matter are not also tolerant of the conditions in a golf putting green. This result is still useful however as it shows that microbial inoculants of PGPR and AMF marketed for thatch reduction are unlikely to be effective.

6.4.2 Disease trial

Different treatments had no effect on the AUDPC for the 2017/18 trial. The 2018/19 trial showed a significant decrease in percentage disease cover for three of the trial months following application of the preventative fungicide as would be expected. AMF however caused an increase in disease cover for two months, and AMF and PGPR an increase for one month. This contradicts both the hypothesis in 6.1.2 and existing research by Gange and Case (2003), where a negative correlation was observed between AMF and *Microdochium*

incidence. This could be because the trial site used had a 40% *P. annua* sward and so the AMF could be antagonising the *P. annua* (as shown in other trials) and therefore making it more susceptible to disease. Therefore, further research using different sward compositions is needed.

The lack of difference in disease incidence for PGPR is also contradictory to existing research, where *Bacillus spp* and *Pseudomonas spp* are shown to provide effective disease control in crops against *Fusarium* diseases. This is thought to be due to induced systemic resistance like AMF and the production of certain antibiotics in some cases (Nguvo & Gao 2019). The lack of difference for iron application also contradicted existing research by Mattox et al (2017) which had shown that increasing iron content decreased *Microdochium* incidence. The lack of effect for the curative fungicide was also surprising compared to the effectiveness of the preventative fungicide, as both are designed specifically to reduce *Microdochium.* This could have been because both had different active ingredients and so there may have been resistance to the propiconazole. The resistance of *M. nivale* to propiconazole in turf grasses has been shown previously in a thesis by Gourlie (2018) in Canada, who linked the resistance to the previous applications of propiconazole. However, as no propiconazole has been applied previously on the trial plot used -or any resistance recorded on the site- it is more likely to be due to the timing of the application in relation to symptoms, time of year and weather conditions. Once damage has occurred it is unlikely to recover over winter, so once symptoms are past a certain point, there will not be a reduction in scarring observed. Hence a preventative spraying approach is often recommended.

Another avenue for further research is the use of the fungi in the genus Trichoderma which have shown efficacy in crops against *Fusarium* diseases (Nguvo & Gao 2019) as well as *Microdochium* in turf grasses (Mattox et al 2016).

6.4.3 Turf Quality and Colour trial

There was no observable difference in turf quality or turf colour following different microbial treatments. This is contrary to findings by Zhang et al (2015) where they found a correlation between turf quality and microbial biomass. The lack of effect could be because the benefits of AMF and PGPR are often associated with increased plant growth and vigour, which are more difficult to observe in grass when mowed short to replicate a golf putting green. There was a difference between thatch prone and non-thatch prone plots, with thatch-prone turf normally of a higher quality but non-thatch prone of a better colour, however this could be related to cultivar rather than thatch.

6.4.4 Drought trial

Dry biomass increased with water application across all four grass species and following inoculation with AMF. This is unsurprising as irrigation is known to be a limiting factor in plant growth and has been well documented in crop plants due to the need for water in photosynthesis (Chen et al 2018). It was previously hypothesised that AMF would aid drought tolerance in all grasses which was shown to be upheld. However, AMF colonisation also caused an increase in dry biomass for all four grass species in all watering conditions except for *A.*

capillaris which experienced no change for waterlogged conditions. AMF have been shown previously to aid drought tolerance by reducing the effects of drought so that no symptoms are seen until a lower soil moisture content (Augé 2001). In the bioenergy grass *Saccharum arundinaceum* AMF colonisation was also found to reduce drought stress by causing the accumulation of non-enzymatic antioxidants and osmolytes (Mirshad & Puthur 2016). Therefore, the beneficial effects in drought conditions shown in this trial are concurrent with existing research.

P. annua decreased in dry biomass as expected based on other trial results, however it experienced an increase in dry biomass under drought and waterlogged conditions. It was previously suggested by Gange (1999) that the uptake of phosphate by AMF is not enough compared to the loss of carbon to be beneficial to *P. annua.* This trial suggests that that is only the case under normal water conditions and that drought stress causes this to change. Jupp & Newman (1987) found that drought conditions caused *L. perenne* to stop phosphate uptake and experienced reduced phosphate uptake even after the soil was rewetted. He & Dijkstra (2014) also found negative effects for nitrogen uptake in periods of drought though found no ill effects after rewetting. Therefore, if the same is true in *P. annua,* its relationship with AMF could become beneficial for phosphate and nitrogen uptake during drought. This would be especially pertinent as *P. annua* is especially prone to hydric stress (Cordeau et al 2018).

Mycorrhiza showed an increase in dry biomass even during waterlogged conditions for three grass species, despite percentage root length colonisation

being consistently lower than for other watering regimes. This increase in dry biomass has been well documented in wetland plants or those associated with regular flooding, including citrus plants which observed greater biomass and leaf catalase activity when colonised with AMF during flooding (Wu et al 2013). It has also been observed in semi-aquatic grasses that the longer time spent underwater the lower the AMF colonisation (Miller 2000) and flooding has been shown to change the AMF communities in the roots of wetland plants (Wang et al 2011), suggesting certain AMF are more tolerant to waterlogged conditions than others. While there is no previous putting green specific research on drought or waterlogging tolerance from AMF inoculation, the mechanisms and correlations could be similar.

6.4.5 *Poa Annua* trial

P. annua decreased in dry biomass following colonisation with all five monocultures of AMF tested as well as the consortium, thus upholding the hypothesis in 6.1.4. The most pronounced decreases were for *Glomus spp A* and the consortium. *Glomus spp A, Funeliformis spp A* and *Glomus spp B* experienced increases in inequality suggesting that these AMF are antagonistic to *P. annua* growth. This is in line with research by Gange (1998) and Gange (1999) and could provide an effective way to reduce *P. annua* prevalence in a golf putting green.

PGPR application in general interacted far better with *P. annua* than AMF, with all causing an increase in dry biomass except for *Azospirillum spp.* Of the PGPR tested, only *Pseudomonas* spp *A* significantly reduced inequality to give more consistent plant size between pots. PGPR have successfully been used

as a biocontrol for *P. annua* in the past (Zhou & Neal 1995), however, these trial results suggest that the effects depend greatly on the PGPR species used. For those golf courses who want to maintain *P. annua* swards they may prefer to consider certain PGPR instead of AMF when using microbial inoculants.

6.5 Conclusion

PGPR are more effective at enhancing *P. annua* than mycorrhiza, though mycorrhiza can help under drought conditions. AMF can be used to suppress *P. annua.* AMF have no effect on thatch degradation or soil decomposition. Colonisation with AMF can be effective at increasing tolerance to extreme watering conditions including drought and flooding. AMF can increase *Microdochium* patch disease in *P. annua* heavy swards with the only effective treatment in these cases being preventative fungicides. More research is needed on the disease tolerance effects of AMF colonisation on different sward compositions.

Chapter 7: General Discussion

7.1 Summary of outcomes

The outcomes of this thesis are summarised below in Table 20.

Objectives	Outcomes
Chapter 3: 1. To establish what microbes are already present in a range of golf courses around the UK. 2. To consider what management practises and environmental conditions may have influenced this microbial population.	• Golf putting greens had different microbial populations both within the same course and between courses. • PLFA analysis showed that the microbial population was dependent on the environmental factors of soil type, coastal or non-coastal location, age and course location. • The management practises that impacted microbial biomass were the number of active ingredients used in a year and biostimulant usage. • Sequencing showed that there were common abundant species between golf courses suggesting a golf course "species pool". Most species richness was comprised of the less abundant species. • AMF populations were present in golf putting greens but were largely unidentifiable to a species level.
Chapter 4: 1. To determine the impact of different pesticides on the microbial biomass of golf putting greens.	• Pesticide usage made little difference to microbial biomass though fungicides did increase the proportion of fungi to bacteria in some cases. • Biostimulants increased microbial biomass when observed on a golf course however showed no impact in the controlled field trial.

2. To determine the impact of different biostimulants on golf putting greens. 3. To determine the impact of garlic products on golf putting greens.	• Garlic treatments significantly reduced mycorrhizal colonisation for both grass species trialled and so are not compatible with a microbial management scheme.
Chapter 5: 1. To determine the most effective way to apply microbial inoculants. 2. To determine if any management practises can affect the application of microbial inoculants.	• Grass species interact differently with monocultures of AMF, PGPR or a consortium of AMF. In general AMF show beneficial effects. • High fertiliser rates decrease percentage root length colonisation of AMF; however, this does not always affect dry biomass. • AMF colonisation occurs at dose rates as low as 2kg ha^{-1} but optimal colonisation occurred with dose rates of 32kg ha^{-1} or 64kg ha^{-1}.
Chapter 6: 1. To determine the benefits of using microbial inoculants in a golf putting green	• AMF can aid drought and waterlogging tolerance in multiple grass species, helping grasses to cope with water stress. • AMF can antagonise *P. annua* making it a good option as a method to reduce the amount of this weed in swards. • Fertiliser usage can be reduced and still provide the same growth effects when coupled with a microbial inoculant.

Table 20: A summary of the objectives and outcomes of this thesis.

7.2 Existing microbial populations of golf putting greens

Golf putting greens had different microbial populations both within the same course and between courses. Through PLFA analysis it was shown that sand-based greens and younger golf courses had a wider variation in microbial biomass. It was also shown that location could affect microbial variation but that there was cross over between all locations. Coastal and non-coastal locations also had different microbial biomass, with coastal courses showing more bacteria and non-coastal more fungi, thought to be due to changes in salinity (Sardinha et al (2003), Juniper & Abbott (2006)). PLFA also showed changes in microbial biomass from increased pesticide active ingredient applications in a year and biostimulant usage. However, none of the PLFA results considered microbial species diversity, but the biomass and proportions between bacteria and fungi. While microbial biomass and the ratio of fungi: bacteria are often used as an indicator of soil health within the industry (Pitchcare 2015), it doesn't always show all effects. For example, overall total biomass cannot tell you if there are beneficial species present and how these species particularly could be affected by different treatments.

Sequencing trials were used to look more into microbial species richness in golf putting greens. This trial showed that there were common abundant species between golf courses suggesting a golf course "species pool". But there was also high species richness of less abundant species allowing each golf course to have its own microbial community. This is consistent with other microbial studies where there were few abundant species but a larger number of rare species (Gange et al 2018). This suggests inoculants should aim to use those common abundant species to ensure they are compatible with a wide range of

golf courses rather than just niche species. Decreased species richness is not always an issue if ecosystem processes and community function are maintained. Allison & Martiny (2008) found that this could be due to microbial communities being resistant or resilient to change, or that when changes occur the remaining species may be functionally similar to the originals. The similar communities demonstrated in these results would suggest that the species pool for microbes in golf putting greens may be adapted enough that they are resistant to most of the common factors that would change microbial community structure. Therefore, while species richness can be informative, biomass must still be accounted for to show the proportions of the overall community, which is why both weighted and unweighted unifrac PCoA plots were utilised.

For these reasons, many studies -as well as this one- choose to utilise both sequencing and PLFA to look at both biomass and species richness. Chen et al (2019) found that PLFA and sequencing were similar to each other when characterising microbial communities, though while sequencing provided greater depth to a species level, PLFA responded quicker to changes in population. This could be because PLFA uses membrane phospholipids and so only detects living organisms (Zelles 1999). It is subsequently recommended that green keepers and companies consider microbial species richness alongside microbial biomass in soil testing.

7.3 How applicable will microbial inoculants be on golf putting greens

Microbial inoculants are already popular in the sports turf industry but there has been little UK specific research to show they could be effective in this habitat.

One of the main aims of this thesis was to provide some of this research and establish a way to utilise microbial inoculants within a management program.

Inoculants have previously been shown to be effective at changing the microbial community despite the presence of an indigenous community in both pot (Janouškova et al 2013) and field trials (Niwa et al 2018). The experiments within this thesis showed that microbial inoculants can colonise a range of different grass species, both in a consortium and as monocultures. This is in line with existing research showing that grasses are often mycorrhizal (Gange (1994), Vandenkoornhuyse et al (2003), Gustafon & Casper (2006)). This thesis also utilised inequality to show how well different microbial species interacted with each grass species tested, demonstrating which would be best as microbial inoculants in the field. The differing interactions for grass species with different AMF and PGPR support previous research on host specificity (Gustafon & Casper 2006, Gadhave et al 2016).

Golf putting greens were shown to have an existing AMF population as part of the golf putting green species pool, though of those that could be identified to a species level there were six AMF species found (though including different strains of each). In general, microbial species richness was shown to be good in golf putting greens for bacteria and fungi, with an average number of 25.8 OTUs of AMF identified per golf putting green. However, this number varied significantly between greens with one green showing no AMF at all. Also, as 68% of AMF could not be identified to a species level there is still a lot more research needed to isolate these AMF and determine the beneficial effects they could have.

Van der Heijden et al (1992) showed that lower diversity AMF populations caused more disparity between the dry biomass of different grass species in a community. This is because plants show host preference to different AMF and so with a smaller range available only the plants with their preferred species present will experience the greatest benefits (Van der Heijden et al 1998). This suggests that a diverse AMF population has the greatest potential to help a mixed sward but supports that tailored inoculums could provide competitive benefits to desired grass species. In this thesis this effect was shown for *P. annua* which showed a decrease in dry biomass resulting from AMF colonisation, while all bentgrasses, ryegrasses, and fescues trialled increased in dry biomass.

It is often seen as beneficial to apply a mixed inoculum of microbial species, to have the greatest chance of providing a species that will colonise well and be beneficial. Alkan et al (2006) showed that co-inoculation with multiple species can be effective, with different hosts specifying which AMF of the inoculant to allow to colonise. Consortia of species also provide the greatest chance of success for AMF species surviving potentially different environmental conditions, such as salinity and phosphate content (Alkan et al 2006). Application of an AMF consortium in this thesis did provide more consistent benefits for the grass species tested than monocultures. This is consistent with findings by Gadhave et al (2016) who found that when inoculums contained species common to the root system they increased in population in the rhizosphere. Similarly, Crossay et al (2019) found that beneficial effects of AMF were greatest in a native consortium and suggested there may be “functional complementarity” between distantly related mycorrhizal species.

The application of PGPR alongside AMF can allow for plant benefits if AMF do not colonise effectively due to environmental conditions. In wheat, host plant pathogen defences were improved upon the addition of compatible PGPR and AMF (Pérez-de-Luque et al 2017). Raklami et al (2019) showed that a combination of AMF, PGPR and rhizobia increased plant productivity and nutrient contents of the shoots in wheat. In some cases, PGPR can also stimulate AMF growth and development making them more effective (Toro et al (1997), Barea et al (1998)). Schutz et al (2018) showed in a meta-analysis of biofertilisers that a combination of AMF and PGPR specialising in phosphate solubilisation and nitrogen fixing were the most beneficial inoculants. They indicated that a combination of the three species contributed the most to yield with the lowest variation in results, allowing for a more consistently effective inoculum (Schutz et al 2018).

Despite consortia being a better option for microbial inoculants, these can still be tailored to the plant species and environmental conditions required, especially as individual plants can be colonised by up to twenty different AMF species (Fitter 2005). Hortal et al (2013) demonstrated the root exudates in the soil determine the PGPR population, and so different inoculants must be tailored to the target plant in order to be effective. Engelmoer et al (2014) showed that different AMF species can compete -especially at high phosphate levels- so the competitiveness of the species used must also be considered, as well as potential interactions with existing microbial populations. Conn & Franco (2004) showed that non-tailored inoculants halved microbial species diversity in wheat, and so non-tailored inoculants could have detrimental effects to the existing community. Similarly, Gadhave et al (2018) showed that just one

species of PGPR has the potential to disrupt the rhizosphere population. Another significant reason to tailor inoculums is to ensure native species are used. As the trading of inoculants increases there is little research on the potential negative impacts of introducing foreign species to the soil and the consequences if these were to become invasive (Schwartz et al 2006).

The most important factor affecting the application of microbial inoculants to golf putting greens was the management technique used. The fertiliser trial in this thesis showed that high levels of fertilisation decrease percentage root length colonisation but that this did not always correlate with the effects on dry biomass. Pesticide usage had no effect on microbial biomass other than fungicides increasing the proportion of fungi, though this is thought to be due to a lack of competition allowing a few species of fungi to overpopulate. However, the analysis of existing golf courses by PLFA did show that the more active ingredients of fungicide applied in a year the lower the microbial biomass of the greens. This suggests that the impacts of fungicides on golf greens is more to do with the number of active ingredients used than the specific type of fungicide. Despite this even the highest numbers of active ingredient applications still had a fungal community and so there must be a population that can tolerate fungicide usage. Green keepers should therefore not forego fungicide use when necessary and preventing disease resistance to fungicides must remain a priority.

The most interesting result was shown for garlic treatments, which are the only commonly used treatments for nematodes and are often viewed as a “biologically friendly” option. Garlic treatments caused a significant reduction in

AMF colonisation both when tested in pot trials and in the field. This is especially concerning as a lot of green keepers would not consider the harmful effects of this treatment if they are using microbial inoculants, and so could be counteracting any work done to improve soil biology. To avoid these harmful effects greenkeepers should avoid using garlic products where possible. They should also leave time between treatments of garlic or fungicides and microbial inoculants to try and avoid negative interactions.

As the management techniques of a golf course could have serious impacts on the effectiveness of an inoculant, the following recommendations can be made to improve the likelihood of them establishing.

1. Inoculants must be tailored to specific swards and conditions where possible in order to achieve the desired effects.
2. Fertiliser usage can be lower when applying AMF and the same growth effects still be achieved. This also allows the AMF a greater chance to colonise.
3. Fungicides can still be used with microbial products, but time should be allowed between applications of the two to give maximum benefit.
4. Garlic products however should be avoided if green keepers are trying to encourage AMF.

7.4 Could microbial inoculants feasibly replace any existing products

Despite some very promising results, it is unlikely that microbial inoculants will replace chemical management in the foreseeable future, and instead should be used in conjunction with them through integrated pest management. This is primarily because pesticides have been proven to be reliable regardless of

grass species and most other management techniques, in managing disease. The same cannot be said for microbial inoculants, as shown by the increase in disease incidence shown from AMF application to a *P. annua* heavy sward (see section 6.3.2). Despite other research showing that AMF can help reduce disease incidence in other grass species, due to the presence of *P. annua* there was a negative effect. Of all the treatments assessed, the preventative fungicide was the only effective treatment, however this trial should be repeated for different sward compositions. PGPR have shown promising results in reducing disease however they do not always perform well in the field (Cordiki et al (2004), Herrmann & Lasueur (2013), Gadhave et al (2016)). Voříšková et al (2019) determined that AMF performance was dependent on abiotic conditions of the habitat, further stressing the need for tailoring to particular environments to avoid negative effects. Therefore, until microbial inoculants can be made more consistent in the field and between grass species, they are unlikely to replace pesticides. Microbial inoculants do have a place within integrated pest management to improve plant health, stress tolerance, nutrient cycling and other benefits, while still using plant protection products when necessary.

There were multiple benefits of microbial inoculants identified in this thesis outside of disease suppression. Firstly, as a biocontrol for *P. annua,* which was proven to be antagonised by a range of AMF and PGPR. This does however mean that *P. annua* is more susceptible to disease so this should be a consideration for green keepers planning on utilising this method. Currently there are few methods to control *P. annua* so an effective control would be very important to the industry. A concern with the use of AMF to control *P. annua* could be the formation of gaps, particularly in *P. annua* heavy greens. AMF

antagonism would hopefully occur over a period by allowing other grasses to outcompete and so should avoid gaps in grass coverage. Alternatively, a good approach could be applying AMF with bentgrass seeds to fill in any gaps if they do form and encourage that competition against *P. annua*.

Drought and waterlogging resistance in several grass species -including *P. annua*- has the potential to be very useful as water stress becomes more prevalent with climate change (Arnell 1999). The potential for a reduction in fertiliser usage would also prove a useful benefit and cost saving measure in many golf courses. Based upon the findings of this thesis it is recommended that microbial inoculant use be encouraged to enable better plant tolerance to reductions in water and fertiliser use, as well as to control the growth of *P. annua* and allow other grass species within a green to outcompete it.

Biostimulants aim to stimulate existing microbial communities and aid proliferation of what is already present. The main benefit of these products would be that there would be no potential of introducing exotic species, and thus increasing competition and reducing the existing microbial population (Gadhave et al 2016). Alternatively, inoculant applications allow you to only increase populations of those microbes you know are beneficial, however they may not be compatible in the existing community. While field trials didn't show any effects of biostimulant application, there was an observed difference in microbial community within the observed golf courses. This suggests biostimulants could be effective given the correct existing microbial community, so more research is required on their application in conjunction with inoculants rather than on their own.

7.5 Conclusions

- Golf putting greens have specific microbial communities which are affected by multiple factors, including inoculant usage, age, location and pesticide usage.
- In specific field trials there was little difference shown upon pesticide use, backing up the concept of a golf putting green “species pool” that is tolerant of the unusual conditions.
- Other management practises that impacted microbial biomass were choice of grass species which could increase or decrease colonisation, and application of fertilisers which decreased AMF colonisation.
- Garlic treatments reduced AMF colonisation so would need to be avoided for those hoping to promote AMF or significant time given between treatments to avoid negative effects.
- Microbial inoculants have the potential to aid drought resistance, reduce fertiliser usage and help to antagonise *P. annua* growth.
- Inoculants must be tailored to the grass species in order to have the desired effects.
- Further research is needed to help improve this tailoring and further examine the potential benefits to microbial management in sports turf.

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Chapter 9: Appendices

9.1 FAMES/BAMES List for PLFA

BAMES:

- Methyl undecanoate
- Methyl-2-hydroxydecanoate
- Methyl dodecanoate
- Methyl tridecanoate
- Methyl 2-hydroxydodecanoate
- Methyl 3-hydroxydodecanoate
- Methyl tetradecanoate
- Methyl 13-methyltetradecanoate
- Methyl12-methyltetradecanoate
- Methyl pentadecanoate
- Methyl 2-hydroxytatradecanoate
- Methyl 3-hydroxytetradecanoate
- Methyl 14-methylpentadecanoate
- Methyl cis-9-hexadecanoate
- Methyl hexadecanoate
- Methyl 15-methylhexadecanoate
- Methyl cis-9,10-methylenehexadecanoate
- Methyl heptadecanoate
- Methyl 2-hydroxyhexadecanoate
- Methyl cis-9,12-octadecanoate
- Methyl cis-9-octadecanoate
- Methyl trans-9-octadecanoate

- Methyl octadecenoate
- Methyl cis-9,10-methyleneoctadecanoate
- Methyl nonadecanoate
- Methyl eicosanoate

FAMES:

- Methyl butyrate
- Methyl hexanoate
- Methyl octanoate
- Methyl decanoate
- Methyl undecanoate
- Methyl laurate
- Methyl tridecanoate
- Methyl tetradecanoate
- Myristoleic Acid Methyl Ester
- Methyl pentadecanoate
- cis-10-Pentadecenoic acid methyl ester
- Methyl palmitate
- Methyl Pamitoleate
- Methyl heptadecanoate
- cis-10-Heptadecanoic acid methyl ester
- Methyl octadecenoate
- trans-9-Elaidic acid methyl ester
- cis-9-Oleic acid methyl ester

- Linolelaidic acid methyl ester
- Methyl Linoleate
- Methyl Arachidate
- gamma-Linolenic acid methyl ester
- Methyl cis-11-eicosenoate
- Methyl linolenate
- Methyl heneicosanoate
- cis-11,14-Eicosadienoic acid methyl ester
- Methyl docosanoate
- cis-8,11,14-Eicosatrienoic acid methyl ester
- Methyl Erucate
- cis-11,14,17-Eicosatrienoic acid methyl ester
- Methyl tricosanoate
- Methyl cis-5,8,11,14-Eicosatetraenoic
- cis-13-16-Docosadienoic acid methyl ester
- Methyl lignocerate
- Methyl cis-5,8,11,14,17-Eicosapentaenoate
- Methyl Nervonate
- All cis-4,7,10,13,16,19-Docosahexanoate

9.2 Grass Species Application Rates

Species name	Common name	Brand name	Sowing rate/gm^{-2}
Lolium perenne 2n	Perennial Ryegrass	Poseidon	35
Lolium perenne 4n	Perennial Ryegrass	Tetragreen	40
Festuca rubra rubra	Strong Creeping Red Fescue	Hastings DLF FRR 9559	30
Festuca ruba trichophylla	Slender Creeping Red Fescue	Turner 1	30
Festuca rubra commutata	Chewings Fescue	Greenmile	30
Festuca arundinacea	Tall Fescue	Essential	50
Poa pratensis	Smooth Stalked Meadow Grass	Greenplay	15
Poa annua	Annual Meadowgrass	Naturescape	15
Agrostis capillaris	Browntop Bentgrass	Egmontwo TAT 721	9
Agrostis stolonifera	Creeping Bentgrass	Cobra nova (Cobra 2 USA)	8

Table 21: showing the different brands of grass species used and their respective sowing rates.

9.3 Golf Course Management Survey Questions

What is the yearly footfall of your golf club?

What soil type are your greens?

How old is your club?

What (approximate) grass mix is each of your greens?

Have your greens been renovated in the past 5 years? If so when?

Have you had any kind of soil testing completed before? If yes, would you be willing to share the results of these tests with me?

What diseases have affected your greens in the past 12 months? Please specify which disease affected which green/s if possible.

How often do you apply the following fungicide active ingredients?

1. Iprodione:
2. Chlorothalonil:
3. Fludioxonil:
4. Azoxystrobin:
5. Pyraclostrobin:
6. Trifloxystrobin:
7. Myclobutanil:

8.Propiconazole:

9.Tebuconazole:

10.Prochloraz:

Have you applied any of these chemicals within the 2 months before the soil samples were collected? If yes which one?

Do you use iron products on your soil? If yes which product and how often?

Do you use any biostimulants on your course? If yes which products do you use and how often?

Do you use any microbial products on your course? If yes, which products and how often?

Any other issues your club faces often? (Nematodes, Poa, Insect pests?) If so how do you treat them?

9.4 Golf Course Characteristics

9.4.1 PLFA golf course characteristics

Golf Course	Age bracket	Soil type	Coastal or non-coastal	Location
A	100-150	Soil	Non-coastal	Middle
B	100-150	Sand	Coastal	South
C	100-150	Soil	Non-coastal	South
D	100-150	Soil	Non-coastal	South
E	100-150	Sand	Non-coastal	Middle
F	0-50	Sand	Non-coastal	Middle
G	0-50	Sand	Coastal	North
H	100-150	Sand	Coastal	Middle
I	50-100	Sand	Coastal	North
J	150-200	Soil	Non-coastal	North
K	150-200	Sand	Coastal	North
L	100-150	Soil	Non-coastal	Middle
M	50-100	Soil	Non-coastal	South
N	50-100	Soil	Non-coastal	Middle
O	100-150	Soil	Non-coastal	North
P	50-100	Soil	Non-coastal	Middle
Q	0-50	Sand	Non-coastal	North
R	0-50	Sand	Non-coastal	South
S	0-50	Sand	Non-coastal	South

Table 22: The characteristics of different golf courses included in the PLFA trial

9.4.2 Sequencing golf course characteristics

Golf Course	Age Bracket	Grass composition	Soil Type	Pesticide active ingredients applied	Biostimulant use
A	100-150	Poa/ bentgrass	Soil	2	Seaweed, enriched compost, phopshite
B	100-150	Poa/ bentgrass	Soil	1	Seaweed, phopshite, enriched fertiliser, compost tea
C	0-50	Poa/ bentgrass	Sand	4	Amino acids
D	0-50	Poa/ bentgrass	Sand	4	Amino acids

Table 23:The characteristics of different golf courses included in the sequencing trial

9.5 Graphs for NDVI, Chlorophyll and Ground hardness of different applications of fungicide and herbicide

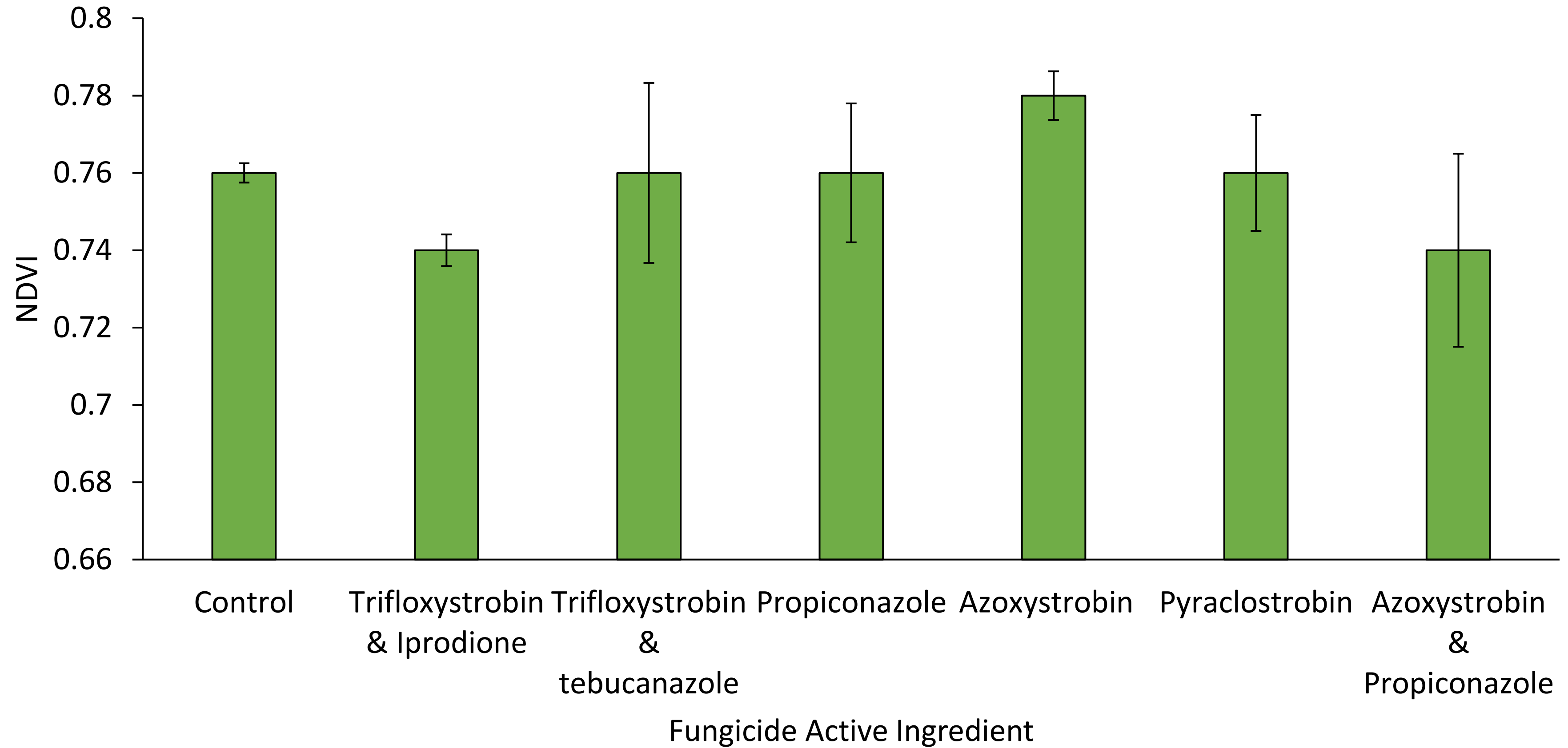

Figure 82: The effect of different fungicide active ingredients on the mean NDVI of trial plots. The error bars shown are one standard error.

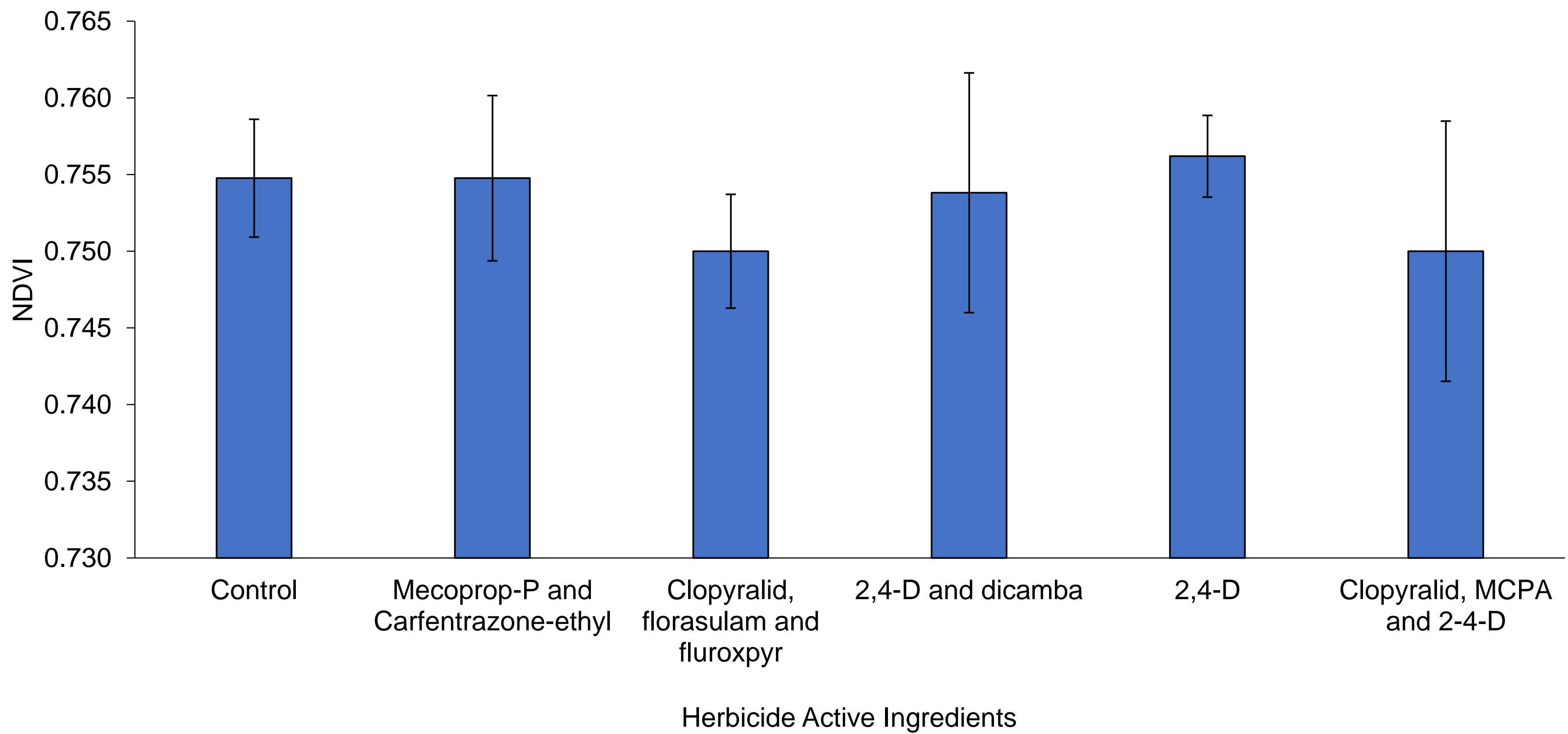

Figure 83: The effect of different herbicide active ingredients on the mean NDVI of trial plots. The error bars shown are standard error.

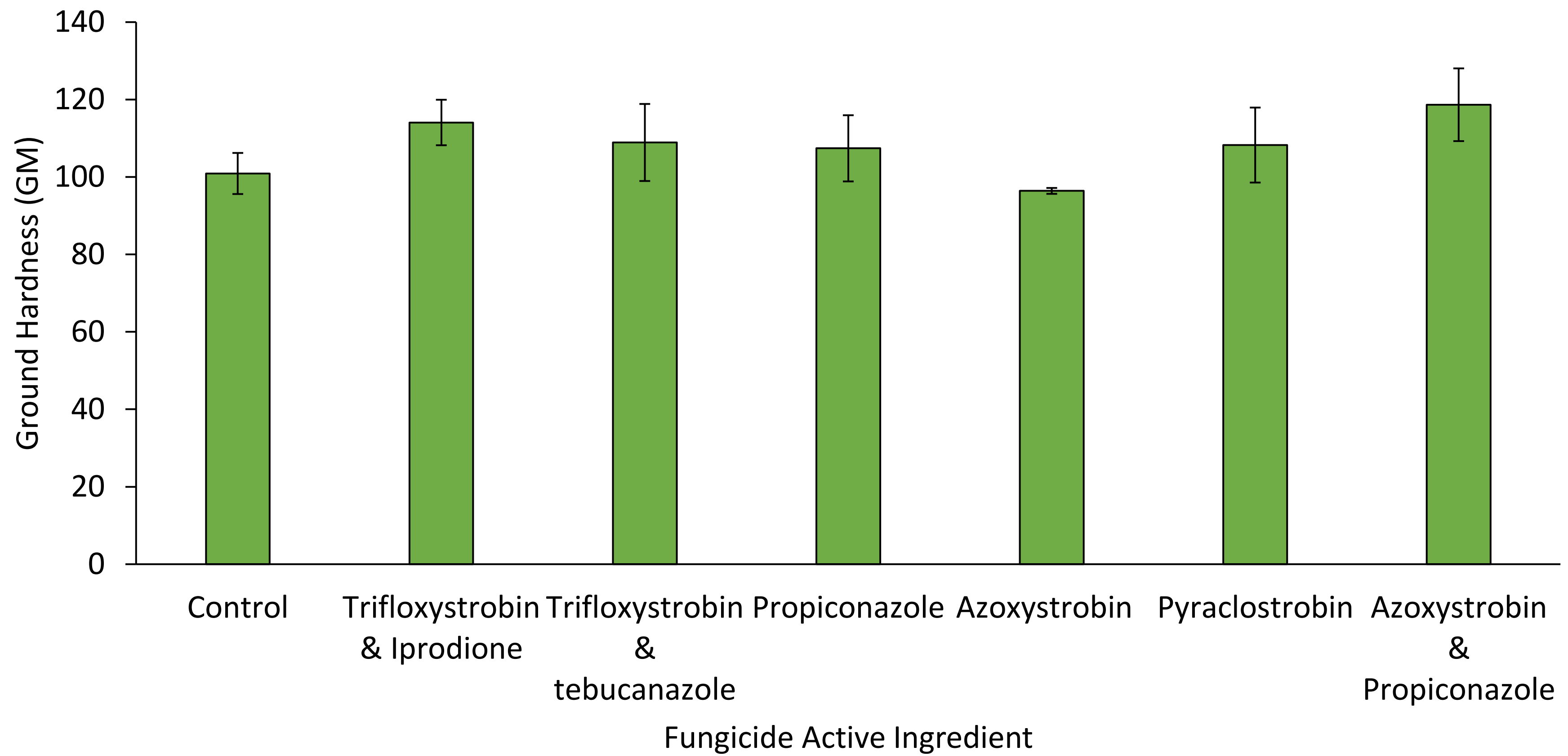

Figure 84: The effect of fungicide active ingredients on the mean ground hardness of trial plots. The error bars shown are standard error.

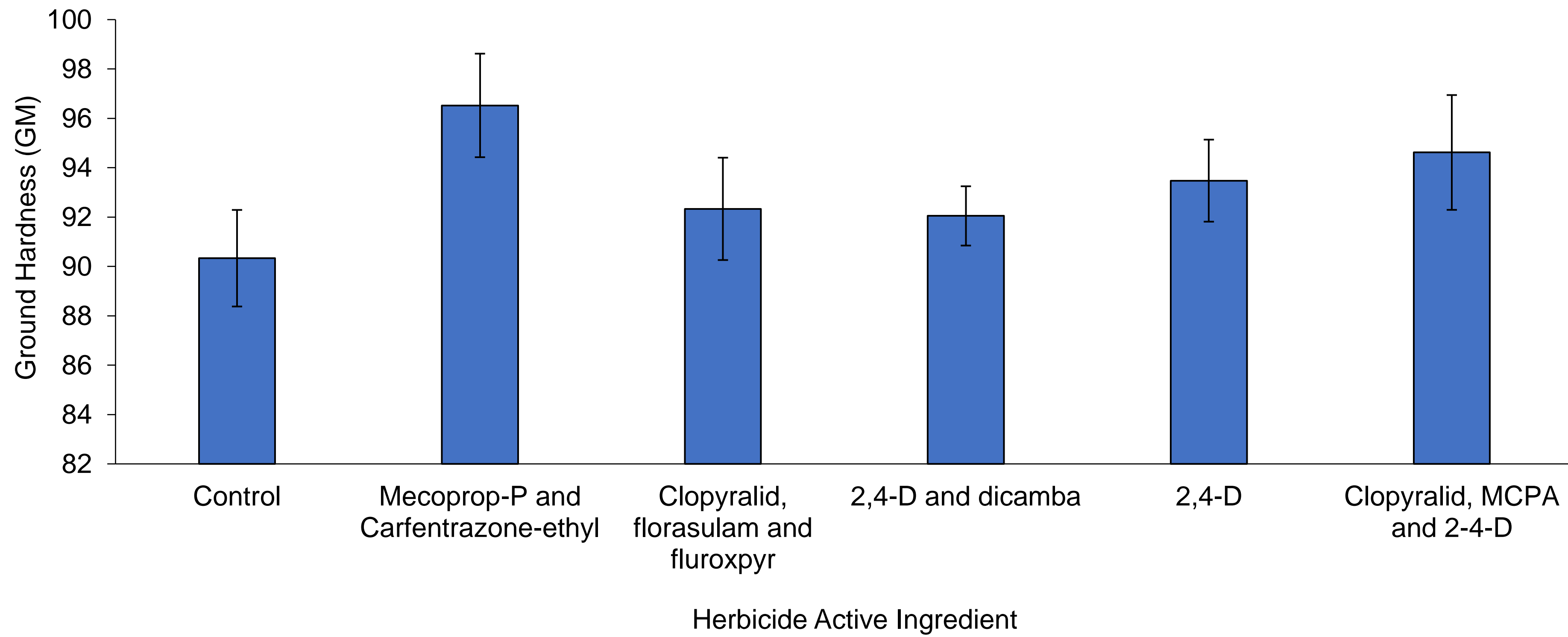

Figure 85: The effect of different herbicide active ingredients on the mean ground hardness of trial plots. The error bars shown are standard error.

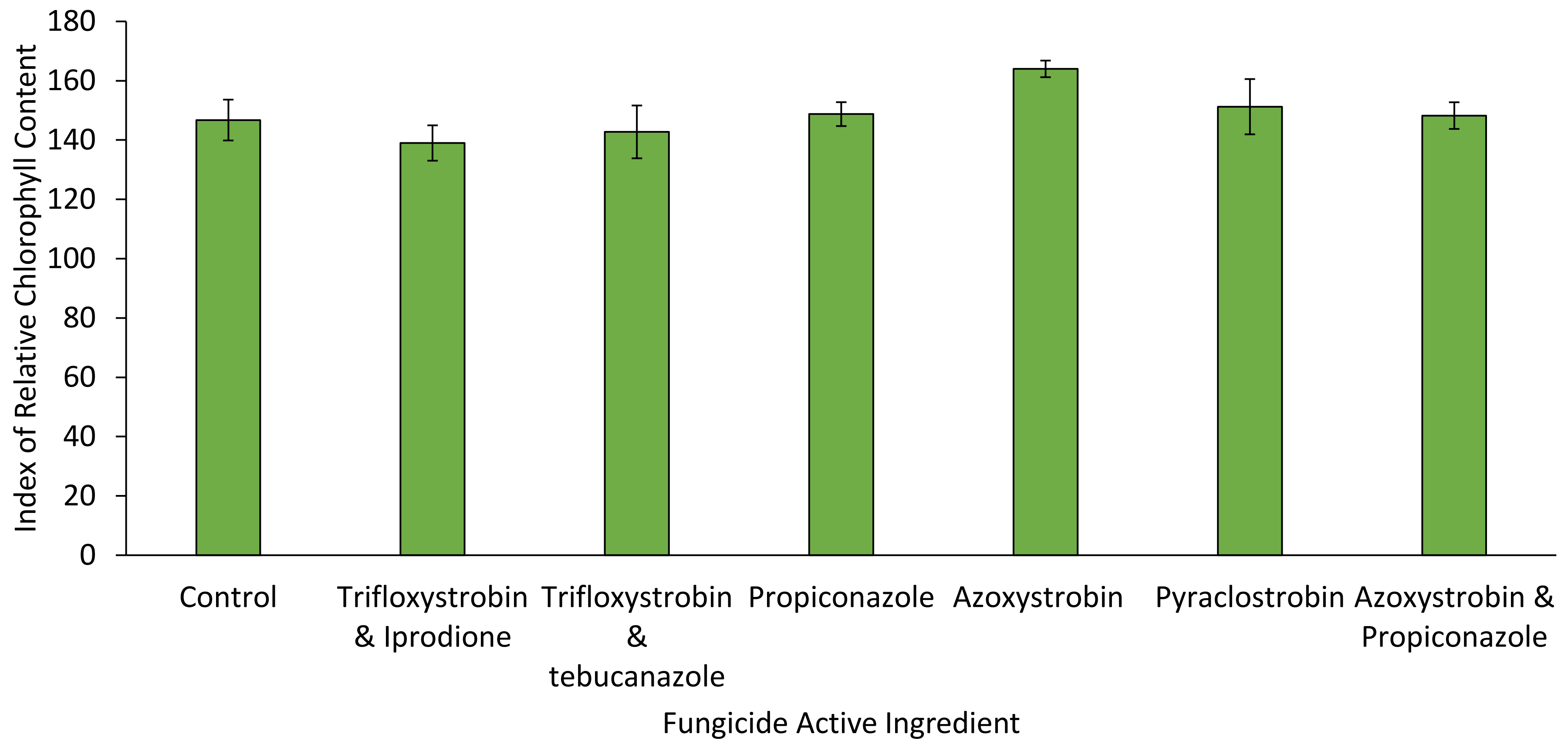

Figure 86: The effect of fungicide treatments on the mean chlorophyll content of trial plots. The error bars shown are standard error.

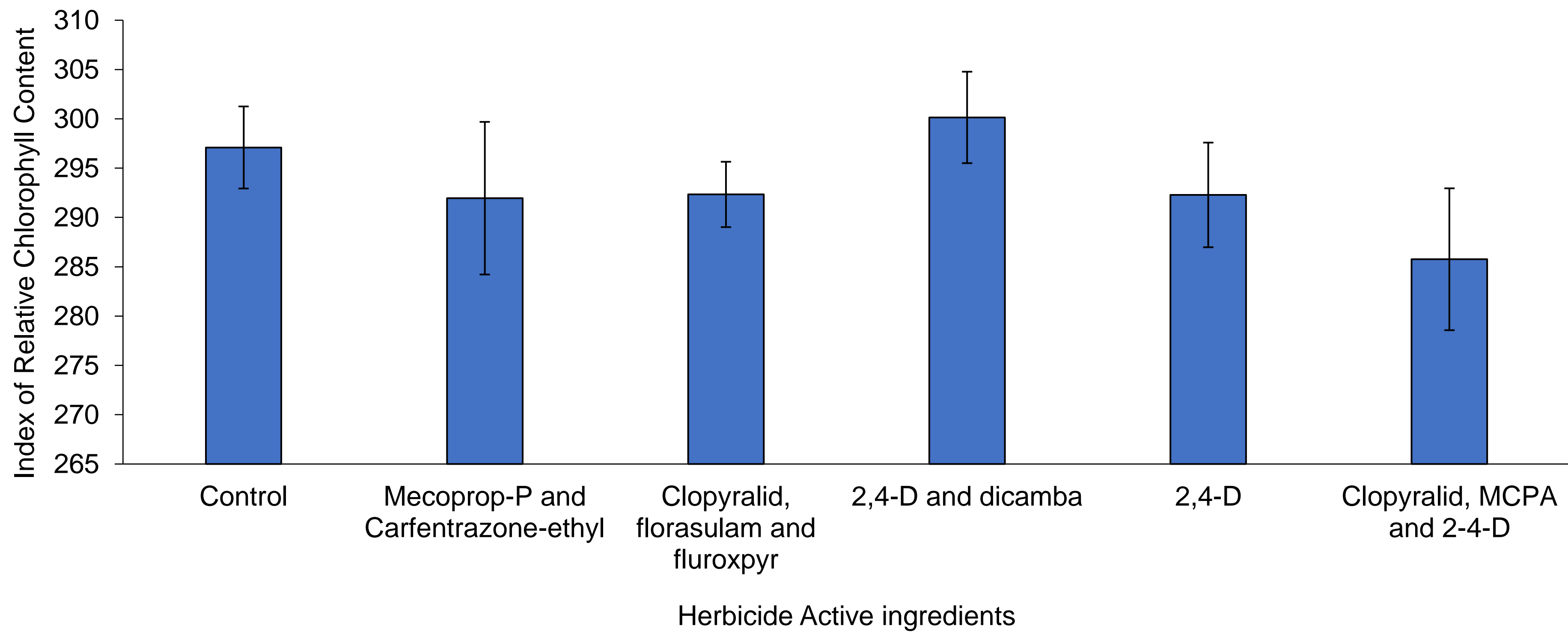

Figure 87: The effect of different herbicide active ingredients on the mean chlorophyll content of trial plots. The error bars shown are *standard error*.

9.6 The ingredients of the J.Arthur Bower Liquid Lawn food

Ingredient	Concentration in ml/L
Ammoniacal nitrogen	21
Ureic Nitrogen	123
Phosphorous Pentoxide	72
Potassium Oxide	72

Table 24: The ingredients of the J. Arthur Bower liquid lawn food used for the fertiliser trial.

9.7 Remaining graphs for changes in dry biomass of different grass species treated with PGPR

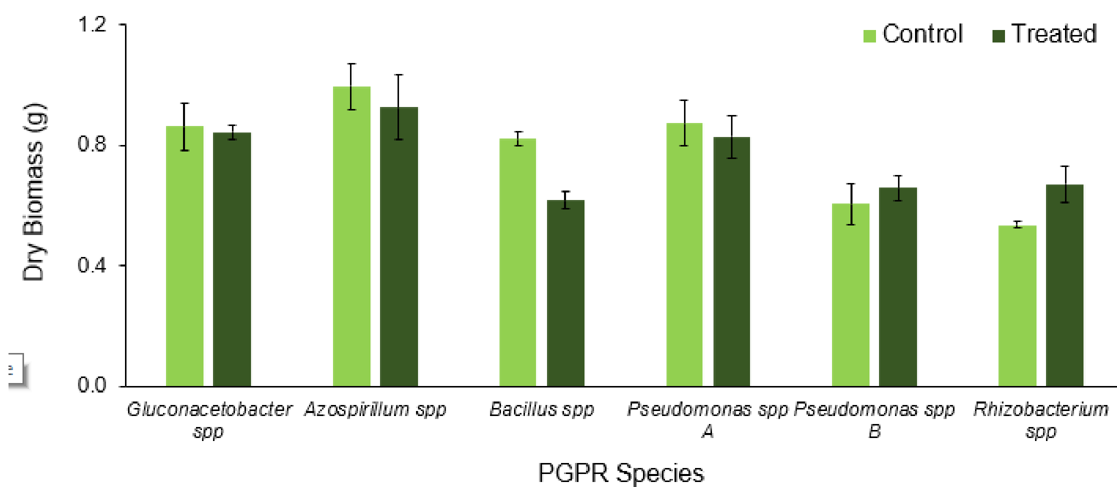

Figure 88: The change in dry biomass when PGPR species were added to *S.phoenix*. Error bars show standard error

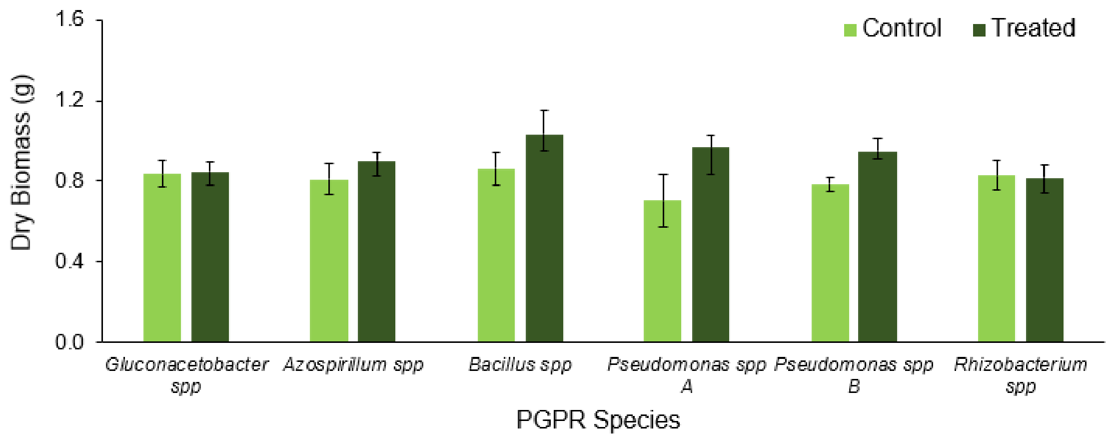

Figure 89: The change in dry biomass when PGPR species were added to *F. r. commutata*. Error bars show standard error

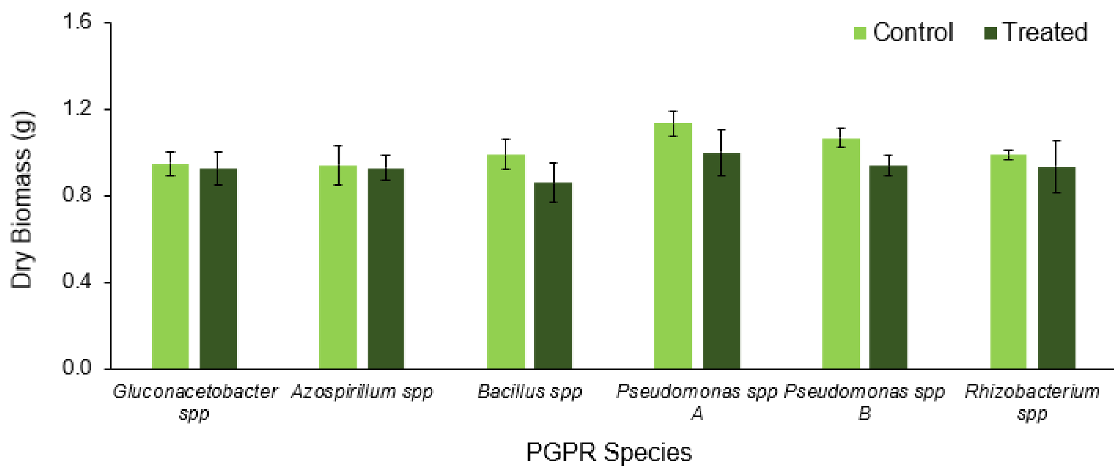

Figure 90: The change in dry biomass when PGPR species were added to *F. r. rubra*. Error bars show standard error

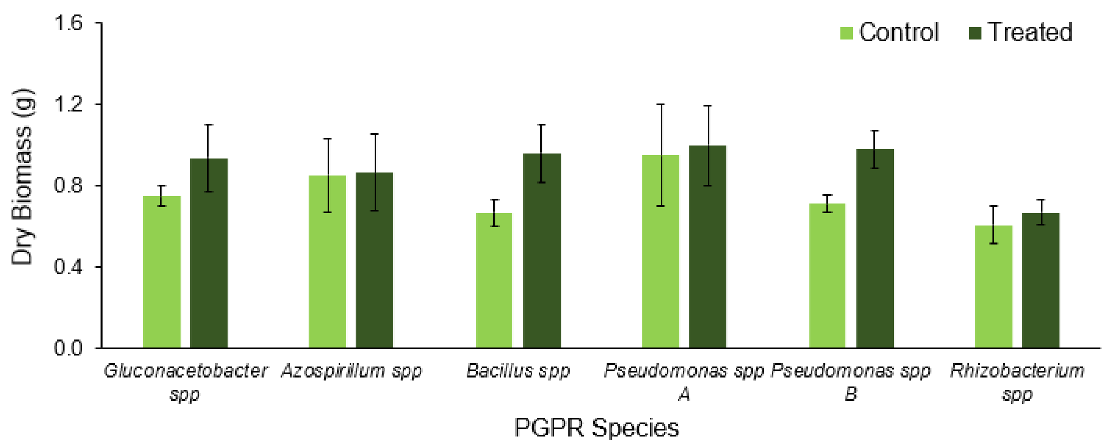

Figure 91: The change in dry biomass when PGPR species were added to *P. pratensis* Error bars show standard error

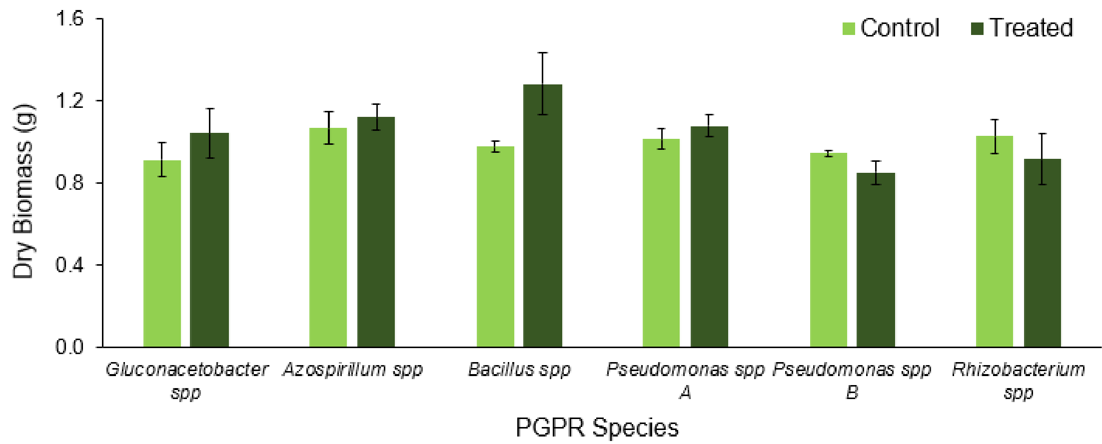

Figure 92: The change in dry biomass when PGPR species were added to *F. r. trichophylla*. Error bars show standard error

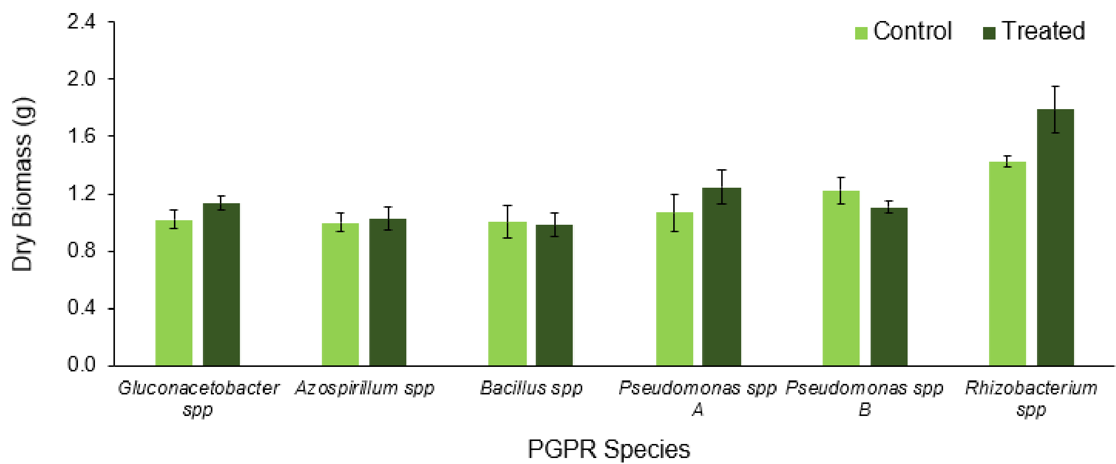

Figure 93: The change in dry biomass when PGPR species were added to *L. perenne (4n)*. Error bars show standard error

9.8 The Gini Coefficients and Lorenz asymmetry coefficients for different grass species treated with PGPR

Grass Species	PGPR Species		Gini Coefficient (95% confident interval)	LAC
Lolium perenne (2n)	*Gluconacetobacter spp*	Treated	0.167 (0.0915-0.335)	1.464
		Control	0.103 (0.0758-0.172)	1.238
	Azospirillum spp	Treated	**0.0153** (0.0087-0.0297)	1.403
		Control	0.1216 (0.0584-0.230)	0.903
	Bacillus spp	Treated	0.128 (0.0566-0.247)	0.906
		Control	0.0740 (0.0437-0.133)	0.980
	Pseudomonas spp A	Treated	0.0541 (0.0412-0.0883)	1.158
		Control	0.0518 (0.0228-0.101)	0.769
	Pseudomonas spp B	Treated	0.0921 (0.0490-0.175)	1.476
		Control	0.0578 (0.0441-0.0945)	1.136
	Rhizobacterium spp	Treated	0.139 (0.0972-0.237)	1.302
		Control	0.129 (0.0679-0.238)	0.915
Lolium perenne (4n)	*Gluconacetobacter spp*	Treated	0.0557 (0.0388-0.0953)	1.091
		Control	0.0892 (0.0688-0.146)	1.181
	Azospirillum spp	Treated	0.0919 (0.0466-0.172)	0.913
		Control	0.0748 (0.0441-0.137)	1.428
	Bacillus spp	Treated	0.119 (0.0790-0.206)	1.261
		Control	0.129 (0.0531-0.266)	1.557
	Pseudomonas spp A	Treated	0.131 (0.0869-0.224)	1.172
		Control	0.155 (0.0946-0.284)	1.432
	Pseudomonas spp B	Treated	0.0534 (0.0328-0.0959)	1.361
		Control	0.101 (0.0642-0.177)	1.245
	Rhizobacterium spp	Treated	**0.118** (0.0756-0.208)	1.119
		Control	0.0362 (0.0248-0.0619)	1.119
Poa pratensis	*Gluconacetobacter spp*	Treated	0.219 (0.130-0.412)	1.486
		Control	0.0861 (0.0537-0.153)	0.975
	Azospirillum spp	Treated	0.221 (0.105-0.454)	1.502
		Control	0.210 (0.0951-0.434)	1.499

	Bacillus spp	Treated	0.209 (0.159-0.345)	1.205
		Control	0.135 (0.0986-0.226)	1.281
	Pseudomonas spp A	Treated	0.271 (0.203-0.449)	1.113
		Control	0.330 (0.222-0.593)	1.410
	Pseudomonas spp B	Treated	0.127 (0.0948-0.212)	1.249
		Control	0.0776 (0.0476-0.140)	1.382
	Rhizobacterium spp	Treated	0.119 (0.0874-0.199)	1.258
		Control	0.188 (0.107-0.358)	1.504
Festuca rubra trichophylla	*Gluconacetobacter spp*	Treated	0.219 (0.130-0.412)	1.486
		Control	0.086 (0.0541-0.151)	0.975
	Azospirillum spp	Treated	0.221 (0.104-0.451)	1.502
		Control	0.210 (0.0944-0.434)	1.499
	Bacillus spp	Treated	0.209 (0.160-0.342)	1.205
		Control	0.135 (0.0989-0.227)	1.281
	Pseudomonas spp A	Treated	0.271 (0.204-0.448)	1.113
		Control	0.330 (0.223-0.591)	1.410
	Pseudomonas spp B	Treated	0.127 (0.0955-0.211)	1.249
		Control	0.0776 (0.0480-0.139)	1.382
	Rhizobacterium spp	Treated	0.119 (0.0876-0.199)	1.258
		Control	0.188 (0.107-0.356)	1.504
F .r. rubra	*Gluconacetobacter spp*	Treated	0.115 (0.0676-0.205)	1.007
		Control	0.0768 (0.0596-0.124)	1.216
	Azospirillum spp	Treated	0.0768 (0.0575-0.127)	1.075
		Control	0.117 (0.0752-0.206)	1.078
	Bacillus spp	Treated	0.147 (0.0957-0.254)	1.032
		Control	0.0951 (0.0705-0.157)	1.066
	Pseudomonas spp A	Treated	0.146 (0.108-0.243)	1.218
		Control	0.0711 (0.0543-0.116)	1.199
	Pseudomonas spp B	Treated	0.0729 (0.0534-0.121)	1.065
		Control	0.0567 (0.0436-0.0924)	1.212
	Rhizobacterium spp	Treated	0.153 (0.0170-0.334)	0.671
		Control	0.0305 (0.0158-0.0576)	1.589

F. r. commutata	*Gluconacetobacter spp*	Treated	0.0750 (0.0485-0.132)	1.154
		Control	0.0922 (0.0233-0.193)	0.721
	Azospirillum spp	Treated	0.0554 (0.0368-0.0961)	1.122
		Control	0.105 (0.0277-0.216)	0.761
	Bacillus spp	Treated	0.149 (0.0869-0.274)	1.457
		Control	0.123 (0.084-0.215)	1.331
	Pseudomonas spp A	Treated	0.0828 0.0421-0.159)	1.579
		Control	0.227 (0.0086-0.499)	0.632
	Pseudomonas spp B	Treated	0.0865 (0.0511-0.158)	1.434
		Control	0.0569 (0.0353-0.101)	1.047
	Rhizobacterium spp	Treated	0.106 (0.0751-0.180)	1.306
		Control	0.108 (0.0527-0.210)	1.569
Schedonorus phoenix	*Gluconacetobacter spp*	Treated	**0.0406** (0.0253-0.0723)	1.292
		Control	0.124 (0.0835-0.212)	0.991
	Azospirillum spp	Treated	0.144 (0.109-0.235)	1.140
		Control	0.0886 (0.0528-0.161)	1.429
	Bacillus spp	Treated	0.0703 (0.0536-0.116)	1.232
		Control	0.035 (0.0183-0.0666)	1.495
	Pseudomonas spp A	Treated	0.105 (0.0562-0.199)	1.555
		Control	0.116 (0.0747-0.208)	1.379
	Pseudomonas spp B	Treated	0.0793 (0.0456-0.153)	1.164
		Control	0.135 (0.0564-0.273)	0.974
	Rhizobacterium spp	Treated	**0.119** (0.0723-0.216)	1.353
		Control	0.0218 (0.0107-0.0475)	1.490
Agrostis capillaris	*Gluconacetobacter spp*	Treated	0.106 (0.0690-0.198)	1.327
		Control	0.0856 (0.0568-0.158)	1.254
	Azospirillum spp	Treated	0.0687 (0.0519-0.113)	1.184
		Control	0.107 (0.0730-0.181)	0.997
	Bacillus spp	Treated	0.261 (0.137-0.519)	1.486
		Control	0.302 (0.186-0.562)	1.438
	Pseudomonas spp A	Treated	0.116 (0.0790-0.196)	0.995
		Control	0.0637 (0.0296-0.122)	0.761

	Pseudomonas spp B	Treated	0.0977 (0.0514-0.185)	1.565
		Control	0.136 (0.0936-0.236)	1.295
	Rhizobacterium spp	Treated	0.121 (0.772-0.215)	1.328
		Control	0.182 (0.135-0.304)	1.258
A. stolonifera	*Gluconacetobacter spp*	Treated	0.107 (0.0758-0.183)	1.274
		Control	0.0695 (0.0501-0.116)	1.158
	Azospirillum spp	Treated	0.132 (0.0947-0.222)	1.009
		Control	0.110 (0.0471-0.221)	1.583
	Bacillus spp	Treated	0.145 (0.0789-0.276)	1.512
		Control	0.220 (0.146-0.395)	1.356
	Pseudomonas spp A	Treated	0.0565 (0.0390-0.0967)	1.211
		Control	0.0385 (0.0191-0.0730)	0.770
	Pseudomonas spp B	Treated	0.0802 (0.0591-0.134)	1.296
		Control	0.103 (0.0744-0.173)	1.201
	Rhizobacterium spp	Treated	0.150 (0.0883-0.280)	1.424
		Control	0.0734 (0.0208-0.0940)	1.108

Table 25: The Gini coefficients (with 95% confidence intervals) and Lorenz asymmetry coefficients for different grass species treated with PGPR. Bold results represent treatments significantly different from their control.

9.9 The Gini Coefficients and Lorenz asymmetry coefficients for different grass species treated with mycorrhizal monocultures

Grass Species	AMF Species		Gini Coefficient (95% Confidence Level)	LAC
Festuca rubra rubra	*Glomus spp A*	Treated	0.118 (0.081-0.203)	1.320
		Control	0.0526 (0.0385-0.0879)	1.302
	Rhizophagus spp	Treated	0.134 (0.0822-0.239)	1.210
		Control	0.0977 (0.0523-0.180)	1.077
	Funeliformis spp A	Treated	**0.0866** (0.060-0.147)	1.258
		Control	0.245 (0.187-0.398)	1.151
	Glomus spp B	Treated	**0.0567** (0.0414-0.0946)	1.068
		Control	0.168 (0.103-0.296)	1.007
	Funeliformis spp B	Treated	0.120 (0.0913-0.198)	1.234
		Control	0.112 (0.0546-0.210)	0.983
Agrostis stolonifera	*Glomus spp A*	Treated	0.259 (0.167-0.441)	0.961
		Control	0.180 (0.103-0.321)	0.973
	Rhizophagus spp	Treated	0.113 (0.0806-0.190)	1.028
		Control	0.207 (0.125-0.383)	1.352
	Funeliformis spp A	Treated	0.118 (0.0719-0.210)	1.064
		Control	0.348 (0.163-0.738)	1.448
	Glomus spp B	Treated	**0.266** (0.197-0.449)	1.228
		Control	0.0465 (0.0340-0.0776)	1.063
	Funeliformis spp B	Treated	0.162 (0.115-0.270)	1.051
		Control	0.0718 (0.0387-0.134)	1.547

Agrostis capillaris	*Glomus spp A*	Treated	0.119 (0.0728-0.216)	1.402
		Control	0.130 (0.0922-0.219)	1.010
	Rhizophagus spp	Treated	0.242 (0.151-0.431)	1.236
		Control	0.113 (0.0501-0.227)	1.570
	Funeliformis spp A	Treated	**0.083** (0.0589-0.140)	1.016
		Control	0.364 (0.267-0.622)	1.226
	Glomus spp B	Treated	0.180 (0.118-0.316)	1.099
		Control	0.102 (0.0500-0.194)	1.565
	Funeliformis spp B	Treated	0.117 (0.0653-0.224)	1.065
		Control	0.168 (0.0894-0.307)	1.130

Table 26: The Gini coefficients (with 95% confidence intervals) and Lorenz asymmetry coefficients (LAC) for different grass species treated with monocultures of AMF. Bold results represent treatments significantly different from their control.