UNIVERSITY OF SOUTHAMPTON

FACULTY OF NATURAL AND ENVIRONMENTAL SCIENCE

Biological Sciences

Volume 1 of 1

USING NEXT GENERATION SEQUENCING TO ASSESS SOIL MICROBIAL COMMUNITIES ASSOCIATED WITH DROUGHT, BIOCHAR AND LAND USE CHANGE TO BIOENERGY

by

Joseph R. Jenkins

Thesis for the degree of Doctor of Philosophy

July 2016

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF NATURAL AND ENVIRONMENTAL SCIENCES

Biological Sciences

Thesis for the degree of Doctor of Philosophy

USING NEXT GENERATION SEQUENCING TO ASSESS SOIL MICROBIAL COMMUNITIES ASSOCIATED WITH DROUGHT, BIOCHAR AND LAND USE CHANGE TO BIOENERGY

Joseph Richard Jenkins

Metagenomics and amplicon sequencing of soil from two long-term environmental manipulations sites (treated with biochar or drought) revealed no significant difference between control or treated taxonomic or functional community structure. Due to the small sample size it was not possible to conclude whether this was due to limited statistical power, or the lack of an effect.

European 16s and ITS metabarcoding field experiments assessed biochar induced microbial community change. Significant shifts in bacterial and fungal β diversity occurred, whilst α diversity remained unchanged. Biochar treated bacterial communities showed significant long-term shifts in β diversity, whilst fungi exhibited short-term community change. *Gemmatimonadete* and *Acidobacteria* were enriched in UK biochar samples after application after one year, whilst control plots exhibited enriched *Gemmataceae*, *Isosphaeraceae* and *Koribacteraceae*. *Proteobacteria* and *Gemmatimonadetes*, were elevated in Italian biochar samples, and no change was noted in French biochar samples.

Soil respiration monitoring of short rotation coppice (SRC) assessed biochar stability. Total respiration was significantly higher in biochar plots during the initial sampling effort (June-December 2012), whilst no significant difference was detected in heterotrophic respiration. An extended dataset (December 2012- June 2014) confirmed this trend. Enzyme activity and nutrient leachate exhibited elevated alkaline phosphomonoesterase activity and decreased activity of acid phosphomonoesterase, indicating a possible switch in the source of these enzymes from fungi and plants to bacteria. Phosphate and ammonium leachate was elevated in biochar plots, which may be linked to changes in the cycling of N and P.

The difference between grassland and SRC willow microbial diversity was compared using 16s and ITS metabarcoding. Significant decreases in fungal and bacterial α and β diversity occurred. Increased abundance of *Nitrospirae* (associated with the oxidation of nitrite) and oligotrophic bacteria occurred. Increases in the *Cortinariaceae*, known ectomycorrhizal fungi (EMF) were also detected. Mechanisms of nutrient cycling in grassland may differ from SRC as a result, specifically through enriched EMF abundance and elevated proportions of the *Nitrospirae*.

Metagenome simulation from metabarcoding data using PiCrust (Langille *et al.* 2013) revealed no significant difference in function due to biochar treatment. It therefore appears that PiCrust may not be appropriate for use with complex soil communities.

Table of Contents

Outputs from the research described in this thesis:

1. Oral presentations
    a. Soil metagenomics: Impact of biochar –Centre for Genomic research Workshop (2012)
    b. Introduction to metagenomics – First year research presentation, Southampton (2012)
    c. Metagenomics to assess microbial diversity – EuroChar meeting, Paris (2012)
    d. Metagenomics from the soil for biodiversity from re-sequencing – Second year research presentation, Southampton (2013)
    e. Metagenomics of the soil for biodiversity from re-sequencing – Expeer annual meeting, Florence (2013)
    f. EuroChar campaign presentation, Paris (2013)
    g. Metagenomics – a tutorial, lab seminar Southampton (2013)
    h. Using NGS technologies to assess soil microbial diversity under future climate scenarios – Expeer work-package meeting, Montpellier (2013)
    i. Biochar: Understanding the impact of geoengineering on microbial communities – 4th Annual Next generation sequencing symposium, Southampton (2014)
    j. Next generation 'omic' techniques- what can they do for ecosystems research? – Expeer Final meeting and conference, Paris (2014)
    k. The impact of Biochar on microbial communities, results from three European field sites- Environmental biosciences seminars, Southampton (2014)
    l. Biochar alters the soil microbiome: results from amplicon surveys of three European field sites – PAG, San Diego (2015)
    m. Biochar alters the soil microbiome: final results from metabarcoding of three European sites – Postgraduate Symposium, Southampton (2015)

2. Poster presentations
    a. Environmental manipulations experiments and metagenomics – EuroChar Conference, Como (2013)
    b. Why is soil important? – outreach event, Southampton (2013)
    c. What is metagenomics? – outreach event, Southampton (2013)
    d. Metagenomics from the soil for biodiversity from re-sequencing – Bioenergy away day, Southampton (2013)
    e. Using metagenomics to assess soil microbial diversity under future climate scenarios- International Environmental 'omics synthesis conference, Cardiff (2013)
    f. Using metagenomics to assess soil microbial diversity under future climate scenarios – 3rd annual Next Generation Sequencing Symposium, Southampton (2013)

g. Using metagenomics to assess soil microbial diversity under future climate scenarios- Showcasing industrial collaborations and transnational science poster session, Southampton (2013)
h. Implications of biochar application for microbial soil ecology: a metagenomics approach – Institute for Life Sciences Molecular Biosciences poster session, Southampton (2014)
i. Biochar: understanding the impact of geoengineering on microbial communities- Complex Systems Sciences poster display, Southampton (2014)
j. Biochar alters the soil microbiome: initial results – 3rd Early Career Biomass Network Symposium, Southampton (2014)
k. Biochar alters the soil microbiome: initial results – Postgraduate Symposium, Southampton (2014)
l. Using metagenomics to assess soil microbial diversity in biochar treatments – IUFRO TreeBiotech2015, Florence (2015)
m. Using metagenomics to assess soil microbial diversity in biochar treatments- Institute for Life Sciences poster session: Global change: Systems and Cycles - Southampton (2015)
n. Biochar alters the soil microbiome: results from amplicon surveys of three European field sites – Postgraduate open day, Southampton (2015)

3. Papers in peer review publications
    a. Biochar mineralization and priming effect on SOM decomposition in two European short rotation coppices, Ventura M, Alberti G, Viger, M, Jenkins JR, Girardin C, Baronti S, Zaldei A, Taylor G, Rumpel C, Miglietta F and Tonon G, *GCB Bioenergy* 2015
    b. Bioenergy reduces soil biodiversity, Jenkins JR, Harris ZM, Alberti G, Edwards RJ and Taylor G *GCB Bioenergy* (in submission)
    c. Biochar alters the soil microbiome and soil function: results of next generation amplicon sequencing across Europe, Jenkins JR, Viger M, Harris ZM, Ventura M, Miglietta F, Girardin C, Edwards RJ, Rumpel C, Fornasier F, Zavalloni C, Tonon G, Alberti G and Taylor G *GCB Bioenergy* (in submission)

4. Other
    a. Next generation sequencing: Environmental DNA – Outreach presentation, Southampton (2013)
    b. EuroChar Final report, Jenkins JR, Taylor G – Section on WP6 (2014)
    c. Biochar for Carbon sequestration and large-scale removal of greenhouse gases (GHG) from the atmosphere, Deliverable 6.2, Jenkins JR, Viger M, Taylor G (2014)
    d. Expeer Final Summary Report Jenkins JR, Taylor G - Section on WP7 (2015)
    e. Expeer Final results Brochure – Metabarcoding to assess microbial response to environmental change, Jenkins JR, Taylor G (2015)

List of tables

List of figures

DECLARATION OF AUTHORSHIP

I,.....

declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

.....

.....

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. Parts of this work have been published as: see above “Outputs from the research described in this thesis”

Signed:.....

Date:.....

Acknowledgements

The work within this thesis would not have been possible without the funding provided by both the ExPEER network and the EuroChar project. My gratitude to my supervisors Prof Gail Taylor and Dr Richard Edwards for their advice, feedback and encouragement.

I am indebted to Dr Maud Viger, for her help in organising and collecting samples. My thanks to Dr Maurizio Ventura, Dr Giorgio Alberti, and Dr Cyril Girardin for their assistance collecting samples, and their methodological advice, and to Dr Flavio Fornasier for carrying out enzymatic analysis. I would like to acknowledge Stacey Selmes, Mike Allwright, Billy Valdes, Frankie Rouse and Dr Caitriona Murray for their help collecting samples from UK sites. Discussions of this work with Dr Adrienne Payne, Dr Suzanne Milner, Dr Zoe Harris, Dr Hazel Smith and the rest of the TaylorLab team have been invaluable.

My gratitude to Dr Tsiloon Li, Dr Florence Garty, Mr John Spry and Dr Davide Zilli, for their support throughout the past four years, and listening to my microbial pontifications.

To Verity, my parents George and Maggie, my brother Pete, my grandparents and the Pinkney cohort, thank-you for your support both emotionally and financially throughout my PhD journey. I wouldn't have completed this thesis without you.

Definitions and Abbreviations

16SrRNA gene- 16S ribosomal RNA gene

AMF – arbuscular mycorrhizal fungi

amoA genes – gene coding for the ammonia mon-oxygenase subunit A

amoB genes – gene coding for the ammonia mono-oxygenase subunit B.

Biochar – black carbon produced by pyrolysis from biomass or organic waste products, applied to the soil environment

Bp – base pairs

BLAST – basic local alignment search tool

BLAT – BLAST like alignment tool

CEC – Cation exchange capacity

COI – mitochondrial C oxidase gene

DGGE – Denaturing gradient gel electrophoresis

EMF – Ectomycorrhizal fungi

FASTA files – formatted sequence information files

FASTQ files – formatted sequence information quality files

ITS – internal transcribed spacer region

LCA – lowest common ancestor

LUC- land use change

Metabarcoding – extraction and sequencing of taxon specific DNA amplicon regions from environmental samples

Metagenomics – extraction and shotgun sequencing of all DNA from environmental samples

MG-RAST – Metagenomic rapid annotation using subsystems technology

NGS –Next generation sequencing

OTU- operational taxonomic unit

PE - Paired-end

PCoA – Principle coordinate analysis

PCR -polymerase chain reaction

PLFA -Phospholipid fatty acid analysis

QIIME- quantitative insights into microbial ecology.

qPCR -quantitative polymerase chain reaction

SFF files – standard flowgram format files

SMS – single molecule sequencing

SOC -Soil organic carbon

SOM -Soil organic matter

SRC – short rotation coppice

Chapter 1: General Introduction

1.1 Overview

Climate change will cause severe detrimental global effects by the middle of this century (Dai 2011; IPCC 2014). Predictions for climate change indicate the increase in drought events, which are damaging to crop production and soil quality (Lipiec *et al.* 2013). Other than the obvious decline in available moisture, drought can increase soil erosion (Defra 2009), lead to decreased soil aeration, change soil albedo and disrupt soil ecology (Sheik *et al.* 2011; Eisenhauer *et al.* 2012). These effects have additional social and economic impacts (Barker 2007), and mitigation is vital.

Biochar has been suggested as a potential method to geoengineer the environment, sequestering carbon within soils whilst improving soil quality, nutrient content, and water holding capacity (Lehmann 2007). Biochar is based on the discovery of *terra preta* soils, black earths found in the Amazon basin. *Terra preta* were produced by indigenous Amazonian Indian peoples, who added charcoal and burnt organic matter to soils (Glaser *et al.* 2001). *Terra preta* soils are highly fertile compared with surrounding soils and dating of the pyrolysed material present suggests centurial to millennial timescales. This inspired the concept of mass application of pyrolysed biomass (biochar) for carbon sequestration and soil improvement (Lehmann 2007; Glaser & Birk 2012).

The reliance on dwindling fossil fuel reserves, coupled with the negative impacts of climate change has increased the prevalence of lignocellulosic biomass crops such as *Populus, Miscanthus, Eucalyptus* and *Salix* (Volk *et al.* 2004; Hinchee *et al.* 2009; Hastings *et al.* 2014). Biomass crops have been mooted as a method to increase soil organic matter (SOM) whilst providing a low carbon resource for energy production, either through conversion to biofuels or via use as biomass in power generation (Volk *et al.* 2004; Naik *et al.* 2010). Increasing utilisation of such biomass crops could reduce reliance on fossil fuels, whilst simultaneously increasing soil quality and carbon stocks.

In each of the above scenarios, there is little understanding of the implications for microbial communities. Whilst rarely considered, these soil ecosystems are significant terrestrial carbon sinks (Trivedi *et al.* 2013) and play pivotal roles in biogeochemical cycling of nutrients (Falkowski *et al.* 2008; Hanzel *et al.* 2013).

Until recently studies made use of low throughput methods providing ratios of fungi to bacteria, falling short of describing changes in individual taxa after treatment. Many soil microbes cannot be cultured using current techniques, limiting the usefulness of laboratory based community studies in assessing true community structure (Rajendhran & Gunasekaran 2008; Thomas *et al.* 2012). A multitude of techniques have been used to estimate community structure, diversity and activity but these methods often result in detailed information about a single taxon within the soil, or a very general overview (Steiner *et al.* 2008; Rillig *et al.* 2010; Landesman & Dighton 2010; Jentsch *et al.* 2011; Castaldi *et al.* 2011). Whilst useful, such studies cannot provide the data which are needed to understand soil ecology at high resolution.

Next generation sequencing (NGS) methods have allowed for sequencing of environmental DNA from the soil, capturing the diversity and community structure associated with climate change scenarios. This chapter is concerned with outlining existing research into microbial ecology associated with the aforementioned biochar, drought and short rotation coppice (SRC) associated land use change. Sections outline dominant bacterial and fungal phyla found within the soil environment, their roles within soil ecology; biochar production, drought, and land use change and their impacts on microbial communities. Subsequent sections will cover the use of next generation sequencing techniques in microbial ecology, detailing some of the methods available and the bioinformatics pipelines which can be used.

1.2 The Soil Microbiome

Soil microbes are key to a wide range of biogeochemical processes (Figure 1), including nitrogen, carbon, and phosphate cycling (Falkowski *et al.* 2008; Fierer *et al.* 2012b; Hanzel *et al.* 2013). Many of these cycles are shifting due to anthropogenic impacts, including increased soil nitrogen and phosphate content through fertiliser use (Vitousek *et al.* 1997; Bennett *et al.* 2001). Production of fertilizers is energy and carbon intensive, further contributing to climate change, or reliant upon limited mineral deposits (Snyder *et al.* 2009). It is therefore imperative that research addresses changes to nutrient cycles, so that sustainable methods of agriculture can be developed.

Microbes constitute a large proportion of global biomass and biodiversity, with 10^9–10^{10} microbial cells in each gram of soil (Jansson 2011). Thus, a better understanding of soil microbial ecology is key to accurately modelling climate change and manipulating the nutrient profile of soil sustainably. Furthermore, successful carbon sequestration through geoengineering (such biochar application) requires the understanding the impact of such actions.

1.2.1 Bacteria

The growing viability of 16S ribosomal DNA marker-based studies is dramatically increasing the understanding of bacterial soil ecology. Previous work has identified taxa with critical environmental roles, such as nitrogen fixing, ammonia oxidising and methane oxidising genera (Fierer *et al.* 2007). Bacteria are essential decomposers of organic matter, breaking down organic polymers, making them available to other organisms.

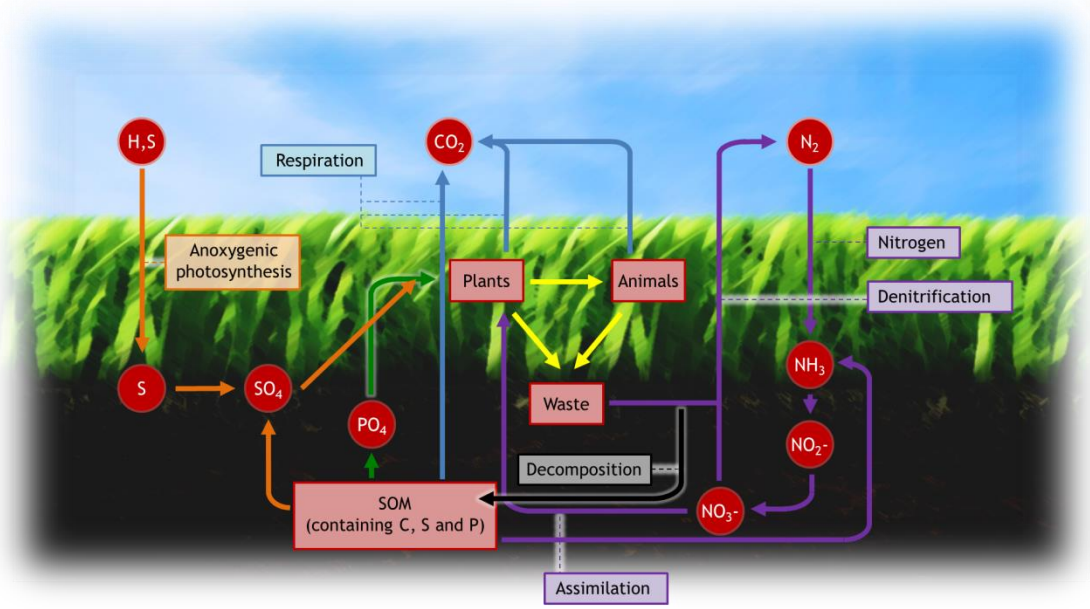


Figure 1 Biogeochemical cycling within the soil environment. Arrows represent microbial processes within cycles. Purple = nitrogen, blue = carbon, green = phosphorous, orange = sulphur. Yellow arrows show interactions between higher trophic levels, whilst black arrows show SOM passing into C, P and S cycles.

Table 1 Bacterial phyla and their ecological roles

Phylum	Functional roles	Section
Acidobacteria	Breakdown of SOM and complex carbon molecules, phosphorous and nitrogen cycle links	1.2.1.1
Actinobacteria	Decomposition of SOM, pathogens and nitrogen cycling	1.2.1.2
Bacteroidetes	Mutualist, pathogens, carbohydrate decomposers	1.2.1.3
Chloroflexi	Hydrocarbon degradation, sulphur cycle links	1.2.1.4
Firmicutes	Carbon and phosphate cycle links	1.2.1.5
Gemmatimonadetes	Unknown	1.2.1.6
Planctomycetes	Nitrogen cycling	1.2.1.7
Proteobacteria	Mutualists and nitrogen cycling	1.2.1.8
Verrucomicrobia	Nitrogen and potassium cycling	1.2.1.9

1.2.1.1 **Acidobacteria**

Acidobacteria are one of the most dominant phyla present within the soil environment averaging 20% of reads detected in soil samples (Janssen 2006). *Acidobacteria* are present across all soil types, but their abundance is increased in low pH conditions (Catão *et al.* 2014). Soils with elevated carbon availability exhibit decreased *Acidobacteria* indicating that the phylum is predominately oligotrophic (Fierer *et al.* 2007; Pascault *et al.* 2013). Oligotrophs thrive in low nutrient environments, being better adapted to utilise

complex nutrient substrates. Due to the additional cost to utilise such substrates, oligotrophs grow and multiply at a decreased rate (Pascault *et al.* 2013; Tapia-Torres *et al.* 2015). Furthermore, it has been suggested that they are associated with breakdown of SOM and complex carbon substrates in nutrient poor soils (Fierer *et al.* 2012a). *Acidobacteria* abundance is correlated with increased ammonium (NH_4) when soil is saturated with phosphorous and nitrogen, which may suggest a role of the phyla in nitrogen and phosphorous cycling (Pan *et al.* 2014).

Soil moisture content also impacts on the proportion of the phylum within soils. In low moisture soils, the proportion of *Acidobacteria* has been demonstrated to decline, increasing after wetting (Barnard *et al.* 2013) However, the specific roles of genera within *Acidobacteria* are still being investigated.

1.2.1.2 Actinobacteria

The phylum *Actinobacteria* consists of G+C rich bacteria found throughout soil environments (Ventura *et al.* 2007). The phylum includes several functional ecological groups, including nitrogen fixers (Ventura *et al.* 2007; Sellstedt & Richau 2013), decomposers (Pan *et al.* 2014) and pathogens (Ventura *et al.* 2007). Previous studies of the genus *Streptomyces* have yielded several antibiotic compounds, suggesting the ability to compete with other bacteria within the environment (Ventura *et al.* 2007). *Actinobacteria* abundance has been shown to increase in carbon rich environments, indicating that they are copiotrophic, and able to utilise high resource environments to rapidly grow (Pan *et al.* 2014). Maximal increases in abundance occur when there is an available source of nitrogen (Fierer *et al.* 2012a). Declines in *Actinobacteria* have been associated with high soil moisture, whilst in dry conditions their abundance increases (Barnard *et al.* 2013). This may indicate an impact of precipitation events and season on the abundance of this phylum (Fierer *et al.* 2007).

1.2.1.3 Bacteroidetes

Current understanding of *Bacteroidetes* is shaped by knowledge of gut commensalists or mutualists. The majority of *Bacteroidetes* studied are associated with the gastrointestinal tract, where they are involved with the

fermentation of complex carbohydrates under anaerobic conditions (Ley *et al.* 2006). Their abundance in a range of environments has been postulated to be as a result of their ability to break down complex carbon molecules and proteins (Thomas *et al.* 2011). A substantial portion of the rhizosphere is associated with the *Bacteroidetes* phylum, possibly indicating interactions with the plant microbiome and SOM breakdown (Buée *et al.* 2009a). *Bacteroidetes* are capable of rapid reproduction and appear to be copiotrophic in their competitive strategies, although pathogenic genera have also been noted (Schimel & Schaeffer 2012).

1.2.1.4 Chloroflexi

Chloroflexi are poorly characterised, due to difficulties culturing isolates. Isolates which have been produced are highly diverse, and have shown a range of interesting attributes including phototrophic, autotrophic and heterotrophic behaviours, anoxygenic photosynthesis and sulphide metabolism (Garrity *et al.* 2001). There are also several taxa within the phylum capable of degrading complex hydrocarbons (Campbell *et al.* 2014). Whilst all identified *Chloroflexi* are obligate anaerobes, they are found in a range of environments including soils, hot springs, marine and animal associated environments (Campbell *et al.* 2014). Interestingly, whilst ubiquitous, they are often rare, comprising of only a small proportion of any given community, which may suggest a specialist niche within the soil environment (Fierer *et al.* 2012b).

1.2.1.5 Firmicutes

Classes within the *Firmicutes* are highly diverse, utilising a range of primary production methods and niches. The phylum includes acid tolerant anaerobes, thermophiles, lithotrophs, aerobes and pathogens. The presence of *Firmicutes* within soils has been correlated with acidic soils and high phosphate abundance (Kuramae *et al.* 2012), although they do not appear to respond in an oligotrophic or copiotrophic manner when provided with changes in carbon availability (Fierer *et al.* 2007). This is probably as a result of the diverse ecology of organisms within the phylum, and whilst functional patterns are not discernible at the level of function, they may be present in lower taxonomic groups. In addition, the classes *Bacillus* and *Clostridium* form spores which can remain in the environment even when exposed to extreme environmental

stresses, which may increase their ability to adjust to environmental change (Setlow P. *et al.* 2012).

1.2.1.6 **Gemmatimonadetes**

To date, two strains have been isolated and characterised from the phylum *Gemmatimonadetes* (DeBruyn *et al.* 2014). However, *Gemmatimonadetes* are present within soils at comparatively high levels (an average relative abundance of 2.2%), suggesting that they are both ubiquitous and likely have a role in soil function (DeBruyn *et al.* 2011). First isolated from wastewater, *Gemmatimonadetes aurantica* was described as aerobic, with a tolerance to alkaline environments, and growing optimally in temperatures of 20–37°c. (Zhang *et al.* 2003). No specific ecological roles have been determined.

1.2.1.7 **Planctomycetes**

Planctomycetes are prevalent in most environments and have been found in soil, marine and gut samples (Fuerst & Sagulenko 2011). Unlike other bacterial phyla, they demonstrate basic compartmentalisation of the cell, and peptidoglycan is absent from their cell walls, making them resistant to several forms of antibiotic (Fuerst & Sagulenko 2011). Samples extracted from wastewater have been shown to have anammox metabolism, converting ammonium to gaseous nitrogen, and as such the *Planctomycetes* may have a vital role in nitrogen cycling (Kuenen 2008). Culturable taxa within the phylum are aerobic chemoheterotrophs, and can survive in acid, alkali and neutral environments.

1.2.1.8 **Proteobacteria**

The *Proteobacteria* are extremely diverse, both morphologically and in the niches they fill. The phylum contains the majority of identified nitrogen fixing bacteria, including many legume forming symbionts. They utilise energy sources ranging from phototrophic, chemoorgantrophic and chemolithotrophic, and include strict anaerobes/aerobes, and facultative anaerobes/aerobes (Philippot *et al.* 2010; Kersters *et al.* 2013). *Proteobacteria* include plant growth promoting rhizobacteria (PGPR), increasing plant growth through inducing resistance to pathogens, changing plant hormone signalling patterns and competing with pathogenic species (Bruto *et al.* 2014). Several *Proteobacteria* classes are correlated with high pH and low C:N environments.

Concurrently, other *Proteobacteria* are associated with high C:N ratios and low pH. (Kuramae *et al.* 2012). *Alphaproteobacteria* are associated amino acids uptake, although they are not correlated with increased protein availability. This may indicate a role of *Proteobacteria* in utilising remains of long chain polymers after primary decomposition by other organisms in the environment (Thomas *et al.* 2011; Pascault *et al.* 2013). Similarly, *Betaproteobacteria* are copiotrophic *r*-strategists, utilising abundant soil organic carbon (SOC) for growth. *Alphaproteobacteria* do not fit into either oligo or copiotrophic categories, which may be due to a range of responses to increased carbon availability at lower taxonomic levels (Fierer *et al.* 2007). Soil moisture appears to have little impact on *Proteobacteria* abundance, with stable proportions present during drying and rewetting treatments (Barnard *et al.* 2013) suggesting the phylum can endure short-term shifts in soil moisture, resisting extreme weather events.

1.2.1.9 **Verrucomicrobia**

Decreased abundance of oligotrophic *Verrucomicrobia* is associated with elevated soil nitrogen and potassium. This is likely to be a result of exclusion by copiotrophic organisms (Bergmann *et al.* 2011; Pan *et al.* 2014). Additionally, soil moisture drives *Verrucomicrobia* abundance, although this may also indicate the effect of soil moisture dependent parameters as drivers of community change, for example, the rate of diffusion of nutrients or the activity of predatory organisms (Buckley & Schmidt 2001). The *Verrucomicrobia* contain methanotrophs capable of living in low pH, high temperature environments (Op den Camp *et al.* 2009), symbiotic associations with mesofauna (Bergmann *et al.* 2011), and to dominate some subsurface soils due to their oligotrophic nature (Bergmann *et al.* 2011).

1.2.2 **Fungi**

Fungi are an essential part of terrestrial ecosystems, decomposing organic matter and making nutrients available to other organisms. Pathogenic fungi present in the soil may be detrimental to crop yield, whilst mycorrhizal associations can augment plant growth, nutrient availability and disease resistance (Rodriguez & Sanders 2014). Interactions between fungi and plants

are known to have implications for community structure, ecosystem function and soil stability (Bever *et al.* 2001).

Mycorrhizal fungi are split into two groups, the ectomycorrhizal fungi (EMF) and the arbuscular mycorrhizal fungi (AMF). The two groups are differentiated by their method of interacting with plants. EMF colonise fine plant root hairs, producing a mantel which covers the root. A network of mycelia which ingress between plant root cells from the mantle then forms (Tedersoo *et al.* 2010). This is known as the Hartig net, whereby photosynthetically derived complex carbons are exchanged by the plant for phosphates and nitrogen from the fungus as part of a symbiotic relationship (Püttsepp *et al.* 2004; Martin *et al.* 2007; Tedersoo *et al.* 2012). EMF are found within both the *Ascomycota* and *Basidiomycota*, with convergent evolution of this trait occurring an estimated 66 times (Tedersoo *et al.* 2010). Whilst other fungi may from mutualistic associations with roots, they do not form a mantle around the root, and a Hartig net. These are morphological indicators of EMF taxa.

Similarly, AMF exchange nutrients with plant roots for products of photosynthesis, although hyphae grow within the cells of the plant root, exchanging phosphate for carbon substrates (Bever *et al.* 2001; Gosling *et al.* 2006).

Due to the difficulty in observing fungal ecology, comparatively little is known about the ways in which they interact with plant and soil environments (Anderson & Cairney 2004). Unlike plants and other macroorganisms which are easily observable, many fungal species are only present as networks of mycelia. However, the use of molecular methods in fungal ecology is beginning to allow for the study of cryptic taxa.

Fungi belonging to the phyla of *Basidiomycota* and *Ascomycota* have been shown to be most prevalent globally, although are most dominant in grass and shrub environments (Tedersoo *et al.* 2014). Phyla contributing a smaller proportion to soil ecosystems include the *Glomeromycota* and *Chrytidiomycota*. Whilst less abundant are also of importance to ecosystems, in terms of diversity of ecological roles (Tedersoo *et al.* 2014). However, it should be noted that due to the increasing use of molecular methods, fungal phylogeny is in flux. Some of the ecological and functional aspects of each of the aforementioned phyla are outlined below.

Table 2 Fungal phyla and their ecological roles

Phylum	Functional roles	Section
Ascomycota	Symbiotic EMFs, saprotrophs, pathogens	1.2.2.11.2.1.1
Basidiomycota	Symbiotic EMFs, Saprotrophs, pathogens	1.2.2.2
Chytridiomycota	Saprotrophs, pathogens	1.2.2.3
Glomeromycota	Hydrocarbon degradation, sulphur cycle links	1.2.2.4

1.2.2.1 **Ascomycota**

The phylum *Ascomycota* is considered almost universal in its abundance, found in urban, rural and aquatic habitats (Schoch *et al.* 2009). Global sampling efforts found *Ascomycota* contributed to nearly a third of all sequences retrieved and almost half of all operational taxonomic units (OTUs) detected (Tedersoo *et al.* 2014). Species richness of several *Ascomycota* showed the highest diversity within tropical regions, whilst others are have become specialised to dominate polar regions (Wardle & Lindahl 2014). *Ascomycota* include several species which are useful to humans, such as truffles and yeasts, symbiotic EMFs; and a range of detrimental pathogens including mildews (Berbee 2001; Tedersoo *et al.* 2013). *Ascomycota* are linked to saprotrophic behaviours, with the ability to degrade lingocellulosic biomass, tannins and humic acids (Osono 2007). Through remobilisation of SOC, *Ascomycota* play a significant role in biogeochemical cycling. *Ascomycota* (together with the *Basidiomycota*) include a range of EMF's, implicating them in symbiotic relationships with plant roots (Grigoriev *et al.* 2011). This further indicates a key role in soil function, ecology and nutrient cycling.

1.2.2.2 **Basidiomycota**

Basidiomycetes contain many of the more recognisable edible mushroom species, as well as rusts and smut fungi (often plant pathogens) (Hibbett *et al.* 2014). Along with *Ascomycota*, the *Basidiomycota* contains several EMF taxa, which form symbiotic mutualist relationships with plant root systems (Ryberg

et al. 2008; Orgiazzi *et al.* 2012). *Basidiomycete* fungi are also saprotrophic, utilising lingocellulolytic compounds to degrade lignin within wood detritus, returning organic matter it to the soil (Osono 2007; Lehmann *et al.* 2011; Crowther *et al.* 2013). Again, as with the *Ascomycota*, changes in *Basidiomycete* abundance are likely to have direct implications for nutrient cycles within forested environments (Crowther *et al.* 2013). Sequencing of *Laccaria bicolor*, a *Basidiomycete* EMF suggests the presence of genes for utilising short-term high nutrient areas of soil, allowing it to rapidly decompose and absorb available nutrients (Martin *et al.* 2007). This saprophytic role is supported by increased rates of glycine and cellulose decomposition correlated Basidiomycete abundance (Crowther *et al.* 2014).

1.2.2.3 Chytridiomycota

Members of the phylum *Chytridiomycota* are unusual in that they have a motile stage, utilising flagella to move in aquatic environments (Jones *et al.* 2011b). They present globally, and whilst found in aquatic environments, often live in water within the soil matrix. *Chytridiomycota* are usually saprotrophic, degrading chitin, keratin and cellulose, although they have come to attention recently due to their parasitic role (James *et al.* 2006). By way of comparison with the *Ascomycota* and *Basidiomycota* their proportional abundance is limited, but contribute substantially in grass and shrubland environments. *Chytridiomycota* richness is driven by increased soil pH (Tedersoo *et al.* 2014). Morphology based methods had led to confusion about the evolutionary history of the group, previously clustering it with *Zygomycota* and several *Ascomycetes*, however, sequencing has revealed it is a separate phylum (Cavalier-Smith 1998).

1.2.2.4 Glomeromycota

Comprising of all of the AMF, *Glomeromycota* form arbuscules, tree like structures within plant root cells, allowing for maximised surface area for nutrient and carbon exchange between the symbiotic fungi and its host (Gosling *et al.* 2006; Rooney *et al.* 2009; Wardle & Lindahl 2014). Presence of *Glomeromycota* influences plant diversity (Schü β ler *et al.* 2001) although distribution of *Glomeromycota* is less reliant on associations with specific plant hosts, with growth and abundance driven by soil pH (Schimel & Schaeffer 2012). However, it has also been noted that the diversity of *Glomeromycetes*

declines with increasing plant age, which may indicate a short-term symbiosis during the initial portion of the host plants life history (Wardle & Lindahl 2014). Diversity of *Glomeromycetes* appears to be at its highest in grassland in global distribution and abundance studies (Wardle & Lindahl 2014), although they have been found to associate with approximately 65% of all plant species, across habitat types (Martin *et al.* 2004). AMF fungi provide phosphate ions for plants, utilising their large surface area to actively collect ions from surrounding soils, prior to transport back to the plant roots (Gosling *et al.* 2006).

1.2.3 The use of NGS in environmental microbiome studies

The decreasing cost of high-throughput, "Next Generation Sequencing" (NGS) of DNA has enabled the surveying of microbial communities in a way not possible ten years ago (Degnan & Ochman 2011; Gilbert & Dupont 2011; Shokralla *et al.* 2012). Changes in community structure and diversity can be assessed by sequencing of taxon specific marker regions (metabarcodes) or through random shotgun sequencing (metagenomics) (Gilbert & Dupont 2011; Yoccoz 2012). Traditional microbiology methods rely on the culturing of samples, which has the disadvantage of missing those taxa which cannot be cultured. DNA based methods are capable of capturing a greater diversity of taxa, and are able to detect small shifts in community structure (Shokralla *et al.* 2012). These techniques have enabled new methods to assess plant/microbe interactions (Knief 2014) and surveying of shifts in soil microbial community structure and function due to environmental change (Sheik *et al.* 2011; Evans & Wallenstein 2014). Larger scale projects such as the Earth Microbiome Project have attempted to accumulate metadata from multiple smaller scale projects to create global models of microbial distribution (Gilbert *et al.* 2010a). They intend to utilise the combined microbial community data, in conjunction with climatic, geographical and chemical data from each site to further refine simulations, with the long-term aim of estimating variation in microbially mediated biogeochemical cycles when given a set of metadata derived parameters. Whilst this process is extremely exciting, the models rely upon smaller scale ventures utilising NGS techniques. The details of these methods are discussed further in Section 1.6, with a focus on

amplicon sequencing of the 16S and ITS rRNA gene (metabarcoding) (Section 1.6.2), and shotgun metagenomics (Section 1.6.1).

1.3 Biochar

Biochar is defined here as any black carbon produced by pyrolysis from biomass or organic waste products, applied to soil environments. The remaining solid matter (which is essentially charcoal) is a highly stable form of carbon with a centurial to millennial residence time (Lehmann *et al.* 2006; Kim *et al.* 2007; Czimczik & Masiello 2007).

Once produced, biochar has a range of potential applications, from the traditional use as a fuel (although of little use with regard to carbon sequestration) to the manufacture of building materials and filters (Lehmann *et al.* 2006; Verheijen *et al.* 2010). However, the main point of interest remains in the use of biochar as a geo-engineering method.

1.3.1 Biochar production methods

1.3.1.1 Pyrolysis

Pyrolysis to produce biochar requires a low oxygen environment, which prevents the production of carbon dioxide, producing stable carbon. Initial feedstock can be varied, leading to various nutrient profiles in the final biochar product. For example, use of poultry manure can provide a biochar rich in nitrogen and phosphorous, whilst use of wood feedstocks results in a low C:N and phosphorous (Lehmann *et al.* 2011). Varying the pyrolysis temperature can be used to further engineer biochar properties. For example, high pyrolysis temperatures can increase total phosphorous within poultry manure feedstocks (Lehmann *et al.* 2011), and increase the rate of carbonisation, forming complex crystalline structures (Verheijen *et al.* 2010). Increasing temperature may be desirable if the requirement is to remove the non-carbon portion of the biochar, and to increase its stability. This is further increased when utilising woody feedstocks, which produce coarse, porous biochars. However, if the required biochar is designed primarily for the purpose of fertiliser, it would be recommended to use poultry litter in order to produce a biochar which is degradable (Gai *et al.* 2014).

1.3.1.2 Gasification

Gasification processes of pyrolysis are designed to produce biochars and syngas, usually requiring high temperatures. These biochar differ in pore structure and texture when compared with those produced by slow pyrolysis (Sohi *et al.* 2009a; Manyà 2012). Syngas is synthesis gas, formed when pyrolysis conditions produce carbon monoxide (CO), methane (CH_4) and water vapour (Naik *et al.* 2010). The rate of syngas production can be increased through the application of steam, increasing CH_4 conversion to hydrogen. Syngas can be fed back into the pyrolysis chamber, providing additional fuel and increasing the efficiency of the reaction whilst reducing emissions from the process (Figure 2) (Sims *et al.* 2010). Alternatively, syngas can be used as a fuel source for traditional gas powered electricity generation (Sohi *et al.* 2009a).

1.3.1.3 Hydrothermal

Hydrothermal methods rely on conversion of material in wet, high pressure, high temperature environments. This has the advantage of being energetically viable even when the feedstock is wet (Lehmann *et al.* 2011). Hydrochars often have markedly lower pH compared to gasification and pyrolysis chars, producing an acid product (Busch *et al.* 2013). Due to high pressure and temperatures involved long chain hydrocarbons formation is increased, potentially causing toxic effects when applied (Busch *et al.* 2013). Furthermore, hydrochars are less stable than their pyrolysis based counterparts, and increase degradation of native SOC when applied (Bamminger *et al.* 2014a).

1.3.2 Biochar application methods

Application of biochar can be carried out using three common methods:

1) Topsoil. The primary application method is to topsoil. This represents the most viable method in an agricultural setting. Topsoil application requires biochar to be applied to the soil surface, prior to rotary hoeing or tillage to a depth of 15 to 30 cm (Maraseni 2010; Verheijen *et al.* 2010). This has the advantage of homogenising biochar within the soil environment, whilst reducing the rate of biochar erosion compared with surface application alone. However, it fails to distribute biochar into

deeper soil horizons which may have more direct contact with roots (Maraseni 2010).

2) Deep. The second method is to apply biochar at depth, although this requires specialist tools and is difficult to undertake. Deep trenches must be dug prior to biochar addition, before repeat ploughing of the soil to homogenise the application (Williams & Arnott 2010). Whilst this places the biochar directly into the deep root rhizosphere, and reduces weathering effects, it is comparatively expensive and difficult, and reduces the quantity of biochar applied (Major 2010).
3) Surface. Finally, in scenarios where application at depth is not viable, surface applications can be utilised. This method is suited to forested environments or short rotation coppice, where incorporation at depth is difficult due to limited space for machinery (Verheijen *et al.* 2010). However, erosion processes will negatively impact on the viability of this method, and the fertiliser effects of biochar application are minimal. Given sufficient time and reapplication rates, it may be possible that applications of this type allow for the gradual mixing of biochar particles through soil formation processes, but the efficiency of the process is expected to be low.

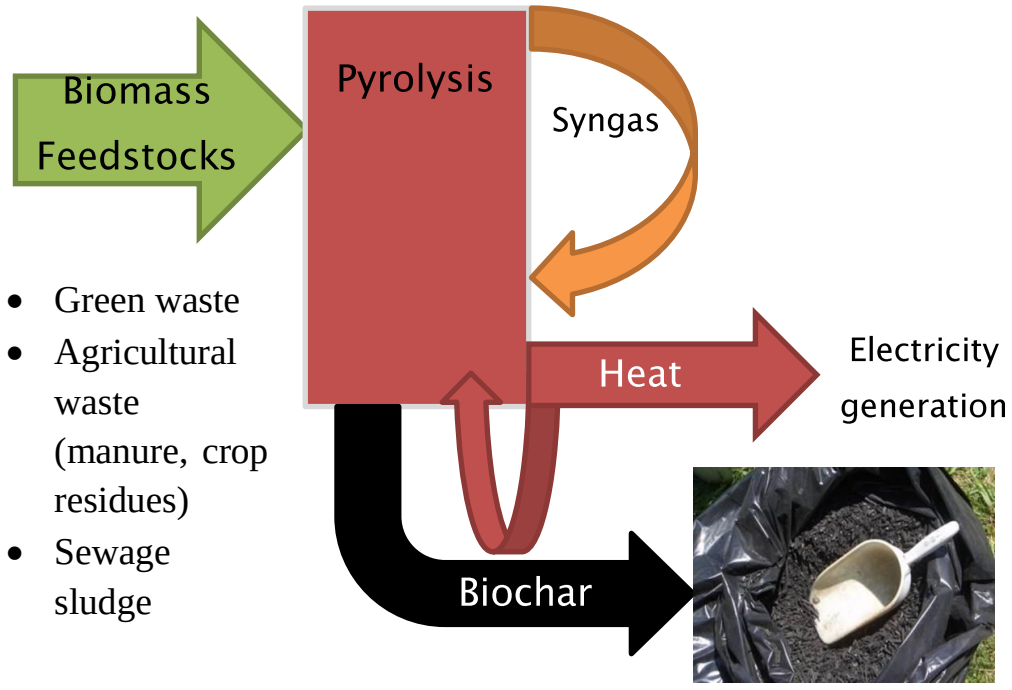


Figure 2 Schematic showing production of biochar through gasification

Table 3 Summary of research examining the mean residence time and priming effects of biochar

Study and Design[1]	Residence time	Soil and Biochar type	Priming
(Cross & Sohi 2011) LI	N/a	Silty-clay loam Bagasse biochar	-
(Bai *et al.* 2013) LI	Hydrothermal char showed half-life of 0.7-2.1 years, Pyrolysis biochar showed half-life of 11.1-110.7 years	3 soils in a sandy-loamy gradient Miscanthus biochar and hydrothermal char	N/a
(Fang *et al.* 2014) LI	44-610 years	4 soils, Inceptisol, Entisol, Oxisol and Vertisol. Eucalyptus biochar	N/a
(Watzinger *et al.* 2014) LI	164-1050 years	Planosol and Chernosem Willow biochar + Wheat biochar	-
(Steinbeiss *et al.* 2009) LI	Between 4 years (glucose hydrochar) and 29 years (yeast hydrochar)	Eutric Fluvisol Hydrothermal char produced from glucose or yeast.	N/a
(Zimmerman *et al.* 2011) LI	N/a	2 Alfisols, 2 Entisols and a Mollisol Oak biochar, pine biochar, bubinga biochar, eastern gamma grass biochar and bagasse biochar	+ and -
(Major *et al.* 2010) F	600-3264 years	Oxisol Mango biochar	N/a

LI= Lab incubation experiment, F= Field experiment

1.3.2.1 Rates of biochar application

Application rates should also be considered, given that biochar amendment is irreversible. *Terra preta* soils have been used as a model for application rates, as these contain approximately 50t C ha^{-1} and maintain increased fertility (Verheijen *et al.* 2010). Repeated biochar amendment at a rate of 50t ha^{-1} at three year intervals has shown an increase in beneficial soil properties (including available phosphate, soil moisture, EMF and microbial growth) when compared with control, low and single dose plots, suggesting an augmentation of the effects at higher does (100t ha^{-1}) (Quilliam *et al.* 2012). Conversely, high application has been linked with decreased AMF colonisation, although phosphorous availability was still increased (Warnock *et al.* 2010).

Repeated applications of lower doses show stabilisation effects on microbial carbon, suggesting the method may prevent large fluxes in microbial populations (Zhang *et al.* 2014). Whilst it is difficult to conclude whether repeated high dosage applications are beneficial in terms of soil fertility, they are suitable for maximising soil carbon content. However, declines in crop yield associated with application rates of 150t ha^{-1} have been noted and further research is required to determine safe maximum dosage (Verheijen *et al.* 2010).

1.3.3 Potential impacts of biochar

Incorporation of biochar into soil may act as a carbon sink (Lehmann *et al.* 2006; Verheijen *et al.* 2010; Woolf *et al.* 2010) and it is postulated that the inability of soil microorganisms to make use of complex and stable forms of carbon present within biochar may increase its residence time within the soil (Table 3).

This is supported by the presence of Amazonian *terra-preta* soils, anthroposols containing large proportions of black carbon hundreds to thousands of years old (Glaser *et al.* 2001). *Terra-preta,* or black earth, is primarily found in South-America, having arisen as a result of native Amerindian domestic practices. These nutrient rich Amazonian soils have augmented levels of SOM compared with adjacent soils and often contain bone and pottery fragments, which suggest they are the remains of ancient middens. In addition, they contain large quantities of charcoal, although the

source of this material is less certain. The presence of black carbon has been attributed to repeated slash and burn agricultural techniques, pottery or food preparation, dumping of waste material or deliberate biochar treatment of cropland (Glaser & Birk 2012).

Whilst there are several virtues to the use of biochar (which will be discussed in detail later in this section) limited information is available regarding its impacts on microbial communities. Existing studies provide conflicting information, due to variation in soil type, pyrolysis method and analytical practise to assess community change (Table 3). Addition of black carbon (in the form of biochar or hydrothermal carbon) to soil can; reduce arbuscular mycorrhizal fungi (AMF) abundance (Warnock *et al.* 2010), stimulate AMF colonisation (Rillig *et al.* 2010), increase rates of microbial respiration (Steinbeiss *et al.* 2009), reduce rates of microbial respiration (Prayogo *et al.* 2013) have no impact on microbial respiration (Steinbeiss *et al.* 2009), cause no increase in microbial biomass (Dempster *et al.* 2011) and increase microbial biomass (Kolb *et al.* 2009; Prayogo *et al.* 2013). Studies have also suggested a significant change in the structure of microbial communities in response to biochar treatment, with fungal communities in rice paddies displaying enriched *Trichoderma* fungi, and elevated proportions of *Actinobacteria* (Hu *et al.* 2014). Conversely, other studies have indicated changes in an array of different taxa (as discussed in Chapter 3 in greater detail). The varied response of taxa detected in previous studies means that is difficult to predict the implications of biochar amendment with regard to microbial community structure, abundance, function and diversity and is an area where more research is clearly necessary. By addressing this issue, it may be possible to determine whether biochar is ecologically acceptable to introduce into the soil environment, whilst simultaneously understanding the potential agronomic benefits and bacterial mechanisms which have been concluded from other studies (Graber *et al.* 2010; Vaccari *et al.* 2011; Jones *et al.* 2012; Schulz & Glaser 2012).

Coupled with the carbon sequestration potential (Lehmann *et al.* 2006), agricultural application of biochar has been shown to increase the biomass, yield and growth rate of plants (Graber *et al.* 2010; Baronti *et al.* 2010; Jones *et al.* 2012; Schulz & Glaser 2012; Viger *et al.* 2015). The issue is further complicated by the effects of multiple applications. Analysis of application sites after several years show no significant difference in soil nutrition,

microbial growth and fungal colonisation between treated and control plots. However, after subsequent secondary biochar application, soil nutrients, moisture, SOM, microbial growth and fungal colonisation were increased (Quilliam *et al.* 2012). This suggests that the nutritive effects may be transient, whilst the carbon sequestration benefits may be long-term. Several mechanisms have been suggested to explain the beneficial effects of biochar incorporation which are discussed in Sections 1.3.3.1 to 1.3.3.3. Microbial utilisation of biochar associated fine particulate carbon and bio-oils can cause increased soil respiration, which may represent increased microbial activity as labile carbon compounds are utilised (Steiner *et al.* 2008). This can cause "priming" effects, whereby elevated microbial activity augments mineralization of extant soil organic carbon (SOC) (Cross & Sohi 2011). Similarly, negative priming can occur, whereby microbes preferentially degrade biochar, preventing the degradation of existing SOC. This is accompanied by a lower rate of soil respiration and activity, leaving much of the biochar intact, whilst preserving the existing SOM (Zimmerman *et al.* 2011). However the majority of carbon within biochar is not thought to be directly accessible to plants and soil microorganisms due to the abundance of polycyclic hydrocarbons, which are responsible for its stability within the soil (Glaser *et al.* 2001).

Conversely, microbial activity and biogeochemical processes may be disrupted through the influx of complex hydrocarbons, several of which (formaldehyde, formic acid and acetic acid) have antimicrobial properties at high concentrations (Steiner *et al.* 2008). Analysis of *terra-preta* and pristine Amazonian forest soil revealed 25% greater species richness in t*erra-preta* soils. All taxa found within forest soil were represented within the *terra-preta* sample, whilst the opposite was not true, indicating novel microbes are present within a charcoal and SOM rich environment (Kim *et al.* 2007). A similar effect may be seen in biochar treated soils, with changes in bacterial diversity being a possible explanation for the nutritive effects noted.

1.3.3.1 **Liming and pH effects**

Incorporation of biochar frequently increases soil pH, known as "liming". pH is known to be a limiting factor in the diversity and richness of bacterial communities (Fierer & Jackson 2006), and has implications for the abundance of several bacterial phyla (see Section 1.2.1). For example, higher pH soils typically contain greater abundances of *Actinobacteria* and *Bacteroidetes*,

whilst exhibiting decreased *Acidobacteria* abundances (Fierer *et al.* 2012b). Therefore the alkali nature of biochar may vary selection pressures acting upon taxa within soil communities, and the abundance of the taxa present (Griffiths *et al.* 2011). This in turn may indirectly impact on microbial activity and biogeochemical cycling (Zimmerman *et al.* 2011; Cayuela *et al.* 2013). Liming can be useful when conditioning acidic soils, although may prove detrimental to those which are already basic. Biochar pH is dependent upon a range of variables, including pyrolysis temperature, the method used during production, the rate of the reaction and feedstock (see Section 1.3.1) (Steiner *et al.* 2008; Warnock *et al.* 2010; Rillig *et al.* 2010) . This makes it difficult to ascertain the precise degree of liming that can be expected from any given biochar (Table 3).

Liming has implications for nutrient availability throughout the soil. Changes in soil pH influence cation exchange capacity (CEC), the ability of a soil to adsorb cations of elements such as calcium, magnesium, potassium and sodium (Lehmann *et al.* 2011; Beesley *et al.* 2011). Increased pH and associated escalation in CEC has a conditioning effect on soils, adsorbing nutritive cations and preventing them from leaching into lower soil horizons (Sohi *et al.* 2009b; Ventura *et al.* 2013). It is possible that CEC may have further effects, influencing the availability of allelopathic chemicals, residual toxins within the soil (such as pesticides), heavy metals and plant signalling hormones (Beesley & Marmiroli 2011; Beesley *et al.* 2011; McCormack *et al.* 2013).

These changes in the chemical environment of soil undoubtedly influence structure and abundance of microbial communities. Changes in the nutritive quality of soils, such as shifts in C:N ratios and availability of nutrients may favour particular taxa within the soil (Fierer *et al.* 2007). For example, decreased abundance of arbuscular mycorrhizal fungi (AMF) due to soil pH variation may reduce phosphorous availability (Warnock *et al.* 2010). Subsequent AMF decline may result in further changes in soil community due to trophic cascade effects causing fluctuations in competition, grazing and predation interactions (McCormack *et al.* 2013). Furthermore, decreased AMF presence within the soil may have further implications for plant growth. AMF's form intracellular symbiotic relationships with plant roots, providing otherwise inaccessible nutrients in exchange for basic sugars (Warnock *et al.* 2010). The

decline in this relationship could feasibly decrease nutrient availability, with direct effects upon plant growth.

Equally, it has also been shown that increased root colonisation by AMF may occur as a result of biochar addition (Rillig *et al.* 2010). The role of AMF in phosphate transfer in plants is primarily mediated by their ability to produce acids, which can chelate phosphorous, making it biologically available (Mendes *et al.* 2014). Therefore, whilst biochar mediated increases in soil pH may select against many AMF, leading to a decline in their abundance, it is possible that the increased rate of root colonisation may relate to a shift in their ecology. As biochar increases soil pH, which will abiotically decrease the rate of P chelation, it is feasible that the increased rates of colonisation, and subsequent phosphate solubilizing behaviours of AMF may increase lead to the biotic mobilisation of P. Phosphorus is also directly affected by changes in microbial communities, with elevated abundances of phosphate solubilizing bacteria and fungi in biochar treated samples (Fox *et al.* 2014; Hammer *et al.* 2014). Therefore changes in abundance of taxa related to the P cycle may lead to elevated mobilisation of phosphates from biochar and surrounding soils (Anderson *et al.* 2011; Fox *et al.* 2014; Hammer *et al.* 2014).

Application of biochar is linked to changes in nutrient availability, through decreasing denitrification and increasing the quantity of nitrogen remaining in the soil (Anderson *et al.* 2011; Ventura *et al.* 2013; Cayuela *et al.* 2013; Prommer *et al.* 2014). In these cases, nitrogen availability has been improved by reducing the rate at which nitrogen leachate percolates into deeper soils, or through slowing the rate of conversion of organic N to ammonium.

With regards to fungal saprotrophic activity, reductions in SOM degradation may occur. The multicellular nature of many fungi can be advantageous in the face of environmental stress, enabling fungal species to cover larger ranges, improving their access to nutrients and water in their environment. However, this comes at the cost of slower growth rates, and a requirement for greater quantities of energy sources and nutrients. Bacteria, being single celled can rapidly respond to environmental change by increasing growth when resources are readily available, but have the disadvantage of low motility and are less robust under stress. Individual bacteria are therefore unable to move out of areas in which environmental change has resulted in conditions which are no

longer suitable. Bacterial populations are more reliant upon the availability of soil nutrients, and react rapidly to nutritive influxes (Fierer *et al.* 2007). As such, pH changes and the associated increased availability of nutrients may favour bacterial communities, resulting in decreases of the fungal:bacterial ratio within the population (Watzinger *et al.* 2014). Therefore, liming effects of biochar application may lead to a rapid shift to bacterially dominated communities, although the phosphate solubilizing ability of AMF and EMF may be augmented.

Table 3. Summary of research examining liming effects on soil properties and microbial populations

Author and Year	Soil and treatment type	Soil properties effected	Impacts on microbial communities
(Lorenz *et al.* 2001)	Haplic Podzol 4Mg ha^{-1} of pelletized limestone	pH significantly increased compared with control, although this was confined to the surface organic matter. No significant effect on C or N stocks due to soil heterogeneity.	Liming decreased microbial biomass growth, with a potentially negative impact on communities which had developed to thrive in low pH soils. Increased protease activity in limed plots, suggesting a shift to fungal rather than bacterial decomposition of proteins. Decreased N-mineralization noted, potentially decreasing organically available N.
(Beesley *et al.* 2011)	N/a, review paper	pH increases lead to a decrease in cationic metal mobility. Similar effects may occur in relation to dissolved organic carbon (DOC). Such decreases in cation and DOC mobility may increase the availability of uncommon nutrients	Increased access to soil nutrients due to reduced leaching may have implications for microbial respiration and growth rates. Changes in nutrient availability may in turn favour specific taxa which are better adapted to compete in the new soil environment.

(Cayuela *et al.* 2013)	14 soils, covering a range of types and pH's 9 biochars with a range of C:N ratios and pH's	Significantly decreased N_2O emissions of up to 90%. Decreases in denitrification were also detected in several soils by up to 232%.	Possible "electron shuttle" effect, providing electrons to microorganisms, leading to the reduction of N_2O to N_2, decreasing N_2O emissions.
(Lehmann *et al.* 2011)	N/a, review paper	pH variation (both increased or decreased), varied nutrient content due to altered CEC. Sorption of inhibitory chemicals	Potential to increase ammonia oxidising bacteria within communities due to decreased inhibitory chemicals, community shift away from Acidobacteria, increased microbial biomass and stimulated soil respiration.
(Chen *et al.* 2013)	Aquept Wheat straw biochar	pH increased by 0.56, decreased nitrates present	Microbial 16S rRNA gene copy correlated with pH values

1.3.3.2 Porosity, sorption and hydrodynamic characteristics

The high porosity and volume of biochar generates several properties in soil structure when incorporated. Biochar produced through pyrolysis decreases soil bulk density, intensifies aeration and increases total water holding capacity (Kinney *et al.* 2012). Increased air spaces between soil particles have the potential to change microbial community structure and function, as well as having further implications for water retention (Warnock *et al.* 2007).

Amplified soil porosity has the additional characteristic of increasing soil surface area, through decreasing the packing of soil particulates (due to the comparatively large size of biochar) and through the pores within the biochar itself. As such, available microbial habitat is increased, providing additional opportunities for the formation of microbial communities (Lehmann *et al.* 2011). Furthermore, it has been suggested that the porosity of biochar may have implications for microfauna and mesofauna, changing the extent of predator/prey and grazing interactions (Warnock *et al.* 2007; Lehmann *et al.* 2011).

The small size of biochar micropores may act as a haven for microfauna, preventing their predation by mesofauna. Additionally, fungal hyphae colonise these pores without the threat of grazing from other organisms (Warnock *et al.* 2007; Hammer *et al.* 2014). However, shifts from bacterial to fungally dominated decomposer communities may occur when soils dry, as fungal hyphae are capable of reaching deeper, isolated pockets of soil moisture (Yuste *et al.* 2011). The elevated soil moisture content produced through biochar amendment may limit the switch to fungally dominated communities.

Through a combination of varied pH, CEC and soil porosity, sorption characteristics of the soil may be changed. This may be beneficial, increasing the availability of advantageous nutrient cations or the retention of otherwise harmful heavy metals and toxins, limiting their bioavailability. However, retention of toxins within the soil may be harmful if they are not rendered unavailable to soil organisms. Several pieces of research have assessed the implications of biochar inclusion and bioavailability of various biocidal compounds (Beesley *et al.* 2010; Graber *et al.* 2011; Cabrera *et al.* 2014; Oleszczuk *et al.* 2014). Biochar addition to soil affects biocides differently.

Trace metal contaminants in biochar treated soil exhibited reduced cadmium and zinc concentrations after water extraction, implying that the rate of heavy metal leachate to the soil matrix is reduced. However, copper and arsenic were found in higher concentrations in treated soil (Beesley *et al.* 2010). Therefore thought should be given to application depending upon the metal profile of the soil, as undesirable metals may be retained.

Polycyclic aromatic hydrocarbons of high atomic weight can also be immobilised by biochar, reducing bioavailability and subsequently limiting biocidal effects (Beesley *et al.* 2010). Therefore biochar may be capable of limiting detrimental effects caused by some biocidal compounds. Herbicide and pesticide transportation and bioavailability may also be mediated through biochar incorporation. Reductions of pesticide concentration added to soil have been noted, with negative implications for biochar use in conjunction with pesticides in agriculture. Sorption of pesticide and herbicide agents by biochar reduces the rate of mineralisation of the substances within the soil, as well as decreasing the rate of migration through leaching (Jones *et al.* 2011a). Therefore, whilst biochar application can limit undesirable leaching of pesticides, it may require drastic changes in agricultural management regimes due to the reductions in pesticide efficiency (Jones *et al.* 2011a). Interaction effects between biochar and pesticide application could result in substantial variation in soil communities, although further research is required within this field to establish the likely implications.

Inclusion of biochar within a tillage regime can increase soil porosity, and as a result increase field capacity. Coupled with surface charges of biochar particles, soil hydrophobicity can vary with incorporation. Soil water content after drainage (field capacity) in a range of biochars (produced from different feedstocks at several temperatures) showed field capacity can be manipulated through changing pyrolysis temperature (Kinney *et al.* 2012). In addition, the initial porosity and surface charge of the feedstock may be used to influence field capacity. An interaction effect between the two variables can be used to engineer biochars with desired hydrological characteristics. In fact soil amended with only 7% mass of biochar increased field capacity by between 25 and 36%, irrespective of biochar feedstock or pyrolysis temperature. However, increases of this type were not measured in all soil types, with one poor quality soil showing no significant benefit (Kinney *et al.* 2012). Designing biochars

with specific field capacity could be utilised to regulate soil moisture content, decreasing irrigation practises in some soils.

Furthermore, the same study noted that hydrophobicity of biochars rich in C-H functional groups was considerably higher. This attribute can be controlled through treatment of pyrolysed materials by sonication in water, further optimising biochar qualities (Kinney *et al.* 2012). Whilst hydrophobicity and field capacity can be manipulated, the implications of these variables are not well understood with regard to microbial communities.

Soil moisture is the second greatest factor driving soil microbial distribution and abundance after pH (Fierer & Jackson 2006; Serna-Chavez *et al.* 2013), and variation in hydrological properties can therefore impact on microbial ecology. Greater capacity for water may provide pockets of habitat for bacteria and mesofauna in drying soils, although this is dependent upon the exact properties of the biochar used (Lehmann *et al.* 2011). Theoretically, soft organisms such as nematodes and protozoans may better survive drought events through increased availability of water, and reduction in rates of soil drying (Manzoni *et al.* 2012). As such, trophic effects should be expected throughout the soil foodweb, with implications for the predators and prey of these species. Increased soil moisture may lead to decreased oxygen availability in lower soil horizons, favouring the dominance of anaerobic bacteria. This in turn may have implications for biogeochemical cycles, due to the reliance of many anaerobic bacteria on alternative electron acceptors (nitrate or sulphate based metabolisms, for example.).

Fungal abundance and distribution may be reduced through biochar amendment in soils which are regularly subjected to drought conditions. Owing to the extended structure of fungi, they are capable of reaching pockets of moisture within the soil unavailable to smaller organisms. This makes fungi more successful in periods of drought leading to communities dominated by fungi during these events (Manzoni *et al.* 2012). Introduction of biochar and the improved hydrological properties associated with it may prevent fungally dominant communities occurring under dry conditions, with implications for soil community trophic interactions.

1.3.3.3 Implications for soil respiration, soil organic matter, and biogeochemical cycles

Integration of biochar into soil increases carbon stocks. This is the mechanism behind the use of biochar for carbon sequestration (Lehmann *et al.* 2006). However, the success of this method remains a contentious point. Whilst biochar is generally accepted as a form of stable, recalcitrant carbon, this has been called into question by several studies, discussed below.

Biochars can cause a short-term stimulation effect on microbial activity, with increased respiration and decomposition of existing SOM (Steinbeiss *et al.* 2009; Khodadad *et al.* 2011; Zimmerman *et al.* 2011; Castaldi *et al.* 2011). In order for biochar to be a viable method for carbon sequestration, total carbon released from soils must be less than the additional carbon present within the biochar. If after application, soil respiration increases, then this may pose difficulties for utilising biochar as a geoengineering method. This may occur through increased decomposition of native SOM, or through the rapid mineralisation of biochar by bacteria, fungi and plant roots. In situations where addition of biochar produces an efflux of CO_2 (either through release of labile C from the biochar itself, or through promotion of biotic or abiotic mediated C evolution from extant SOC) the effect is termed "positive priming". For those scenarios where the presence of biochar reduces C evolution from extant SOC, a "negative priming" effect is occurring (Zimmerman *et al.* 2011). Positive priming may occur as a result of abiotic effects, such as pH variation, through increased porosity and aeration or changes in soil water content (Zimmerman *et al.* 2011) or through biotic factors such as increased microbial activity, or changes in the abundance of specific taxa. Similarly, negative priming occurs through changes in microbial community composition (utilising more labile C within the soil)(Ventura *et al.* 2014), or through sorptive effects of the biochar itself (Zimmerman *et al.* 2011). Whilst biochar may have short-term positive priming effects, there may also be a longer term reduction in CO_2 evolution, or no change from untreated plots once biotic and abiotic effects conclude. Similarly, a slight increase in soil respiration can occur, but is inconsequential when factoring in the net addition of carbon to the soil environment.

Soils containing biochar have higher levels of SOM (Glaser & Birk 2012). Whilst biochar can be considered SOM, its inclusion may retain extant SOM within the soil through interactions with biochar surface charges (Beesley *et al.* 2010).

SOM levels are closely linked with total water holding capacity, which in turn is correlated with reduced nutrient leaching, and soil fertility (Baronti *et al.* 2010). Decreased rates of SOM decomposition, and increased rates of SOM build-up occur in biochar treatments, suggesting application could improve soil quality (Dempster *et al.* 2011). SOM comprises of both SOC and any other associated elements (such as biologically available P, N, K and S) (Zimmerman *et al.* 2011). Therefore, by retaining additional biological material within the soil matrix, there may be long-term implications for nutrient availability.

There is some discussion about the level of recalcitrance to be expected from biochar associated SOC. Whilst some studies estimate biochar to sequester carbon for hundreds or thousands of years (Steiner *et al.* 2008; Grossman *et al.* 2010; Warnock *et al.* 2010; Major *et al.* 2010; Zimmerman *et al.* 2011), others suggest that it may be decadal (Steinbeiss *et al.* 2009; Rillig *et al.* 2010), which would reduce the viability of the technique for carbon sequestration. However, both studies suggesting a decadal turnover are based on hydrothermally produced biochars, which are comprised of smaller particles. Due to their increased surface area and higher labile portions, they are liable to rapid utilisation by microbial communities. Whilst aforementioned SOC, SOM and soil respiration rates suggest biochar amendment may reduce degradation of SOC in the long-term, there are no studies in excess of ten years which can support these assertions. Historic biochar has been collected and dated (Lima *et al.* 2002), but the mechanisms of interaction between the biochar and the surrounding soil at the point of introduction and the time afterwards can only be estimated. Solid evidence showing the long-term recalcitrance of biochar are currently lacking from the literature, predominantly due to the time frames required to study C sequestration.

Addition of Eucalyptus biochar in coarse soil can cause a reduction in microbial biomass, and nitrogen mineralisation (Dempster *et al.* 2011). Three biochar treatments, (0%, 0.45% and 2.27%) and three nitrogen fertiliser treatments (organic, inorganic and basal N) were applied to plots. Analysis of biomass, chloroform extraction of ^{14}C labelled CO_2 and *amoA* diversity (an ammonia oxidising gene) within the samples showed decreased microbial carbon, net nitrification and nitrogen mineralisation, indicating microbial activity declined. Analysis of *amoA* genes in the samples showed no change in diversity due to biochar, but revealed variation with addition of nitrogen fertilizers, indicating

differences in population due to shifts in resource availability and redistribution of the community to reflect the different niches available.

Similarly, changes in soil phosphorous due to biochar incorporation have been suggested to have negative implications for AMF. Reduced phosphorous levels found in both incubation and field experiments were correlated with reductions in AMF colonisation, which may result in a breakdown of plant-AMF symbiotic relationships (Warnock *et al.* 2010).

As described above, a wide range of changes in soil nutrient profile can occur through biochar amendment. These represent a plethora of potential means to affect microbial communities, and whilst research has been undertaken to assess the likely changes, existing research concentrates on low resolution community profiling. This includes low resolution genetic methods such as terminal restriction fragment length polymorphisms (TRFLP) or molecular methods targeting fatty acids such as phospholipid fatty acid analysis (PLFA). Both tools have proven useful in understanding in microbial communities (Section 2.4.2 and *Table 16*) but at a low level of resolution, looking at changes in fungi:bacteria, or noting the change in Gram-positive to Gram-negative bacteria (Hirsch *et al.* 2010; Frostegård *et al.* 2011).

In order to gain a real understanding of the changes in soil community as a result of biochar amendment, high resolution, mass throughput methods such as metabarcoding and metagenomics are required.

1.4 Drought

Prevalence of extreme weather events will increase in future due to climate change (IPCC 2014). Elevated global temperatures, coupled with shifts in weather patterns will increased prevalence and severity of drought events (Barker 2007). Decreases in available water lead to water stress, which has direct implications for crop productivity (Lipiec *et al.* 2013). There is now growing interest in the effect of drought on biogeochemical cycles, and in particular, the effects on microbial communities. Whilst there are multiple definitions of drought (Dai 2011), for the purpose of this review drought will be used to describe sustained unseasonal water stress.

As previously discussed in Section 1.3.3.2, soil moisture is the second largest explanatory variable for bacterial biogeography (Fierer & Jackson 2006; Serna-Chavez *et al.* 2013). Therefore increased duration, severity and prevalence of drought events are expected to have implications for soil microbes.

1.4.1 Potential impacts of drought on soil microbial communities

Soil microbial communities are drivers for decomposition of organic matter, which is intrinsically linked to biogeochemical cycling. Abiotic changes in soil environments result in new selection pressures on soil organisms, forcing shifts in community structure (see Section 1.3.3.2). In turn, activity and growth rates of microbes will vary and as a result, changes in the mechanisms and rates of biogeochemical cycles will occur. These mechanisms are described below, with reference to the interplays between microbial activity, community structure and associated nutrient cycles under drought scenarios.

1.4.1.1 Microbial activity and biomass under drought

Decreased soil moisture reduces the rate at which cellular exudates and enzymes can travel throughout the soil environment (Manzoni *et al.* 2012). As such, drought is expected to reduce both fungal and bacterial activity. Drought experiments in Mediterranean shrubland caused declines in fungal and bacterial biomass (Yuste *et al.* 2011). Reduced total microbial biomass may affect soil respiration rates and biogeochemical cycling. Activity of a range of enzymes including phenol oxidase and acid phosphatase are decreased by drought (Toberman *et al.* 2008; Baldrian *et al.* 2010), whilst microbial biomass and respiration is reduced, indicating a decline in growth rates and activity (Jentsch *et al.* 2011).

Reduction of microbial activity by drought is further supported by a meta-analysis which concluded microbial respiration and decomposition of SOM declines with water availability, regardless of biome or climatic conditions (Manzoni *et al.* 2012). However, other studies have revealed no significant effect of drought on microbial biomass (Kreyling *et al.* 2008; Landesman & Dighton 2010). This suggests that the impacts of drought vary, and may depend of the soil and initial community structure present. Soils which are

highly porous with limited water retention are unlikely to have high soil moisture, and microbial communities present may be pre-adapted for low soil moisture and drought events.

1.4.1.1 Drought driven change in community composition

Greater variability in soil moisture can impact portions of microbial communities unable to adapt (Evans & Wallenstein 2011). Drought causes significant declines in fungal species abundance, changing dominant taxa (Toberman *et al.* 2008). Declines in the rate of mycorrhizal colonisation in droughted soils indicate that while fungi may become the dominant kingdom in the community, their growth and activity may still be reduced (Jentsch *et al.* 2011). Additionally, reduced abundance of herbivorous soil arthropods may have added implications for community composition through top-down trophic interactions (Jentsch *et al.* 2011).

In the case of free-draining soils, PLFA analysis has shown no change in bacteria:fungi ratio, or Gram-positive:Gram-negative ratio, suggesting limited effect of drought events on pre-adapted low moisture communities (Landesman & Dighton 2010). Forest soils have yielded increased microbial biomass in wet compared with associated dry soils (Baldrian *et al.* 2010). Conversely, analysis of 16s rRNA from soil samples treated with precipitation manipulations (representative of a range of scenarios) showed no change in community composition between treatments over a five year window (Cruz-Martínez *et al.* 2009). Differences did occur during the year six, although only during two months of sampling (April and July), and did not continue into subsequent sampling months. Whilst changes occurred in the aboveground flora and fauna, this appeared to have no effect on the structure of the soil community. Furthermore, seasonality appeared to have a greater impact on soil bacterial communities than treatment, suggesting bacterial and archaeal species may be more resilient in the face of climate change than macrofauna, due to their rapid generation time, species turnover and flexibility in niche requirements. However, it should be noted that other drought studies have suggested decreased abundance of *Proteobacteria* and increased proportions of *Acidobacteria* in droughted plots, possibly due to their preference for oligotrophic conditions (Castro *et al.* 2010).

In contrast, fungal communities are demonstrably more robust after ten years of drought when compared with bacterial communities. Fungal mycelia pass through air filled pores into water and nutrient filled regions of the soil leading towards communities dominated by fungi (Yuste *et al.* 2011). Similarly, soil community samples pre-exposed to a long period of severe drought show greater resilience to heat-drought events (Bérard *et al.* 2012) with the potential of diminishing the community effects of drought events.

1.4.1.2 Potential implications of drought on biogeochemical cycles and soil respiration

Changes in soil moisture will undoubtedly have implications for biotic and abiotic aspects of biogeochemical cycles (see Section 1.3.3.2). Decreased activity of phenol oxidase is associated with declining rates of lignin and plant litter decomposition within soil, whilst decreased acid phosphatase activity reduces the rate phosphate hydrolysis from organic soil components (Toberman *et al.* 2008; Baldrian *et al.* 2010; Jentsch *et al.* 2011). This may indicate that drought driven declines in activity could modify the mechanisms associated with C and SOM cycling. Declines in fungal diversity lead to decreased rates of carbon mobilisation from leaf litter. Subsequently, limited litter breakdown reduces the rate of nutrients such as nitrogen, phosphorous and potassium being recycled to the soil. Stress events are expected to shift nitrogen cycles, through selection of taxa capable of storing and utilising otherwise unavailable nitrogen sources (Schimel & Balser 2007). To further compound the issue, dry soils decrease the diffusion of substrates, as indicated by the accretion of ammonium in droughted treatments (Landesman & Dighton 2010). Whilst nitrogen is still present in the soil environment in the form of ammonium, the lack of diffusion due to limited water availability prevents it from being utilised by plants and microorganisms (Landesman & Dighton 2010).

Soil respiration reductions occur during droughted periods, limiting the carbon released from the soil environment (Yuste *et al.* 2011; Selsted *et al.* 2012; Barnard *et al.* 2013). A shift from bacterial to a fungally dominated ecologies may increase the rate of carbon sequestration as C is incorporated into fungal mycelia (Yuste *et al.* 2011). However, fungal communities suffering from drought are likely to show decreased ability to fix carbon (Toberman *et al.* 2008) with implications for carbon sequestration in the long-term.

Associations between native plants and AMF species may further complicate the picture by varying plant productivity, and in turn modifying carbon input associated with plant growth (leaf litter, root growth, etc.).

Conversely, some cases exhibit no significant change in enzyme activities associated with carbon and nitrogen cycles (Kreyling *et al.* 2008; Steinweg *et al.* 2013). This may indicate a greater stability in soil communities than expected, and that drought events alone may not significantly impact nutrient cycling. However, meta-analysis of nutrient cycles under drought noted communities exhibited decreased enzyme activities due to decreased diffusion rates, suggesting strong, seasonal responses to precipitation events, and stagnation of biogeochemical cycles throughout extended drought periods (Manzoni *et al.* 2012).

1.5 Short Rotation Coppice

The requirement for low carbon, renewable sources of energy has increased the interest in bioenergy crops, with particular focus dedicated to second generation short rotation coppice (SRC) systems. SRC crops are planted in rotations, allowing for repeated harvest of woody biomass once every 3-5 years, over a 15 to 30 year time period (Department for Environment Food and Rural Affairs 2004; Aylott *et al.* 2008; Hinchee *et al.* 2009). This relies on fast growing trees, such as poplar (*Populus sp.*), and willow (*Salix sp.*) (Hinchee *et al.* 2009; Rowe *et al.* 2009). SRC has the added advantage of being grown on marginal land, preventing competition with food crops for high quality soils. Biomass produced by SRC is destined for co-fired power-plants, to supplement existing fossil fuel sources (Aylott *et al.* 2008; Hinchee *et al.* 2009), although methods for biofuel production have been developed (Gomez *et al.* 2008). SRC has a range of potential benefits. Growth of woody biomass appropriates C in the short-term (although this is released after biomass incineration), and can reduce reliance upon fossil fuels (Volk *et al.* 2004). During the growth of SRC, complex root systems form, remaining within the soil environment throughout the rotation, sequestering C on a decadal scale. In addition, litter fall from SRC can increase the availability of SOM in soil surface, cycling nutrients back to the surface from deeper horizons (Yannikos *et al.* 2014).

However, in order to achieve the most progressive targets for the UK, 2.7 million hectares of SRC requires planting (Rowe *et al.* 2009). Whilst plantation of this scale is unlikely to occur, it raises the question of the implications of large scale land use change for biomass production.

1.5.1 Impacts of land use change for SRC: soil environment

Planting of SRC upon marginal land is likely to result in changes in the soil environment. SRC fields are prepared by subsoiling and ploughing, ensuring the top 60 cm of soil is aerated (Department for Environment Food and Rural Affairs 2004). This tillage method decreases the total water content of the soil, whilst increasing oxygenation (Linn & Doran 1984). However, in the long term, SRC sites do not require regular tillage, and as such may be a valid method for carbon sequestration and build-up of SOC (Harris *et al*, submitted). By reducing annual tillage to a no-till method, it SOC containing approximately 57 g C m^{-2} per year could be sequestered in the time between planting of new SRC stands (West & Post 2002). Once planting has been carried out, the increased abundance of roots elevates belowground biomass, causing changes in hydrology (Rowe *et al.* 2009; Holland *et al.* 2015). The associated increased uptake of water can dry surface soils, again increasing the proportion of aerated pores (Linn & Doran 1984).

Conversion of marginal or grass land into high density SRC leads to variation in phenology. For example, SRC species have elongated growth rates compared to grasses, continuing to grow until later in the year, which may influence soil biota (Dimitriou *et al.* 2009). Furthermore, the transport of water via evapotranspiration occurs at a greater rate in trees compared with arable crops/grasses. Increased root depth allows them to access resources at a greater depth compared with grasses. As previously mentioned, when combined with litter fall, SRC establishment can cycle nutrients back to surface horizons, with the subsequent availability of nutrients selecting for copiotrophic ecologies (Dimitriou *et al.* 2009; Yannikos *et al.* 2014).

Transition from grassland to SRC willow shows no evidence of decreasing soil nutrient levels, due to the recycling of nutrients from leaf litter via mineralisation by microbes (Volk *et al.* 2004). However, for commercial SRC to

remain commercially viable, nitrogen fertilisers are added during spring. Failure to apply nitrogen fertilisers after harvest result in gradual declines in soil nutrient levels as biomass containing nitrogen, phosphorous and potassium is removed from the field after harvest (Ens *et al.* 2013; Stauffer *et al.* 2014). Conversion of grassland to SRC appears to have no effect on total organic matter or CEC, although phosphorous, potassium and C:N ratio decrease in SRC compared to Grassland, suggesting a greater rate of turnover of organic matter in SRC plots (Stauffer *et al.* 2014). After 4 years of rotation, soil available nitrogen can decrease by up to 24% and phosphorous by 105%, although much of this is retained in root systems (Hangs *et al.* 2014). Decomposition of root structures and subsequent reintegration of nutrients may provide for recovery of biogeochemical cycles with time (Hangs *et al.* 2014).

As coppice becomes established and canopy height increases, albedo effects may begin to affect summer soil surface temperatures. The increased density of summer foliage may reduce ambient temperatures, which may have implications for evapotranspiration, diffusion rates, and in turn, biogeochemical cycling (Tiwary *et al.* 2013). As indicated throughout this section, variation of the environment from grass or marginal land may result in changes in the soil environment, with implications for the microbial communities living within them. Section 1.5.2 will discuss the likely effects of land use change due to SRC establishment in the context of microbial ecology.

1.5.2 Potential impacts of SRC on microbial communities

The transition to SRC from grassland can impact the soil environment, as discussed in Section 1.5.1. As discussed in Section 1.3.3.1, pH and soil moisture are primary drivers of microbial diversity, also causing shifts in community structure (Fierer & Jackson 2006). Abundance, quality and vegetation type can all influence microbial communities (Garbeva *et al.* 2004; Chen *et al.* 2007), and as such SRC establishment may result in variation in associated bacterial and fungal communities (Rooney *et al.* 2009). This in turn may have implications for soil community function.

Whilst the literature suggests fungal dominance will increase in SRCs (Rooney *et al.* 2009; Xue *et al.* 2015), there is little research documenting the effects of

SRC on bacterial communities. The increased aeration of the soil may reduce the prevalence of anaerobic bacteria associated with N cycling and methanogenesis, but this is speculation based upon the known abiotic effects (Linn & Doran 1984). Recent bacterial and fungal metabarcoding of *Populus* and *Salix* SRC sites in the US have shown elevated abundances of *Nitrospirae* post-conversion, which may indicate augmentation of nitrate levels (Xue *et al.* 2015).

Nitrification rates appeared unaffected three years after transition and no change in basal rates of soil respiration occurred (Stauffer *et al.* 2014). However, enzyme activity within SRC soils promotes laccase activity and total soil organic matter, whilst no change in alkaline phosphatase activity occurred (Stauffer *et al.* 2014). Laccase is an enzyme associated with fungal lignin degradation whilst alkaline phosphatase is involved in the breakdown and cycling of phosphorous in the soil environment. Increased biological activity of laccase suggests carbon cycling within SRC soils may be increased when compared to arable land, due to increased fungal activity.

In cases where tillage is undertaken, increased aeration of soil and decline in water filled pore space may favour aerobic bacteria (Linn & Doran 1984). Given that many denitrifying bacteria are anaerobic, rates of denitrification may decrease (Sanford *et al.* 2012; Guo *et al.* 2013). Therefore, tillage may disrupt portions of the nitrogen cycle, decreasing N_2 emissions and N_2O production, a potent greenhouse gas (Snyder *et al.* 2009). Equally, declining nitrate conversion by bacteria may elevate bioavailable nitrate for plant growth.

SRC establishment can cause variation in microbial communities. The ratio of bacteria to fungi in SRC soils is similar to those found in forest ecosystems rather than agricultural systems (Stauffer *et al.* 2014), and may play a role in moderating the mechanism of phosphorous and potassium mobilisation and subsequent availability (Püttsepp *et al.* 2004). Similarly, the ratio of fungi to bacteria in traditional crops is reduced, along with total fungal biomass when compared with SRC sites (Yannikos *et al.* 2014). This may indicate increased decomposition of cellulose and lignin in SRC sites, upregulating the speed at which OM is reincorporated into the soil.

Arable soils have lower abundances of plants hosting EMF taxa, limiting the abundance of EMF. However, the introduction of host plants in the form of SRC

may increase EMF proportional abundances (Hrynkiewicz *et al.* 2012). Growth of SRC crops can lead to the reintroduction of EMF, increasing fungal diversity, although the functional implications of this are not fully understood. Popular SRC species (poplar and willow) can form simultaneous symbiotic associations with both fungal types. Exactly which EMFs become established is a result of multiple factors, including the SRC species, edaphic variables and climatic conditions (Hrynkiewicz *et al.* 2012). Such changes in fungal diversity may influence the rate of litter decomposition in SRC stands (Baum *et al.* 2009; Yannikos *et al.* 2014).

1.6 Next Generation Sequencing Techniques

The usage of accurate DNA sequencing has become commonplace since its discovery (Sanger. F 1977). Sanger sequencing relies upon elongation and termination of single stranded DNA fragments through random incorporation of di-deoxynucleosidetriphosphates (ddNTPs). Resulting sequences are sorted through gel electrophoresis, producing detectable bands representative of the points at which a ddNTP is incorporated. By carrying out the method utilising ddNTPs representative of each base (A, T, C and G) it is possible to determine the sequence present within the sample.

This methodology has been improved since its inception, through automation and increasing throughput of the process. Whilst Sanger sequencing is more accurate per sequence than several NGS methodologies (as discussed below), and produces longer reads (>700bp) it is slower and more costly than its newer counterparts, requires greater quantities of sample DNA and produces a considerably lower data yield.

NGS technology makes use of developments in high-throughput sequencing to rapidly produce gigabases of sequence data (Knief 2014). The removal of vector based cloning used by conventional sequencing techniques dramatically reduces preparation time required. Some techniques utilise an amplification step prior to sequencing by synthesis. Sequencing by synthesis detects nucleotides as they are incorporated into a strand complementary to a single stranded DNA template (Shokralla *et al.* 2012). The exact methodologies vary between platforms, but most produce fragmented reads of 100-800 base pairs

(bp). If reads are from a previously sequenced organism, they can be compared with an existing genome.

Most platforms include a multiplexing protocol, allowing several samples to be loaded into a single flowcell, sequenced, and separated *in silico*. This allows up to 384 samples to be run simultaneously, decreasing the cost per sample. However, multiplexed data decreases total sequence data available for each sample (Glenn 2011; Shokralla *et al.* 2012; Di Bella *et al.* 2013).

NGS technologies are versatile, being able to carry out sequencing of genomes, metagenomes and transcriptomes all at high speed and accuracy, without the need for vector based cloning. However there are still limitations to each NGS method.

Current methods report accuracy of each base using Phred Q scores. These represent the likelihood of an incorrect base call at each position. A Q score of 10 represents a 1 in 10 chance of the base being incorrectly called, a Q score of 20 a 1 in 100 chance, and a Q score of 30 a 1 in 1000 chance. Most NGS methods have inbuilt quality controls which trim low Q scores from the ends of reads, or discard reads altogether if mean Q scores drop below a threshold. Sequences produced using Sanger sequencing usually have higher average Q scores for longer portions of sequence. However, the increased throughput produced by NGS methods allows for consensus measures to correct low quality regions.

Platforms usually undertake sequencing in two steps, library preparation and detection of incorporated nucleotides. Library preparation takes sheared DNA, carries out end repair and attaches oligonucleotide adapters (which are specific to each platform) prior to sequencing (Shokralla *et al.* 2012; Van Dijk *et al.* 2014). Often, a selection process such as Gel Size Selection is carried out at this stage, to purify DNA fragments and ensure reads are of the desired length for further processing.

NGS technology fall into two main categories, platforms utilising PCR amplification (Roche 454 and Illumina) and platforms based on single molecule sequencing (SMS) technologies (PacBio SMRT and Oxford Nanopore) (Table 2). SMS technologies only require a single molecule of DNA, and as such do not utilise an amplification step before sequencing, removing the possibility of PCR bias. Furthermore, SMS technologies produce reads of 30–60 kb in length,

much larger than the short reads produced by Roche and Illumina technologies, or even traditional Sanger sequencing methods. However, they are disadvantaged either through their experimental status (Oxford Nanopore) or their high error rate (PacBio SMRT). In the case of PacBio, this is less of an issue in full genome projects, in which long reads can be scaffolded and errors removed through consensus read comparison.

Table 4 A summary of NGS platforms available or in production. This summary is based on work by Shokralla et al (2012)

Platform	Read length	Method	Max reads per run	2012 Cost per megabase (Mb)	Pros	Cons	Studies
Illumina	50-200	Bridge amplification, sequencing by synthesis and detection through fluorescent markers	$6x10^9$	$0.10	Very high capacity, accurate detection of nucleotide addition, high output, low cost, paired end sequencing	Error rate increases with read length, slower due to deblocking phase, amplification bias, short read lengths	(Degnan & Ochman 2011; Caporaso *et al.* 2012)
Roche 454	400-800	Bead amplification, sequencing by synthesis and detection of light emitted by luciferase reaction.	$1x10^6$	$7.00	Fast due to lack of deblocking phase, long read lengths	Difficulty reading homopolymer regions, expensive, amplification bias	(Tasse et al. 2010; Blaalid et al. 2012; Delmont et al. 2012)

PacBio SMRT	30kb	Single molecule real time sequencing	$6x10^3$	$4.00	Very long reads	Limited accuracy, less useful studies requiring many reads	(Knief 2014)
Ion Torrent	100–200	Sequencing by synthesis, detection of pH changes as bases are incorporated	$11x10^6$	$7.50	Fast run time	Limited use in literature, expensive	(Tonge *et al.* 2014)
Oxford Nanopore	No maximum length	Single molecule sequencing. Reads changes in electrical current as DNA passes through a nanopore.	N/a	N/a	No amplification bias, long read length, no reagents, cheap	Experimental, not yet commercially available, potentially high error rates	(Mikheyev & Tin 2014)

1.6.1 Metagenomics

Emergence of NGS technologies has resulted in a torrent of genetic and genomic research. One new research area arising from the technology is metagenomics. Metagenomics has a wide range of definitions within the literature, with opinion varying on what constitutes true metagenomic research (Xu 2006; Kunin *et al.* 2008; Wooley *et al.* 2010; Thomas *et al.* 2012; Shokralla *et al.* 2012; Esposito & Kirschberg 2014). Several definitions include so called "16S" or "targeted" metagenomics, sequencing of the 16SrRNA region specific Bacteria and Archaea (Taberlet *et al.* 2012a; Esposito & Kirschberg 2014). However, for the purpose of this thesis, metagenomics will not include marker based methods, and will only refer to shotgun metagenomic approaches (Kunin *et al.* 2008). Instead, marker based methods will be termed "metabarcoding" (Taberlet *et al.* 2012b). A breakdown of ways in which metagenomics and metabarcoding can be used, along with the methods and target organisms can be seen in Table 3.

Metagenomic studies have been carried out using Sanger sequencing; an approach which, whilst accurate and capable of producing long reads is very slow, time consuming and expensive (Shokralla et al. 2012; Knief 2014). This approach provides only a small portion of the picture of microbial community assemblage due to its high cost, methodological difficulties and biases, and limited sample throughput and data output. NGS techniques are rapidly increasing the viability of metagenomics, enabling the collection of data from an entire microbial community.

Shotgun sequencing utilises the randomised sonication of DNA, prior to sequencing of a size selected portion of the sample. In contrast to metabarcoding, no amplification occurs prior to sequencing; all DNA passing library preparation is sequenced. Although a vast amount of data can be gleaned using this method, the pipelines for analysis can be complex. Metagenomic sequences are short fragments (100–400 bp), which can be compared with existing gene databases. Fragments of taxon specific 16S sequences are matched with taxonomic databases, whilst gene fragments are compared with KEGG and GO databases to establish their function (Thomas *et al.* 2012; Shokralla *et al.* 2012). Often, to increase accuracy the nucleotide

sequence is converted *in silico* to a coding protein before comparison with functional databases.

1.6.1.1 Previous metagenomics studies

Early attempts at metagenomics involved the ligation of extracted DNA into vectors and transformation into host bacteria. The resulting cloned bacteria could then be assessed for traits such as enzyme or protein production, resistance and antimicrobial properties. Clones representative of these functions could then be sequenced, in order to determine which genes were responsible for the noted effects (Handelsman 2004; Kunin *et al.* 2008). These methods enabled some of the first metagenomics studies to link function to specific taxa, by identifying taxonomic markers flanking functional genes of interest (Handelsman 2004). For example, sequencing of pelagic communities in the Sargasso Sea revealed the presence of over 700 rhodopsin like proteins, approximately only 70 of which were known prior to the study (Venter 2004). However, this study required a large team of scientists, was expensive, and failed to attribute function or taxonomy to the majority of samples (Handelsman 2004; Schloss & Handelsman 2005).

Later studies addressed less complex communities to reduce noisy data. Acid mine drainage studies showed that biofilms were dominated by a *Leptospirillum* species. The lack of complexity allowed for construction of representative genome for the species, and enabled identification of individual strains though use of single nucleotide polymorphisms (SNPs). Furthermore, the presence of complete genomes permitted reconstructions of detailed metabolic maps, suggesting the community capable of both C and N fixation carried out by the dominant *Leptospirillum*, whilst the less abundant *Ferroplasma* species utilised N fixed by other taxa (Tyson *et al.* 2004). Subsequently, comparative metagenomics was attempted, comparing newly collected samples from whale fall and agricultural soil with the pre-existing data from the Sargasso Sea and Acid mine drainage (Tringe 2005). This paper helped establish the method used in the majority of metagenomic studies today, that of using unassembled sequences to produce a “fingerprint” for a habitat, reflecting identifiable functions and taxa.

Subsequent development of metagenomics has raised exciting possibilities. The method has been used in multiple fields; medicine to identify potential

antibiotic compounds (Owen *et al.* 2013) and in human microbiome studies (Turnbaugh *et al.* 2008; Tasse *et al.* 2010; Huttenhower 2012); earth science to elucidate taxa associated with geochemical cycling (Fierer *et al.* 2012b) bioremediation (Mason *et al.* 2012) and oil degradation (An *et al.* 2013); and biology to better understand community ecology and the effects of environmental change (Shokralla *et al.* 2012; Souza *et al.* 2013; Luo *et al.* 2014). For example, comparison of human microbiomes has revealed variation in bacterial communities present between individuals, although several functional groups occur across samples. It was also noted that several functional groups occurred in greater prevalence in samples taken from specific body sites, such as phosphate and saccharide transport in mouth samples, indicating specific roles of bacteria within these environments (Huttenhower 2012).

In environmental studies, samples from oceanic upwellings off the coast of California indicate a correlation between nutrient availability and community diversity. Sites with samples containing an abundance of taxa with small genomes had greater abundances of amino acid, iron and cadmium transporters, whilst sites dominated by larger genome taxa yielded K and NH_3 transporters (Allen *et al.* 2012). This provides insight into the differential impact of taxa upon nutrient cycles within oceanic upwelling ecosystems. Comparisons of metagenomes across biomes indicate similar diversity across tropical forest, temperate forest and grassland environments, whilst hot and cold deserts formed a separate group. Highly vegetated environments and hot and cold deserts have very different microbial ecologies, determined by increased abundances of the taxa *Actinobacteria*, *Bacteroides* and *Cyanobacteria* in desert soils. Likewise, decreased abundances of pathways related to nutrient cycling occurred in desert soils (Fierer *et al.* 2012b).

These studies illustrate the potential power of metagenomic methods, which can provide new insights into microbial ecology and function, even in those taxa which are currently unidentified and unculturable.

1.6.1.2 Limitations of shotgun metagenomics

There are several limitations to be deliberated when considering the use of metagenomics. The shotgun nature of metagenomics may fail to detect rarer taxa and genes in the environment (Shah *et al.* 2011). Furthermore, identifying

whether low abundance reads represent a significant difference between treatments, or merely background noise can pose further problems. However, low abundance reads are unlikely to represent a significant functional role within a microbial ecosystem.

Whilst short sequences provided by many modern platforms can be used for metagenomics, identification of taxa can prove difficult. Short reads, and the multiple regions of the 16SrRNA region which must be covered to accurately determine taxa, means that metagenomics may not be able to identify taxonomic changes to the extent of metabarcoding (Temperton & Giovannoni 2012). As previously mentioned (Section 1.6.1.1), although metagenomic studies can provide useful information, much of the datasets can remain unannotated, as they do not match any existing databases. Similarly, functional regions may also prove difficult to accurately identify with very short reads. This poses such a serious problem that it has been suggested that an effort to annotate data from existing projects should be undertaken prior to further research using the technique to assess the effects of environmental changes (Dini-Andreote *et al.* 2012). However, as more data is accumulated and identified, these problems should be eventually addressed. As the datasets remain archived after analysis, it should be possible to reimplement pipelines after several years to improve the accuracy of analysis as new bioinformatics tools and metagenomic databases are designed.

1.6.2 **Metabarcoding**

Metabarcoding techniques study specific taxa within a sample through use of marker region specific primers. The method selects and amplifies portions of DNA which have a marker region of interest (an amplicon). Amplicon regions consist of a section of hypervariable sequence, flanked by conserved sequence. This allows for primers to be designed to target the conserved regions, permitting amplification of the marker region. A variety of taxon specific target regions exist, including the mitochondrial C oxidase I (COI) gene region for Animalia (Andersen *et al.* 2012), the 16S ribosomal subunit (16SrRNA) gene in Bacteria (Caporaso *et al.* 2010b), the internal transcribed spacer (ITS) region of nuclear ribosomal DNA in Fungi (McGuire *et al.* 2013) and regions of the chloroplast *trn*L intron in Plantae (Hiiesalu *et al.* 2012). Other marker genes can be used to analyse taxonomy at varying phylogenetic depths. Pooling

several markers can be used to assess the full spectrum of environmental DNA (Epp *et al.* 2012). By carrying out metabarcoding studies, it is possible to survey communities from environmental samples, providing not only presence/absence data, but also relative abundances of taxa present (Caporaso *et al.* 2010b; Shokralla *et al.* 2012).

To study microbial change in soils, the 16SrRNA and ITS region (for bacteria and fungi respectively) will be used. These approaches have successfully determined the effects of environmental variation on soil communities (Caporaso *et al.* 2012; McGuire *et al.* 2013; Ramirez *et al.* 2014), and have the advantage of being able to identify and quantify microbial communities (including unidentified taxa) without the need for laboratory cultivation.

1.6.2.1 The 16SrRNA region

The 16SrRNA region codes for the 16S subunit, an essential cellular structure forming part of the bacterial ribosome which is a highly conserved, but hypervariable region (Woese & Fox 1977). Furthermore, it is specific to bacteria and archaea, so use of the 16S amplicon immediately excludes noise from other kingdoms. Whilst the majority of the sequence is conserved, there is sufficient variation in small, hypervariable regions to allow taxa to be distinguished from one another (Figure 3). Coupled with extensive 16SrRNA databases identification and phylogeny of samples can be inferred.

Figure 3 Schematic showing the bacterial ribosomal operon, including the Promotor and Terminator regions (P and T) and the 16S, 23S and 5S ribosomal subunits

PCR is carried out after ligation of 16S specific primers, amplifying portions of the 16SrRNA genes within the sample of interest. Resulting DNA is sequenced, and the output compared with sequence databases. Sequencing of different hypervariable regions can cause variations in results, but the majority of studies use the V3–V4 hypervariable regions as these are effective across the majority of bacterial phyla (Klindworth *et al.* 2013). The V3–V4 region consists of two large hypervariable regions, and can be sequenced successfully using overlapping paired end reads with the Illumina MiSeq. The region is

approximately 460bp, so the use of two overlapping 300bp reads allows for the entire region to be sequenced.

1.6.2.2 **The ITS region**

Comparable to the 16SrRNA subunit in bacterial studies, the internal transcribed spacer (ITS) region is a universal marker for fungi (Schoch *et al.* 2012; Ihrmark *et al.* 2012; Huffnagle & Noverr 2013). The ITS regions flank the 5.8S ribosomal subunit regions, with (ITS1) between the 18S and 5.8S subunits, and ITS2 flanked by the 5.8S and 28S subunits (Figure 4). ITS regions are used due to their high efficacy in identifying fungi across the fungal phyla (Schoch *et al.* 2012).

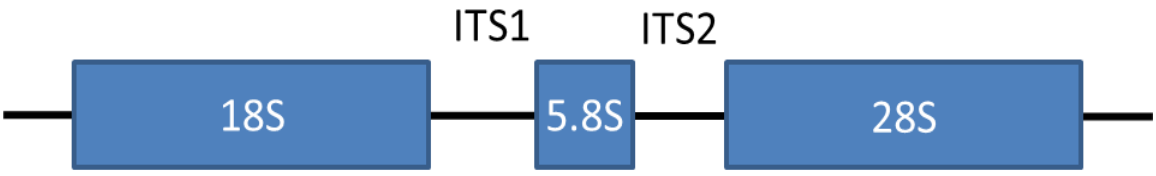


Figure 4 Schematic showing the fungal ribosomal gene, including the ITS1 and ITS2 regions

There are known biases associated with the use of either ITS1 or ITS2, with ITS 2 over representing the order *Lecanorales* and ITS1 detecting slightly lower abundances of *Cortinarius*. However, comparison of analysis using both regions revealed little difference in results (Blaalid *et al.* 2013).

1.6.2.3 **Limitations of metabarcoding**

Metabarcoding provides a useful tool for assessment of microbial communities. Surveys of this type can be used to determine a community baseline, and this can be compared with communities after treatment. Community changes after treatment provides insight into its likely impact on microbial community ecology. It should be noted that primer bias has been detected in several studies, leading to amplification of specific bacterial and fungal taxa whilst neglecting others (Bellemain *et al.* 2010; Bergmann *et al.* 2011; Ihrmark *et al.* 2012; Klindworth *et al.* 2013). However this can be remedied to some extent by careful selection of the universal primers used (Ihrmark *et al.* 2012; Klindworth *et al.* 2013).

Metabarcoding studies cannot directly inform us of ecosystem function. As described in Sections 1.2.1 and 1.2.2, several bacterial and fungal taxa carry out broadly similar functions. The functions of many species or genera are unknown. Bacterial accessory genomes- genetic material which contains a range of functional genes, not associated with bacterial core genome- further complicate the matter. Marker genes can identify the taxa, but are unable to detect variation in accessory genomes, which can vary in their contents by up to 80% within a species (Morales & Holben 2011). Therefore, community change may not accurately depict changes in function. New bioinformatics tools are available which may help to remedy the problem, modelling expected functional diversity of a community using databases of sequenced taxa (Langille *et al.* 2013). This will be discussed further in Chapter 6: .

Table 5 Environmental DNA studies, their target organisms, platforms and pipelines

Name	Aim of study	Sequencing platform	Pipeline	Approach
(Andersen *et al.* 2012)	Vertebrates	Roche 454	Bespoke	Metagenomics and metabarcoding
(Andreote *et al.* 2012)	Bacteria	Roche 454	MEGAN and MG-RAST	Metagenomics and metabarcoding
(Barberán *et al.* 2012)	Bacteria	N/A, downloaded from Global Ocean Sample data	MG-RAST	Metagenomics
(Blaalid *et al.* 2012)	Fungi	Roche 454	CLOTU	Metabarcoding (ITS1)
(Buée *et al.* 2009b)	Fungi	Roche 454	Blastclust and MEGAN	Metabarcoding (ITS1)
(Degnan & Ochman 2011)	Bacteria	Illumina	Bespoke	Metabarcoding (16S)
(He *et al.* 2010)	Bacteria	Roche 454	N/a	Metabarcoding (16S)
(Hiiesalu *et al.* 2012)	Plants	Roche 454	Bespoke and BLAST	Metabarcoding (*trn*L)
(Jorgensen *et al.* 2012)	Plants	Roche 454	OBI-tools	Metabarcoding (*trn*L)

1.6.3 Next Generation Sequencing platforms

Several NGS platforms are available (as summarised in *Table* 6), each with a variety of advantages and disadvantages. Below is a discussion of the methods used by Illumina and Roche 454 sequencers, the two platforms most commonly used during the initial stages of this research during 2012. This is followed by a discussion of two single molecule sequencing (SMS) technologies, PacBio SMRT, a method which is becoming increasingly common, and MinION, a technology which is currently in development, but may have exciting implications for future work.

1.6.3.1 Illumina

Preparation of Illumina sequencing begins with adaptor ligation enabling DNA to be fixed to flow cell channels. Gel Size Selection may then be carried out, selecting fragments of a desired length (Chmolowska 2013; Van Dijk *et al.* 2014). Once the sample is bound to the flow cell, unlabelled nucleotides are added and bridge amplification takes place, whereby each fragment provides a template for another identical fragment. Once denaturation of amplification occurs, each newly synthesised fragment binds to the flow cell. When repeated, millions of clusters of identical fragments are formed, ready for sequencing by synthesis.

During sequencing, each nucleotide is labelled with a different coloured fluorescent marker, (for example, adenine may be red, guanine green, cytosine yellow and thymine blue) to be activated by laser. As each base is incorporated to the fragments in a cluster, a laser activates the fluorescence and this is recorded by the device. Each cluster is monitored, and the changes in colour over time recorded, showing the incorporation of each nucleotide, thus sequencing the DNA fragment (Glenn 2011; Luo *et al.* 2012; Shokralla *et al.* 2012).

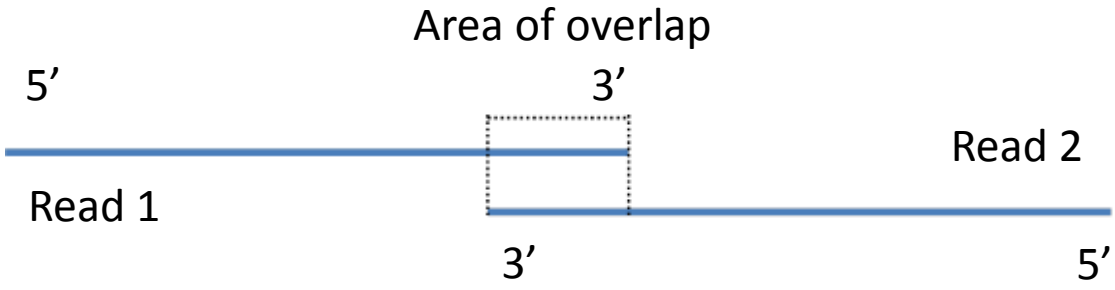
Compared with other NGS methods, Illumina is capable of accurately sequencing homopolymer regions although the accuracy is lower than that of Sanger sequencing (Luo *et al.* 2012). However, they produce relatively short reads (100–300 bp) so bioinformatics approaches are required to produce an entire V3–V4 region (Masella *et al.* 2012). Furthermore, the 5’ end of each

sequence is prone to a decline in quality scores, posing a problem for short read methods. Whilst this can be remedied when paired end reads are utilised, this can be detrimental to studies using unpaired 100bp reads, as quality control can reduce these reads to 80bp in length.

Illumina runs are considerably cheaper than other sequencing platforms, enabling more bases pairs of sequence for the same price. The additional bases per dollar are better spent on increasing sample numbers, as detection of additional taxa is rarely improved by increased sequence depth (Shokralla *et al.* 2012; Caporaso *et al.* 2012). Therefore, multiplexing of multiple samples is the most economical way of utilising the additional data.

Both metagenomic and metabarcoding data can be generated by Illumina technology. In the case of metagenomics, DNA is extracted from samples, sonicated into fragments of random sizes and size selected during library preparation. Metabarcoding sequencing requires a PCR step prior to library preparation, using target specific primers added to DNA samples, so that amplification desired marker region occurs. This generates an amplicon library of short, region specific reads. By ensuring a 50 bp overlap between each pair, bioinformatic methods can correct low quality bases, using the highest quality score from each read whilst combining the two reads together, producing a single long read for the V3-V4 region (Masella *et al.* 2012) (Figure 5).

Figure 5 Schematic showing the overlap between two reads. The low quality portion of R1 can be corrected using the high quality reads from the beginning of R2



1.6.3.2 454 Pyrosequencing

Roche 454 Pyrosequencing utilises a different method from Illumina's approach. Following library preparation (as described in 1.6.3.1) DNA samples are added to beads with complimentary adaptors bound to their surfaces, a single DNA fragment binds to each bead. Amplification results in each bead being covered with copies of the original strand. Once complete, beads without bound DNA are removed, and amplified DNA denatured, producing beads covered with single stranded DNA templates. Each bead is placed with an enzyme bead (covered with luciferase, sulfurylase and DNA polymerase) into a well on a plate, and sequencing begins. One type of deoxynucleoside triphosphate (dNTPs) is added at a time, and as each nucleoside is incorporated a pyrophosphate is released. This is then catalysed by the luciferase, releasing light which can be detected by the instrument (Glenn 2011; Luo *et al.* 2012). Greater light intensity equates to several of the same dNTP being incorporated. 454 pyrosequencing has the advantage of producing long reads within a short period of time. However, accuracy of homopolymer regions is decreased (Shokralla *et al.* 2012). Read length used by 454 can be useful in metagenomics studies although the decline in accuracy can limit downstream bioinformatics, making it difficult to accurately identify reads. Additionally, the increased cost of 454 sequencing reduces the number of samples which can be sequenced. In the case of metabarcoding, increased read length may be useful in sequencing larger amplicons, although the higher price of the method makes Illumina sequencing more attractive.

1.6.3.3 PacBio SMRT

PacBio SMRT (Single Molecule Real Time) sequencing is an SMS technology, based on sequencing by synthesis. Sequencing relies on small wells, known as Zero Mode Waveguides (ZMW), containing a single DNA polymerase placed at its base. Single stranded DNA templates from the sample are introduced to each well, along with fluorescent nucleotides. Each nucleotide has a fluorescent marker bound to the nucleotides phosphate group, which is released as it is incorporated during DNA synthesis. This results in an unlabelled DNA molecule and a brief fluorescence, detected by the ZMW. This enables sequencing of thousands of bases in real time. This is occurring simultaneously across tens of thousands of other ZMWs, rapidly generating

sequence data (Glenn 2011; Shokralla *et al.* 2012; Knief 2014). As DNA do not require fragmentation prior to sequencing, SMRT sequences can be kilobases in length (Glenn 2011). Whilst SMRT technology provides exciting methods for genomic studies, its use in metagenome and metabarcoding studies has been limited due to its comparatively low throughput and higher cost (Knief 2014).

1.6.3.4 **MinION and GridION**

MinION is an SMS system in development by Oxford Nanopore Technologies (ONT), which may massively increase throughput of sequencing with associated reductions in price. MinION is a USB sized device which plugs into a standard laptop. Samples loaded into the MinION are sequenced in real-time, providing data which can be readily accessed and analysed on the laptop. In addition to its small size and rapid sequencing rate, MinION is likely to be considerably cheaper than existing technologies, having a price tag of around $1000 per sequencer, compared with the $1000-1,000,000 of current sequencing units. GridION uses the same technology, but as a scalable system, allowing for the addition of further GridION modules to increase sequencing throughput to the level of existing NGS technologies.

Nanopore detects the charge of each base as it passes through an engineered nanopore membrane. Like other SMS technologies, reads are thousands of bases in length and DNA is sequenced in real time (Di Bella *et al.* 2013). However, sequencing chemistry methods are not used, instead directly measuring the sequence from the molecule itself (Carvalhais *et al.* 2012). Again, like SMRT, ONT has the advantage of having no amplification step, directly measuring the content of the sample, removing PCR and primer biases. The method could be used to sequence full genes in metagenomic studies, or entire amplicon regions in metabarcode studies, giving greater accuracy to datasets (Temperton & Giovannoni 2012). The low rate of throughput provided by the MinION has been addressed through the development of GridION, increasing sequencing capacity, without substantially increasing costs. However, at present neither of the systems is commercially available, and results of the open access MinION tests indicate issues with the device. Currently only 25% of reads from resequencing of a lambda phage can be mapped to its reference genome, suggesting a very low accuracy (Mikheyev & Tin 2014). Whilst the technology may have potential in future, it is still in its infancy.

1.6.4 Analysis tools and software

The rapidly advancing fields of metagenomics and metabarcoding, means new software is constantly in development. For the purpose of this thesis, two existing pipelines will be utilised to analyse the two types of sequence data collected. Whilst the price of sequencing has decreased, the cost of computational services required to analyse the data has not. MG-RAST is a high-throughput cloud based method for data processing and analysis produced by Argonne National Labs, aiming to address this problem (Wilke *et al.* 2013). QIIME on the other hand, is a pipeline which can be installed on any desktop computer, allowing analysis of 16S and ITS metabarcoding datasets. Both pipelines are regularly updated and support is freely available from the web community. Several other pipelines are available, including MOTHUR (Schloss *et al.* 2009) and CAMERA (Seshadri *et al.* 2007). However, the pipelines detailed in the sections below were chosen for their wide prevalence in the literature.

1.6.4.1 MG-RAST

Metagenomics Rapid Annotation using Subsystems Technology (MG-RAST) is an automated pipeline for analysis of metagenomic datasets. Powerful computational hardware is made available through an online portal, to which users can upload data for processing on MG-RAST's servers. The pipeline includes a quality control step, followed by normalisation and subsequent sequence alignment, binning and taxonomic identification. Several tools for in depth analysis, including heat mapping, dendrograms and principle coordinate analysis are included (Figure 6).

MG-RAST will accept a range of common sequence file types, including 454 pyrosequencing SFF (standard flowgram format) files, FASTA (a standard sequencing output consisting of sequence reads, each assigned to a read ID) and FASTQ (similar to FASTA, but including quality scores for each base in each sequence) as commonly used by Illumina machines. Files can be uploaded with metadata, or made publically available to increase the priority of processing.

MG-RAST undertakes a pre-processing step removing low quality reads from the data, or trimming low quality ends from each read. Once initial QC is complete, sequences with k-mers of 20 identical bp are removed.

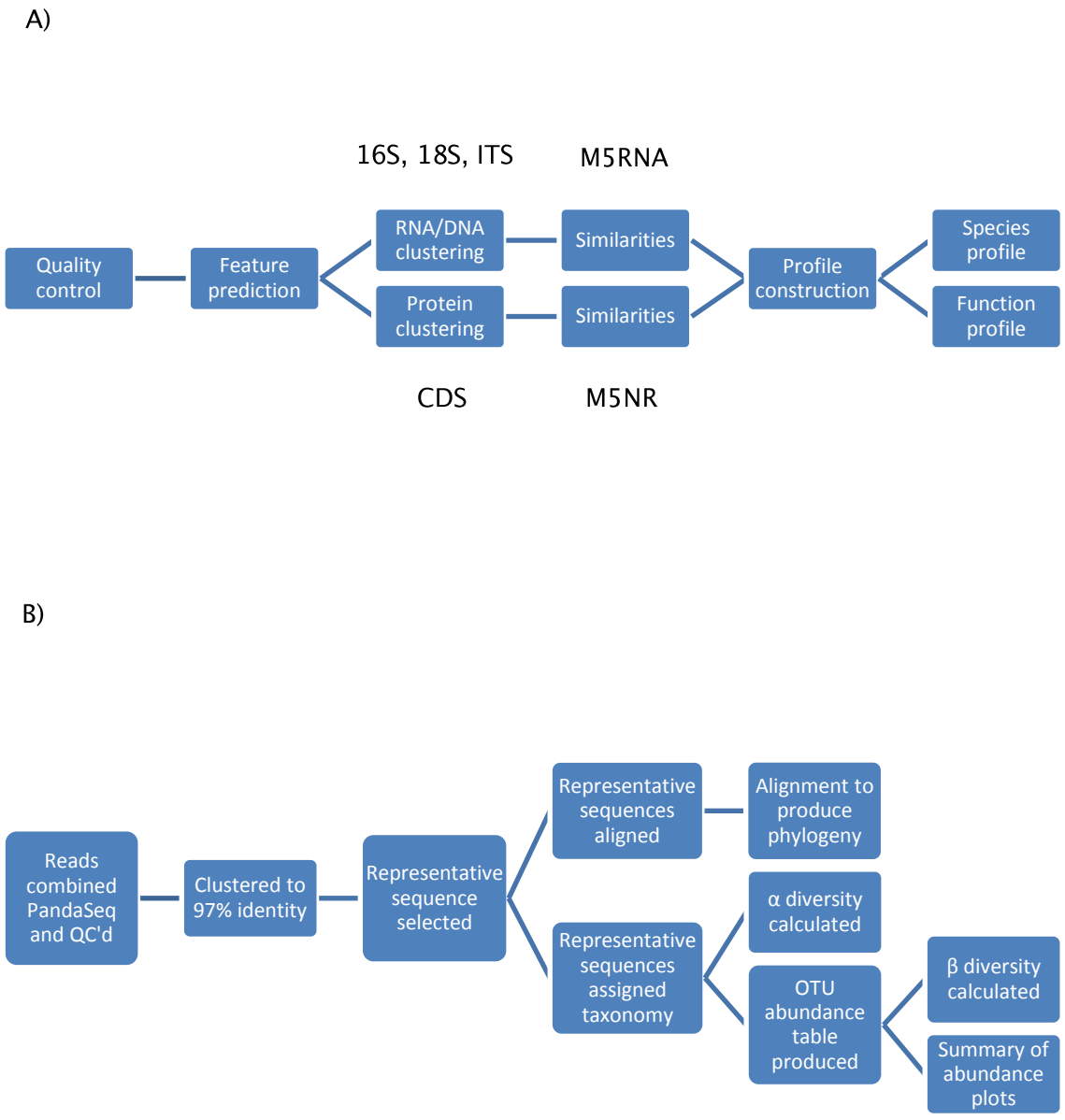


Figure 6 A) MG-RAST pipeline, taken from Wilke et al 2013; B) The main steps in the QIIME pipeline

This dereplicates the dataset. Remaining sequences are screened by alignment via Bowtie (Langmead *et al.* 2009) with several model organisms, to remove non-microbial DNA.

If paired-end sequencing has been carried out on the samples, the merge mate pairs option should be used. This automatically merges paired end reads into a single file, using fastq-join (Aronesty 2011), joining paired ends that overlap by at least 8bp. Pairs failing to overlap are discarded.

Sequences pass through a modified version of FragGeneScan (Rho *et al.* 2010), utilising a combination of prokaryote genetic traits (codon usage bias, start/stop patterns) and models of sequencing error (probability of frameshift errors during sequencing) to estimate open reading frames, and, as a result, protein coding regions. This can identify potential protein coding regions in reads as short as 75bp in length (Wilke *et al.* 2013). Sequences containing possible coding regions are translated to amino-acid sequences. These are clustered to 90% similarity using uclust (Edgar 2010) and each cluster aligned with protein function databases, annotating the clusters. The abundance of reads assigned to each functional group is calculated.

Simultaneously, MG-RAST identifies rRNA candidate genes by alignment at 90% identity (using uclust) with the SILVA database (Quast *et al.* 2013). This step identifies which reads are likely to be rRNA genes. Reads that successfully align are clustered to 97% identity (approximately genus in bacteria). Representative sequences from each cluster are compared using BLAT (Kent 2002) with the M5 RNA database (Wilke *et al.* 2013) to produce taxonomic profiling.

Both the SILVA and M5RNA databases comprise of a combination of databases, they may contain identical sequences labelled as different species (due to their presence across species or strains). Therefore, MG-RAST provides three options for viewing taxonomic data; best hit, (annotation with the highest percentage identity is used for all reads matching it); representative hit (choosing the first match with the highest percentage identity); or the lowest common ancestor (LCA) (assigning the identity of the closest ancestor of the multiple annotations to the read) (Wilke *et al.* 2013). Each method has pros and cons. Best hit artificially increases the results as the same read can be assigned multiple annotations. Representative hit may exclude valid results due to selection of the first ranked annotation, whilst LCA produces difficulties for downstream analyses which require all reads to be annotated to the same taxonomic resolution.

1.6.4.2 QIIME

QIIME (Quantitative Insights into Microbial Ecology), is designed for analysis of metabarcoding data produced by NGS platforms. QIIME processes raw, multiplexed reads, runs quality control, demultiplexes, undertakes OTU picking, alignment, diversity calculations and statistical analysis (Figure 6). By default, QIIME can process 16S rRNA sequences. However, portions of the pipeline which refer to 16S databases, such as GreenGenes (DeSantis *et al.* 2006) can be redirected to other marker specific databases such as UNITE (Kõljalg *et al.* 2013).

Once raw sequence data is available, the user creates a mapping file consisting of sample ID's with associated multiplex barcodes, primer sequence data and sample metadata (control or treatment, additional descriptive data, etc.). QIIME uses the information within the mapping file to split multiplexed reads into individual samples by matching barcodes in the file with sequence present within the reads. Barcodes and primers are then removed, resulting in trimmed sequence data for each demultiplexed sample.

The resulting sequence data is clustered using one of several methods (including cd-hit (Li & Godzik 2006), Blast (Altschul *et al.* 1990), Mothur (Schloss *et al.* 2009) and uclust (Edgar 2010) algorithms) as opted for by the user. Methods exist for *de novo*, closed reference and open reference picking. Closed reference methods compare each read against an existing database (specified by the user) and reads which fail to match at a given percentage (again defined by the user) are discarded. *De novo* methods cluster reads to 97% identity, approximately representative of a genus. A single representative sequence is selected from each OTU, either the centroid sequence (in the case of uclust), the most abundant sequence, the longest sequence or a random sequence. Each representative sequence is aligned with the Ribosomal Database Project (RDP) taxonomic databases (Wang *et al.* 2007). Uclust datasets use consensus taxonomy, the taxon to which 90% of the sequences can be assigned (Caporaso *et al.* 2010b).

If phylogenetic measures of diversity are used, alignment of representative sequences is carried out. This utilises the PyNAST (Caporaso *et al.* 2010a) sequence alignment method, aligning each representative sequence with the GreenGenes 16S core set (DeSantis *et al.* 2006). The alignment is then filtered

prior to construction of a phylogenetic tree. Open reference combines both methods, attempting open reference alignment initially, prior to clustering of reads which fail with those successfully aligned (Goodrich *et al.* 2014).

1.6.4.3 **Analysis methods**

Analysis of communities uses α (alpha) and β (beta) diversity methods. α diversity measures OTU richness present within the sample, in other words the total number of OTU's present within a sample. β diversity assesses the dissimilarity between the identities of the OTUs, and their relative abundances.

Bias is introduced to both methods as the number of reads per sample is increased. As reads in a sample increase, the number of species detected will increase. One way of dealing with this bias is through rarefaction (Caporaso *et al.* 2010b). Subsampling each sample to 90% of the total reads in the smallest sample shows what would have been detected if all samples had been sequenced to the same depth, reducing associated bias. Simultaneously, multiple rarefactions can be carried out at a range of depths, and α diversity calculated for each rarefied table. These are combined and plotted, producing a rarefaction curve. Steep curves indicate that new OTUs are still being detected, whilst a plateau shows that the full diversity of the sample is captured.

α diversity can be calculated using several metrics, including Phylogenetic diversity (PD), Observed species, and chao1. Each is a valid metric, but measures diversity differently. Observed species is the most basic metric, defining α diversity by the total number of species detected in the sample. PD measures the total length of branches for each samples phylogenetic tree resulting in a minor increase in diversity for each closely related OTU, and a larger increase for each distantly related OTU (Faith & Baker 2006). Chao1 weights diversity scores of each sample based on the number of singletons and doubletons found within the dataset. This provides an adjusted estimate of the number of OTUs truly present, after taking rare OTUs into account (Hughes *et al.* 2001). Each metric provides information on the similarity in the number of OTUs present, but none consider which species are present. It is possible to have two samples with identical α diversity values, made up of different species. For this reason, β diversity is also calculated.

β diversity compares diversity between samples, revealing differences in OTUs present and their abundances. Again, the reads per sample may impact the number of OTUs and abundances detected; samples are usually rarefied to 90% of the smallest sample. β diversity calculations produce a matrix comprising of the distance between each pair of samples allowing visualisation of data as a dendrogram, or principle coordinates analysis (PCoA). When plotted in three dimensions, samples containing similar OTUs in similar abundances cluster together, whilst those less similar are plotted apart.

Several metrics exist to measure β diversity. Unifrac distances (Lozupone & Knight 2005) utilise phylogenetic data to produce a distance, whilst Bray-Curtis distances (Bray & Curtis 1957) are non-phylogenetic.

Unweighted UNIFRAC comprises of the phylogenetic information only, providing a presence/absence overview of the dataset. Samples clustering using this method have similar phylogenetic trees. In the case of Weighted UNIFRAC measures, these utilise both the phylogenetic data and the abundances of the taxa present (Lozupone & Knight 2005). Bray-Curtis dissimilarity methods are similar to Weighted UNIFRAC in that they measure changes in diversity, but are not linked to phylogeny. Bray Curtis is calculated as:

$$\bar{C} = 1 - \frac{2w}{a+b}$$

Where w is the sum of the lowest abundance scores for taxa present in both samples, a is the sum of abundances in sample a, and b is the sum of abundances in sample b (Bray & Curtis 1957). This clusters samples containing similar taxa in similar proportions, without utilising phylogenetic methods, making it possible to see shifts in community abundance and structure due to treatment.

QIIME includes several scripts for producing visual output from samples. These include heatmaps, displaying differences in community composition divided by taxon; summary bar charts, containing the proportional abundance of each taxa and PCoA plots, visually summarizing UNIFRAC or Bray-Curtis data. Furthermore, a range of scripts allow for statistical analysis of α and β diversity data. These include Adonis (also known as a permutational ANOVA Anderson

2001), anosim (Chapman & Underwood 1999) and multiple t-tests with Monte-Carlo permutations.

1.7 Aims and objectives

This research aimed to investigate several climate related environmental changes on soil microbial communities using realistic field-based approaches and long-term experimental manipulations. Such approaches are necessary but are limited by variable soil samples requiring careful experimental design. Following an initial metagenomics study on biochar and drought treated field sites, the majority of this thesis focussed on examining the impacts of biochar in the soil environment and its implications for taxonomic diversity in soils. Additionally, a smaller study was undertaken to assess bacterial and fungal community change as a result of land use change from grassland to short rotation coppice (SRC), given the paucity of data on this topic and the high relevance of this LUC for future energy policy, globally .

Chapter two details the first pilot studies assessing impacts of drought or biochar through ecosystems manipulations experiments. These aimed to determine the viability of metagenomic and metabarcoding studies in understanding environmental change and were used to inform the design of subsequent studies.

Chapter three examines biochar treatment across three European sites using metabarcoding. Each site received identical biochar treatments in order to control for variation resulting from the use of different feedstocks or pyrolysis temperatures. The sites were located at Novarra, Italy (an SRC Poplar site in northern Italy); Lusignan, France (a dactylis grassland site in central France) and Pulborough (an SRC Willow site in West Sussex, England) (Figure 7).

An additional time series experiment was undertaken in Pulborough, UK aiming to assess temporal shifts in microbial communities after biochar treatment. Again, both bacterial and fungal primers were used. Samples were taken before, one month and one year after biochar application. The use of a time series approach aimed to understand short and mid-term effects of biochar treatment for soil microbial communities.

Chapter four covers automated soil respiration measurements conducted throughout the time series experiment, to understand biochar stability in the soil, providing further information its C sequestration potential. This was combined with resin lysimeter and enzyme analysis to determine effects of biochar application on soil function.

Chapter five details a study comparing land use change from grassland to willow short rotation coppice (SRC). Grassland samples were collected from Pulborough, UK during Summer of 2012, and compared with SRC samples collected simultaneously.

Finally, chapter six details use of the 16S metabarcoding data collected in the previous chapters to model expected metagenomes. PiCrust (Langille *et al.* 2013) was implemented to compare the results of predictive metagenomes from 16S metabarcode data with metagenomics data in Beano and Tolfa. The method was also used to predict functional variation associated with biochar treatment in chapter three.

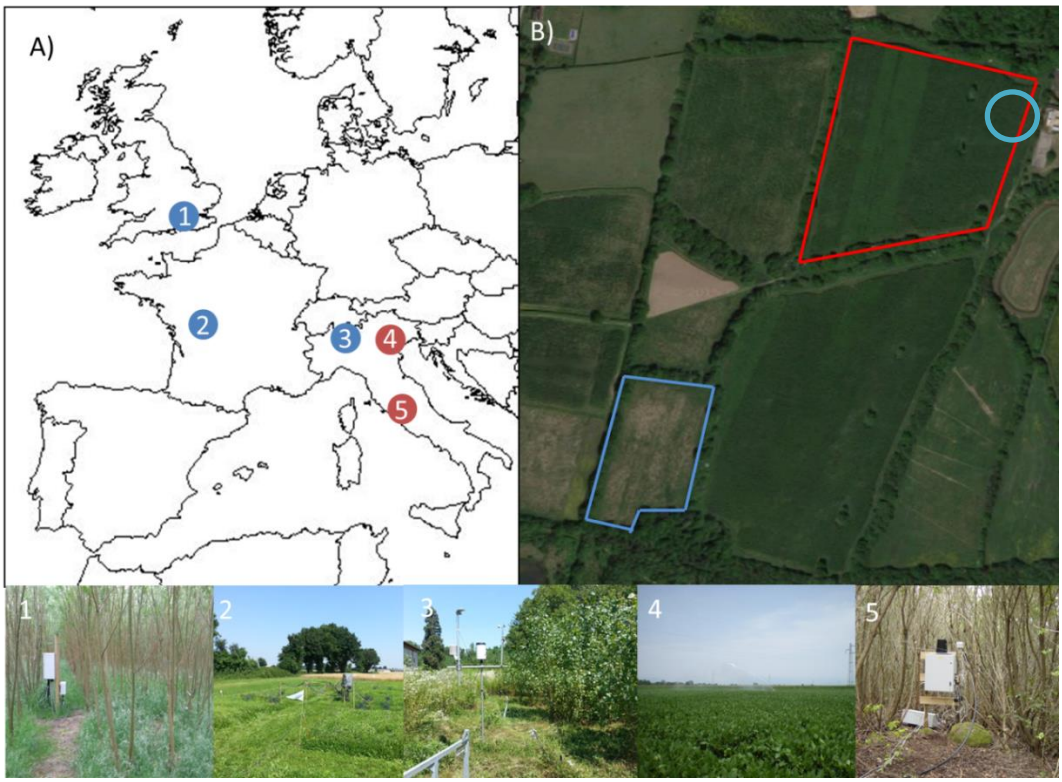


Figure 7 A) Location of metabarcoding (Chapter 3: 1 = Pulborough, UK, 2= Lusignan, FR 3= Novarra, IT) and metagenomics (Chapter 2: 4= Beano, 5 = Tolfa) sites. B) Location of soil respiration equipment (blue circles, Chapter 4:), SRC (red outline) and grassland (blue outline) (Chapter 5)

1.7.1 Objectives of this research

- Utilise metabarcoding and metagenomics in a pilot study to understand the implications of two ecosystems manipulations experiments (drought or biochar) for bacterial communities from a taxonomic and functional perspective
- Assess changes in bacterial and fungal diversity across a European network of biochar sites using 16SrRNA and ITS metabarcoding. Determine the effects of biochar application on heterotrophic and total soil respiration in a short rotation coppice field
- Assess changes in bacterial and fungal community between grassland and SRC crops
- Simulate metagenomes from 16S data using PiCrust to determine potential functional shifts in communities

Chapter 2: Metabarcoding and metagenomics as a tool to determine the effects of environmental change on soil microbes.

2.1 Introduction

Existing environments will be significantly impacted by climate change. Associated shifts in environmental variables will include increased CO_2, elevated mean temperatures and increased risk of extreme weather events (IPCC 2014). Each of these aspects may lead to changes in the biotic environment, due to variation in selection pressures (Sections 1.3 & 1.4). Further complicating the picture is the potential for anthropogenic mitigation methods to further vary the environment (Lehmann *et al.* 2006; Rowe *et al.* 2009). This thesis aimed to use high throughput sequencing technologies to understand changes occurring in the soil microbial communities under two climate change treatments; field-scale, long-term, drought and biochar manipulations.

Drought is to increase in prevalence due to increased rates of evapotranspiration and changes in rainfall patterns resulting from anthropogenic climate change, leading to more frequent soil moisture deficits (Dai 2011; IPCC 2014). Changes in the availability of soil moisture can drive distribution and function in microbial communities (Section 1.4). Previous studies have assessed the effects of drought on soil microbial respiration, enzyme activity or total biomass (Yuste *et al.* 2011; Jentsch *et al.* 2011). Alternatively, low resolution methods have established the impact of drought on microbial communities structure, using methods such as PLFA or DGGE (Toberman *et al.* 2008; Landesman & Dighton 2010). Whilst these methods are essential for understanding general changes in the structure of microbial communities, little is understood about the impacts on individual taxa.

The desire to utilise geoengineering methods to mitigate climate change is increasing, as it does not require divestment from a fossil fuel driven economy. One method which looks particularly promising is the application of biochar to the soil (Section 1.3). Previous work has measured microbial respiration and biomass (Castaldi *et al.* 2011), or used low resolution PLFA, TRFLP or DGGE methods to assess community structure (Kolton *et al.* 2011; Anders *et al.* 2013). These studies provide low resolution information about microbial communities, showing general changes in the ratios of Gram-positive and Gram-negative bacteria, and the ratios of fungi to bacteria (Section 1.3.3). However, they do not provide information regarding biochar's impact on

individual genera or functional gene groups, making it difficult to determine fine scale effects of treatment.

Next generation sequencing (NGS) is able to produce high resolution community profiles (Sections 1.6.1 and 1.6.2). Metagenomic methods provide information about the abundance of genes within in the environment, which can infer changes in the functional diversity present. Using these methods provides an exciting opportunity to study microbial populations, determining both the taxa and gene frequencies present within a population (Gilbert *et al.* 2011; Fierer *et al.* 2014). Metabarcoding determines the proportional abundance of genera present within a sample, without the need for culturing. Furthermore, application of metabarcoding methods can measure total diversity of a community, including the occurrence of unidentified taxa, and their proportional abundance (Fierer *et al.* 2012b; McGuire *et al.* 2013). By comparing the abundance of unidentified taxa which change due to a treatment, it may be possible to detect unidentified taxa which may be of interest for future study.

Here, the effects of drought and biochar treatments on microbial communities were assessed using both metabarcoding and metagenomic methods. This thesis aimed to detect changes in taxonomic differential abundance and identity due to treatment, whilst assessing associated shifts in functional gene profiles.

2.1.1 What are the expected impacts of drought on microbial communities?

Drought is anticipated to increase in frequency; duration and intensity during the next century (Section 1.4). Drought related declines in soil moisture have secondary implications for the soil environment. These include changes in soil texture, increased aeration, and shifts in soil nutrient retention (Section 1.4.1).

The structure of microbial communities appears robust and relatively unaffected by drought (Cruz-Martínez *et al.* 2009; Landesman & Dighton 2010; Sheik *et al.* 2011). Each of these studies detected no effect of drought on community structure when applied as part of a single factor study. Similarly, semi-arid populations exhibit no change in bacterial community composition in drought treatments versus controls, although large fluctuations occurred

after seasonal rainfall. This suggests acclimatisation of the community to low water environments, followed by changes after rainfall events due to r-strategist microbes exploiting newly available resources (Cregger *et al.* 2012). Therefore no change in α diversity (a measurement of taxonomic richness), or unweighted β diversity, (a measure phylogenetic distances between extant taxa) was expected as identities of taxa would remain unchanged. Furthermore, no change in the number of taxa present or their identities was expected but changes in the proportional abundances of the taxa present may occur. Previous studies rarely assess the impact of treatment to genera, and this lack of resolution may miss shifts in proportional abundance at lower taxonomic levels. By combining α and β diversity measurements with differential abundance techniques, this thesis will conclude whether taxonomic distribution and proportions remain unchanged by drought. Furthermore, shifts in taxa with specific functional roles (for example, nitrogen cycling) will be reflected in the abundance of functional genes (such as *amoA* and *nifH*), allowing us to determine the likely effects of treatment on biogeochemical cycling and soil function.

2.1.2 How will drought affect microbial community function and gene abundance?

Measurement of soil respiration indicates a decline in microbial activity in response to drought (Selsted *et al.* 2012; Steinweg *et al.* 2013). This may be due to declines in total biomass, or due to shifts in the method of metabolism in low moisture environments. If changes in respiration are related to declining abundance of biomass in response to treatment, then the proportion of core respiration associated genes may remain the same. In other words, whilst the total bacterial population may decline, the relative proportions may remain unchanged. However, if other forms of metabolism are selected for, then shifts in the abundances of core respiration genes may occur. Drought negatively impacted microbial activity, decreasing water extractable carbon and nitrogen after long-term treatments (180 days) (Hueso *et al.* 2012). These declines suggest decreased mineralisation rates for carbon and nitrogen substrates, leading to a slowing of their biogeochemical cycles. The decline in alkaline phosphomonoesterase, urease and beta-glucosidase, enzymes related to carbon, phosphorous and nitrogen cycling further confirm this theory (Hueso

et al. 2012). As such, similar reductions in activity may be detected in metagenomic datasets through declines in abundance of eDNA homologues and orthologs for the aforementioned enzymes. Taken together, a decline in the abundance of genes related to carbon, phosphorous and nitrogen cycling, with a possible increase in cellobiohydrolase homologues may be expected. Alternatively, drought events may have little impact on functional diversity, although there may be large shifts in community structure (Kreyling *et al.* 2008; Evans & Wallenstein 2014). Functional redundancy may mean that whilst some taxa decline in abundance, the functional diversity and abundance is maintained by another taxon more suited to the role in a low moisture environment (Evans & Wallenstein 2014).

2.1.3 What are the expected impacts of Biochar application on microbial communities?

Prevalence of biochar application is expected to increase, due to its potential as a carbon sequestration method (Section 1.3). However, the exact implication for microbial communities remains unclear (Lehmann *et al.* 2011). Assessment of community level change has suggested increased α diversity in response to biochar application (Xu *et al.* 2014a). Elevated α diversity indicates a proliferation of bacterial taxa, resulting from new ecological niches becoming available for colonisation. Similarly, ancient *terra preta* soils (see Section 1.1) have been found to contain approximately 25% greater diversity when compared with surrounding pristine soils (Kim *et al.* 2007). This may indicate a beneficial effect of pyrogenic carbon in terms of microbial diversity, and subsequently, functional diversity.

In terms of individual taxa of interest, changes due to biochar treatment have been previously detected through use of 454 sequencing, but these were limited to pot studies. These found elevated abundance of *Bradyrhizobiaceae*, *Hyphomicrobiaceae*, *Streptosporangineae* and *Thermomonosporaceae*, and declines in *Streptomycetaceae* and *Micromonosporaceae*. *Bradyrhizobiaceae* and *Hyphomicrobiaceae* are associated with denitrification of nitrate to gaseous nitrogen, whilst increased *Streptosporangium* and *Thermomonosporaceae* may indicate elevated cellulose and lignocellulose degradation (Anderson *et al.* 2011).

Some of the aforementioned shifts in abundances have been detected in other studies. For example, *Hyphomicrobiaceae* have been noted at increased abundances in an independent study of biochar (Xu *et al.* 2014a), which may suggest that this group has a standardised response to biochar treatment. However, in other pot trials treated with biochar, *Chitinophageaceae* and *Sphingobacteriales* have been detected at increased abundances (Kolton *et al.* 2011). These taxa are associated with carbon cycling and SOM decomposition, but were not detected in the Anderson study.

Genera within the *Sphingobacteriales* have been associated with breakdown of aromatic compounds, often present on the surface of biochars (Kolton *et al.* 2011; Xu *et al.* 2014a). *Terra preta* soils have been shown to contain greater proportions of *Verrucomicrobia*, and alpha, beta and gamma *Proteobacteria* (Kim *et al.* 2007; Grossman *et al.* 2010). Therefore application of biochar can cause changes in community abundance, although the exact nature of the shift varies between experiments and very little research on the long-term field sites has been completed. This thesis aims to determine whether changes in microbial diversity occur in a long-term biochar field trial, and whether the changes in taxa are similar to those previously described.

2.1.4 How will biochar application affect microbial community function and gene abundance?

Biochar application leads to changes in soil respiration (Smith *et al.* 2010; Ventura *et al.* 2014). Short-term increases appear to be due to increased microbial utilisation of labile biochar carbon (Jones *et al.* 2011c). In metagenomic studies, it is expected that increased abundance of carbon degrading bacteria may be reflected in greater proportional abundance of genes associated with utilisation of complex carbon molecules. Dehydrogenase and beta-glucosidase activity have been elevated in biochar treated incubation experiments (Jones *et al.* 2011c). If increased activity is a result of greater biomass of organisms, this may be reflected in greater proportions of beta-glucosidase and dehydrogenase genes within the metagenome. In contrast to this, enzyme activity experiments detected a decline in dehydrogenase and beta-glucosidase activity, which is postulated to result from toxic effects of biochar treatment, or through sorbtion of exudates and enzymes to biochar

particles (Ameloot *et al.* 2014), whilst another study noted no impact of biochar on sugar or organic acid breakdown and cycling (Jones *et al.* 2012). Examination of metagenomic profiles should provide further information as to whether the abundance of metabolic genes is affected by treatment.

Increased nitrification has been observed in alkaline biochar studies, coupled with intensified denitrification. If increased abundance of nitrifying and denitrifying bacteria occurs in samples, the nitrous oxide reductase gene (*nosZ)* would be expected to proliferate in abundance (Xu *et al.* 2014a). Additionally, phosphorous transporter gene abundance is also expected to be elevated in biochar samples. AM fungi have been demonstrated to adsorb organic phosphorous substrates from biochar, prior to transportation to plant roots, which may be reflected in gene abundances (Hammer *et al.* 2014). Similarly, phosphate and phosphonate breakdown has been activated by *Rhizobacteria* containing the *phnI* gene (Fox *et al.* 2014). Changes in the abundance of *Rhizobacteria*, and subsequently phosphate transport genes may help to explain some of the augmented growth effects noted in biochar studies.

2.1.5 Why utilise NGS methods in detecting microbial and functional change?

Utilisation of metabarcoding and metagenomic approaches enables surveying of microbial communities at a resolution and scale not previously possible. Diversity may be measured in terms of the species present (metabarcoding or metagenomics) or the profile of functional genes present (metagenomics). These techniques have been used in the past to assess differences in communities in the human microbiome (Ley *et al.* 2006; Gill *et al.* 2006; Huttenhower 2012). Although previous studies have measured changes in community due to biochar and drought, this is often at a coarse resolution, showing overall community shifts. In the case of PLFA studies, these provide only very low resolution information about communities, relating to the ratio of fungi to bacteria, and the proportions of Gram-positive and Gram-negative bacteria (Williams & Rice 2007; Frostegård *et al.* 2011). Whilst essential for understanding changes in ecology, it provides little detailed information regarding the potential effects of such change. By using metabarcoding, determining which genera contribute to community shifts, may be used to elucidate potential changes in function.

Previously, assessments of functional change relied upon detecting variation in a single functional group (Braker *et al.* 2000; Penton *et al.* 2013; Meyer *et al.* 2013). This is often through qPCR of a gene or range of genes associated with a single environmental function, such as the nitrogen cycle. It is hypothesised that drought treatment will have a limited effect on the proportional abundances of taxa, as whilst communities may reduce in size, the proportions of individual taxa are likely to remain intact. Biochar is expected to exert stronger selection pressures on the communities, due to the wide range of environmental shifts occurring due to its incorporation (see Section 1.3 for further detail). Therefore the relative abundances of taxa are expected to shift in biochar samples, which in turn should be reflected in the functional profiles detected by the metagenomic portion of the study.

2.2 Method

2.2.1 Metagenomics sites

Samples were collected from two environmental manipulation experiments in Italy (see Chapter 1: Figure 7), set up as part of the Expeer project (www.expeeronline.eu). Expeer experimental areas include highly instrumented sites where on a range of ecosystems manipulations experiments, including temperature manipulations, land use and fertilisation regimes. We specifically used a precipitation drought experiment in Tolfa, IT assessing the effects of long-term drought in woodland, and a long-term experiment on the effects of biochar application in an agricultural setting.

2.2.1.1 Tolfa (drought samples)

Drought samples were collected from the ExpeER site at Tolfa-Allumiere, in central Italy (42°11'N, 11°56'E), set up for use of a long-term study during 2004 (Cotrufo *et al.* 2011). The site was a long-term woodland drought experiment in which vegetation was characterised as mixed Mediterranean woodland, comprising of *Arbutus unedo L., Erica arborea L., Fraxinus ornus L., Quercus pubescens Willd and Quercus cerris L.* The site is dominated by *Arbutus unedo L.*, which has an average canopy height of 5m (Cotrufo *et al.* 2011).

Soil was characterised as an Andosol, with a pH of 4.0. Mean annual air temperature and precipitation were 16°C and 730mm respectively. Soils were predominately Sand (45%) and Silt (48%), with low levels of clay (6%). Total N was approximately 3.9 g/Kg. Treatments (20% drought, and a wet summer treatment) were initially set-up during April of 2004, whilst control plots (ambient rainfall) were set up during April 2006. Plots were arranged using a randomised block method (3 plots x 3 treatments). For the purposes of this study, only control and droughted plots were sampled. Drought treatment was maintained by through fall methods in which plots were treated with either ambient rainfall control, or with 20% rainfall exclusion. Exclusion was achieved through suspension of drains (20cm in width) 1.8m above the ground, allowing for rainfall to be collected and diverted from the plots towards a drain that directed water away from the site. Control plots received ambient rainfall (Cotrufo *et al.* 2011). Samples were randomly collected from six 8 x 12m drought and control plots during July of 2011

2.2.1.2 **Beano (Biochar samples)**

Biochar treated samples were collected from Beano, Northern Italy (40° 00'N, 13°01'E). At the time of sampling the site consisted of an agricultural field cropped with *Glycine max,* although the site had been previously growing *Zea mays* (Baronti *et al.* 2010; Ameloot *et al.* 2014). The soil was silt loam, with a pH of 7.1 whilst mean annual air temperature was 13.5°C (Baronti *et al.* 2010). Mean annual rainfall was 1216mm per year.

Biochar application was carried out using a randomised block method, with 3 plots per treatment. Plots had an area of $20m^2$. The biochar applied was produced through pyrolysis of coppiced woodland, consisting of hazel, beech, oak and birch, pyrolysed at 500 °C in a charcoal kiln (Baronti *et al.* 2010). Application of biochar was carried out during 2008 at a rate of 10 t ha^{-1} to fields containing residues from the previous seasons maize crop, prior to incorporation to a depth of 35 cm using rotary hoeing. Biochar chemical characteristics are shown in *Table 6* (Baronti *et al.* 2010).

Table 6 Chemical characteristics of the biochar used (dry weight)

Characteristic	Value
Total C (g kg^{-1})	840
Total N (g kg^{-1})	12
Available N (g kg^{-1})	0.03
C:N	70
P (g kg^{-1})	0.5
K (g kg^{-1})	4.3
Ca (g kg^{-1})	2.6
S (g kg^{-1})	1.1
pH	7.2

2.2.2 Soil sample collection at Tolfa and Beano

Stainless steel soil corers, sieves, spatulas and containers were all washed thoroughly with water, before being cleansed with RNAseZap and rinsed with MilliQ water. Sampling equipment was then rinsed with ethanol and left to dry until the ethanol had evaporated. Finally, all equipment was wrapped in aluminium foil and autoclaved to ensure its sterility.

Samples collected in Tolfa were from six plots 8 x 12 m in area (3 wet, 3 drought). In Beano, samples were collected from six plots, three 10m x 10m control plots, and three 5m x 4m biochar treatment plots. All samples were collected during July 2011. Five samples were collected 1.5 m from the centre of each of the plot, to a depth of 15cm. Resulting samples were filtered using a 2mm sieve to remove large particulate matter, stones, and roots, before freezing in liquid nitrogen. Samples for DNA analysis were placed immediately into Eppendorf tubes provided with the PowerSoil DNA extraction kit, prior to transport at -80°C.

2.2.3 DNA extraction

Samples from both sites were processed in the same manner once transported to the lab, following the protocol described in the MoBio PowerSoil DNA isolation kit (for DNA samples). The process separates soil particles, dissolves humic acids which would otherwise inhibit PCR and limits degradation of genetic material within the sample. Subsequent steps lyse the cells present and precipitate out organic, non-genetic materials such as proteins and humic

components. Finally, DNA was filtered from the remaining supernatant after centrifugation, washed with ethanol to remove contaminant material and buffer added. Resulting DNA samples were pooled by plot (5 samples per plot x 3 plots) and sent to the Instituto di Genomica Applicata Technology Services, Italy for library creation and Illumina sequencing.

2.2.4 Sequencing preparation and methodology

Preparation of the library was undertaken through the Illumina TruSeq DNA Sample Preparation protocol. A Biorupter NGS sonication device was used to shear 2µg of DNA, and blunt-ending and A+ addition carried out, according to manufacturer's specifications. Illumina adapters with 6bp Illumina indexes were ligated to fragments before gel recovery. The 400-500bp fraction was extracted and amplified through PCR to produce an enriched library containing fragments which had successfully ligated to adapter molecules. This utilised the standard TruSeq enrichment methodology (Illumina, Sand Diego, CA). Briefly, 25 µl of Illumina Truseq PCR Master Mix and 5µl of Illumina Truseq PCR Primer Cocktail were added to each well of a 96 well plate, already containing 1µg of sample DNA in each well, prior to thermal cycling at 98°C for 30 seconds, followed by 10 cycles of 98°C for 10 seconds, 60°C for 30 seconds and 72°C for 30 seconds. After the 10 cycles complete, there is a 5 minute cycle at 72°C before holding at 10°C. Quantification of libraries and quality control commenced using a Nanodrop ND-1000 UV-Vis Spectrophotometer and Agilent 2100 Bioanalyzer. Libraries were pooled in 6-plex equimolar ratios to yield a concentration of 10nM. 2µmol aliquots of pooled libraries were processed by cBOT for cluster generation according to manufacturer's recommendations. Sequencing was carried out by Illumina HiSeq2000 at 101 cycles per read. Images were processed with Illumina Pipeline version 1.8.2.

A separate 16S sequencing of samples was undertaken. DNA (extracted using the method detailed in Section 2.2.3) was amplified with the 515F and 806R 16SrRNA primers targeting the V4 hypervariable region (Caporaso *et al.* 2012). PCR was undertaken to the specifications specified by the Earth Microbiome Project (http://www.earthmicrobiome.org/emp-standard-protocols/16s/). The master mix consisted of 13.0µL of PCR grade water, 10.0µL of 5 Primer Hot Master Mix, 0.5µl of each of the forward and reverse primers (for a total of

0.10µL of Primer mix) and 1.0µL of template DNA, PCR was then carried out for 1) 3 mins at 94°C, 2) 45 seconds at 94°C, 3) 60 seconds at 50°C and 4) 90 seconds at 72°C. 35 further cycles of steps 2,3and 4 were then undertaken prior to 10 minutes at 72°C, and a final holding step at 4°C. Samples were barcoded and pooled, prior to sequencing using Illumina MiSeq using V2 chemistry (2 x 100bp).

2.2.5 Analysis pipeline

2.2.5.1 Metagenomic pipeline

Data were uploaded by IGATS to the MG-RAST pipeline and trimmed of adapter sequences. Pipeline options for were set to dereplicate sequences, removing those sequences which were identical, before fastq files were trimmed using DynamicTrim method (Cox *et al.* 2010). This trims sequences to the longest contiguous portion, containing minimum Phred scores of 20, and a maximum of 5 bases of a lower value. Due to the non-overlapping nature of the paired end reads, each set was processed individually, providing a dataset for each R1 and R2 read within each pair.

Best hit classification analysis was used to determine taxonomic origin. This assigns taxonomy to ribosomal reads using the M5NR or M5RNA annotation (Wilke *et al.* 2013). The highest scoring annotation is assigned to the read, unless two annotations have an identical score. In this case, both annotations are retained.

Data for taxonomic abundance was filtered using a maximum e-value cut-off of $1e^{-5}$ (representative of a 1 in 100,000 chance of a sequence of length x matching a sequence within a database containing y sequences), a minimum identity of 60% and a minimum alignment length of 15 amino acids (this is equivalent to 45 nucleotides). Datasets for R1 and R2 were processed separately. Each taxonomic table was downloaded in .biom format, for further statistical analysis using QIIME modules (Caporaso *et al.* 2010b). Functional profiles were downloaded using the same settings, annotated using the hierarchical classification option, using KEGG Orthology. For each metagenome, total SEED subsystems were also downloaded for comparison. Results of R1 vs R2 for each dataset (SEED and taxonomic assignment) were compared to one

another via a correlation to assess differences in low resolution functional profiles.

Both functional and taxonomic profiles were analysed using the QIIME pipeline for differences in α and β diversity between treatments. These analyses were undertaken separately for R1 and R2 values to determine whether reads provided similar results. α diversity was assessed via the alpha_rarefaction.py script, rarefying each sample randomly without replacement to a value of 90% of the number of reads in the smallest sample. α diversity values were compared by treatment group via compare_alpha_diversity.py, using a non-parametric t-test with Monte Carlo permutations. 999 permutations were carried out, prior to bonferroni correction.

β diversity was assessed through the non-phylogenetic measure of Bray-Curtis distance. Varying length of ribosomal sequences within reads, made the use of phylogenetic UNIFRAC methods inappropriate. PCoA (Principle coordinates analysis) plots were produced through QIIME's beta_div_through_plots.py script, again subsampled to 90% of the reads within the smallest sample. Treatment versus control was tested for cluster formation with ADONIS via the compare_categories.py script.

Differential abundance of KO functions and genus abundance was measured through use of STAMP (Parks & Beiko 2010), using a two-sided Whites non-parametric t-test (White *et al.* 2009). Corrections for multiple testing used the Benjamini-Hochberg method (Benjamini & Hochberg 1995). Results were filtered to a q-score of 0.05, with >5 sequences and effect size filters of difference between proportions of >0.5%, or a ratio of proportions of 2, prior to generation extended error bar plots. Using a q-score of 0.05 is representative of an expectation that 5% of tests significant at the corresponding *p*-value are false positives.

2.2.5.2 **Metabarcoding pipeline**

Metabarcoding assessed the proportional abundance of bacteria through 16S rRNA sequencing. Primers used were designed to span a 150bp region, ensuring an overlap of approximately 50bp between the reads. Thus sequencing a 150bp region used two overlapping 100bp paired end reads. Paired end Illumina reads were processed using PandaSeq (Masella *et al.* 2012). Given a file containing the identities of primers and adapters used in

sequencing, PandaSeq identifies the location of primers and adapters, removes them and then combines the overlapping portion of paired end reads. In addition, PandaSeq carries out a quality check of each read utilising the result of the highest scoring base call in cases of disagreement between reads. For example, in an area of overlap, where R1 is the sequence GATTACA, all with quality scores of 20 and R2 states the sequence as GCTTACA, with the quality score of 5 at the first C, PandaSeq will replace the C in the combined sequence with an A. Mismatched reads are discarded, providing an additional QC step. This resulted in a .fasta file with R1 and R2 combined into a single read for each sample. Site specific samples were then renamed and concatenated using bespoke software (SeqSuite, http://www.slimsuite.unsw.edu.au/software.php), to ensure read IDs were compatible with QIIME. This resulted in a single file for each site, containing all appropriate samples ready for submission to the QIIME pipeline.

Each site was analysed independently using QIIME. All scripts mentioned with the suffix .py are portions of the QIIME pipeline. *De novo* OTUs were picked using the pick_de_novo_otus.py command, clustering OTUs to 97% similarity. The *de novo* method retains sequences which could not be assigned to existing databases. Representative sequences were selected, aligned against existing databases and assigned taxonomy by UCLUST and USEARCH algorithms (Edgar 2010). This enabled a phylogenetic tree (Price *et al.* 2010) and OTU table to be produced. OTU tables were filtered to remove singletons, sequences only occurring once in a sample using the command filter_otus_from_otu_table.py. Removal of singletons reduces dataset noise and increases manageability, as they are usually a result of a sequencing error (Kunin *et al.* 2008; Wooley *et al.* 2010).

α and β diversity were assessed using the alpha_rarefaction.py and beta_diversity_through_plots.py scripts. In each case, sequences were subsampled to 90% of the number of reads present in the smallest sample, allowing for comparison across samples regardless of the number of reads per sample. Due to the presence of full regions appropriate for alignment (the V3 and V4 16srRNA regions) α diversity for metabarcode data could be compared using phylogenetic methods (Faith's Phylogenetic Diversity or PD (Faith & Baker 2006)). Furthermore, UNIFRAC distances were used to measure β diversity,

measuring phylogenetic diversity within each community (Lozupone & Knight 2005).

Resulting output from alpha_rarefaction was statistically assessed using the compare_alpha_diversity.py script, comparing treatments using a non-parametric t-test with Monte Carlo permutations, resulting in a pseudo p-value. Permutations for the test were set to 999, and Bonferroni corrections for multiple testing applied.

β diversity differences were assessed using the compare_categories.py script, utilising Adonis (a form of PERMANOVA (Anderson 2001)) calculating an effect size and pseudo p-value from a UNIFRAC distance matrix (see Section 1.6.4.2 for further information on the QIIME pipeline).

STAMP analysis of differential abundance of taxa was carried out using the same filters as those defined in Section 2.2.5.1.

2.3 Results

2.3.1 Results of MG-RAST and QIIME quality control

Community structure and diversity were analysed through metagenomic and metabarcoding methods. Shotgun sequencing produced 703,560,020 reads, comprising of 71,059,562,020 base pairs, with an average length of 101bp. These are deposited on the MG-RAST servers. Details of the related MG-RAST IDs, (which serve as accession numbers) can be found in *Table 7*.

Table 7 MG-RAST IDs of sequence data

Site	Drought IDs	Control IDs
Tolfa	Pool2R1:83291	Pool1R1:83288
	Pool2R2:83293	Pool1R2:83289
	Pool4R1:83297	Pool3R1:83294
	Pool4R2:83299	Pool3R2:83296
	Pool6R1:83303	Pool5R1:83300
	Pool6R2:83305	Pool5R2:83302
Beano	Pool8R1:84580	Pool7R1:84576
	Pool8R2:84581	Pool7R2:84577
	Pool10R1:84567	Pool9R1:84582
	Pool10R2:84569	Pool9R2:84583
	Pool12R1:84573	Pool11R1:84570
	Pool12R2:84574	Pool11R2:84571

The Tolfa dataset contained 344,022,068 shotgun reads. Reads failing quality control after filtering ranged from 14.1% – 15.6% for R1, and 31.0% – 33.7% for R2. Reads containing ribosomal genes varied between 0.2% and 0.4% for R1, and 0.1% and 0.3% for R2. Total reads passing QC which were identified as a known protein ranged from 22.3% – 31.8% in R1, whilst in R2 the range was 19.8% – 25.9%. Proportion of reads containing proteins of unknown function ranged from 33.9% – 41.8% for R1 and 29.5% – 36.4% for R2.

For Beano, shotgun sequencing produced 359,537,952 reads, of which between 11.5–12.0% of R1 reads failed QC. Of the remaining reads 0.1% coded for ribosomal genes, 22.6–32.8% coded for proteins with known function and 36.6–47.3% contained sequences coding for proteins of unknown functions. Reads from R2 showed a higher rate of QC failure, ranging between 21.7% and 23.3%. R2 reads successfully passing QC presented similar proportions to R1 for ribosomal genes (0.1%) and regions coding for proteins of known function (21.6%–31.7%). Regions coding for proteins of unknown function varied between 33.4% and 43.3%. Due to the reliance of MG-RAST on prokaryotic coding regions, it is not recommended for use with eukaryotic sequences. Therefore analysis was carried out on those sequences that could be subset as being derived from a prokaryotic source.

In total, the 16S run produced 2,861,221 combined reads, of which 1,271,733 were from the Beano dataset, and 1,589,488 were from Tolfa. Quality control and singleton removal steps in the QIIME pipeline reduced the total to 2,423,645 reads, comprising of 1,356,844 reads from Tolfa and 1,066,801 reads from Beano (a pass rate of approximately 85% and 84% respectively). A breakdown of the number of high quality reads per sample can be seen in *Table 8*.

Table 8 Total reads per sample after QIIME quality control

	Treatment Sample 1	**Treatment Sample 2**	**Treatment Sample 3**	**Control Sample 1**	**Control Sample 2**	**Control Sample 3**
Tolfa	238,540	227,020	213,014	174,126	309,536	194,608
Beano	210,533	209,978	154,813	224,451	97,261	169,765

2.3.2 Impacts of drought on microbial community structure, diversity and function

2.3.2.1 Drought impacts on taxonomic and community composition

Comparison between R1 and R2 taxonomic proportional abundances indicated that the results of the analysis were almost identical for the two datasets (R^2= 0.97–0.99). For this reason, all subsequent MG-RAST results will be described for R1 only. Samples collected from Tolfa were dominated by *Proteobacteria* (37–45%) and *Actinobacteria* (30–41%), with smaller proportions of *Acidobacteria* (5–10%). Other phyla detected at levels above 1% include *Firmicutes*, *Cyanobacteria* and *Bacteroidetes* (Figure 9). Between 3–5% percent of reads in each sample could not be assigned to a previously classified bacterial phylum.

Results of metabarcoding and QIIME analysis suggest communities were dominated at the phylum level by *Proteobacteria* (34.3% – 42.2%), *Actinobacteria* (25.9% – 30.1%) and *Acidobacteria* (21.9% – 33.0%). *Bacteroidetes* and *Verrucomicrobia* were also present at levels above 1% (1.9% – 3.6% and 0.8% – 1.1% respectively). Although the dominant phyla within the samples were similar, the proportions of reads attributed to the *Actinobacteria* were lower in the metabarcoding dataset. Furthermore, *Acidobacteria* 16SrRNA reads appeared to be increased in the metabarcoding dataset when compared with the metagenomic data (Figure 9).

Furthermore, several phyla were detected at levels above 1% in metagenomic data, which were not present within the metabarcoding dataset (*Cyanobacteria*, *Firmicutes* and Other, unidentified bacteria). Of these taxa, reads which were assigned to *Cyanobacteria* were primarily from the genus *Nostocaceae*, whilst *Firmicutes* were derived primarily from *Bacilli* classes (2.3–2.9%) with smaller proportions of *Clostridia* (0.4–0.7%). Differences in taxa are likely to arise due to different pipeline methods. For example, MG-RAST uses a best hit approach to assign taxonomy to any portion of a 16S region it detects, whilst QIIME uses an entire V3–V4 region. Similarly, MG-RAST makes use of the M5NR database (Wilke *et al.* 2013), combining multiple sources of information into a single

searchable database, whilst QIIME utilises a 16S specific database, GreenGenes (DeSantis *et al.* 2006).

α rarefaction of metagenomic taxonomic data showed no significant difference in diversity due to treatment for any metric, indicating the number of species present remained unchanged. This appears to confirm the hypothesis that species richness is unaffected by drought. Metagenome derived taxonomic rarefaction curves yield a steep slope for the Observed Species metric, as new species continue to be found at higher sampling depths (Figure 8). However, the Shannon index still plateaus at around 1000 reads, indicating the majority of diversity has been sampled by this point. α diversity results from the metabarcoding dataset also produced no significant difference by treatment. Due to the presence of the entire V3-V4 16S region, it was possible to produce a phylogenetic metric of diversity (PD). PD compared the phylogenetic diversity between samples, looking at the total length of branches within the phylogenetic tree from each sample. Trees with similar total branch lengths contain a similar range of diversity. Observed species, phylogenetic diversity (PD) and chao1 metrics indicated no significant difference between treatment and control (*Table 9*). Again, this confirmed the hypothesis that there is no difference in taxonomic richness between droughted and control plots.

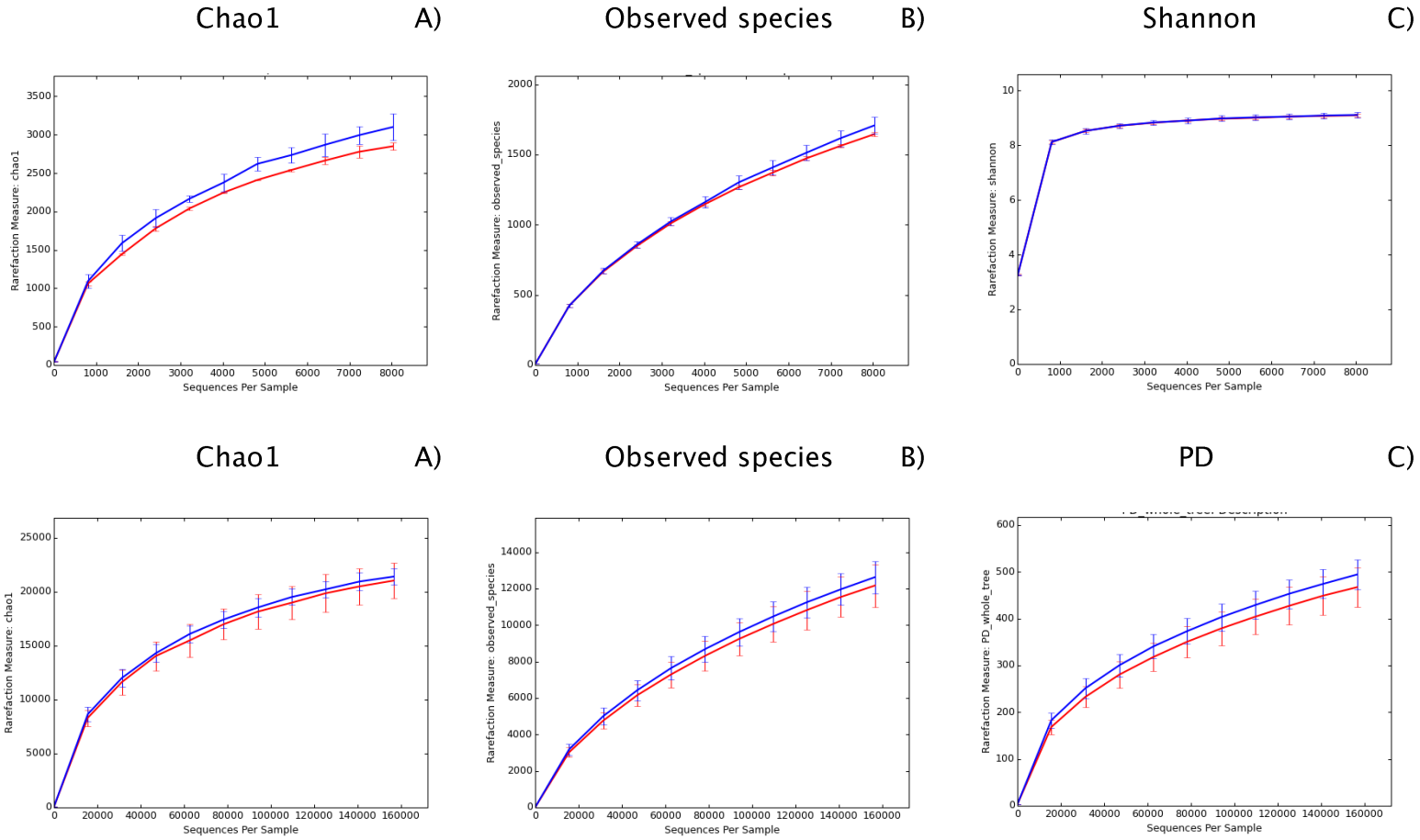


Figure 8 Rarefaction of metagenomic (top row) and metabarcoding (bottom row) taxonomic α diversity metrics at different sampling depths. A) Chao1 values; B) observed species values; C) Shannon (or for metabarcoding, Phylogenetic Diversity–PD) values. Red represents droughted samples, blue represents control samples. Error bars show the std dev of each rarefaction point.

Table 9 Results of t-tests with Monte-Carlo permutations for α diversity in Tolfa (drought) for taxonomic results from metabarcoding and metagenomic methods.

	Drought Mean	Control Mean	Drought StDev	Control StDev	t-stat	p-value
Metabarcoding	Obs= 12164.5	12624.0	1161.96	885.28	-0.45	0.503
	Chao1= 21019.9	21392.5	1665.28	725.59	-0.29	0.754
	PD= 467.1	494.2	41.92	31.13	-0.73	0.474
Metagenome	Obs= 1644.9	1707.3	15.9	60.9	-1.40	0.127
	Chao1= 2849.0	3099.7	50.6	174.9	-1.95	0.199
	Shannon=9.1	9.1	0.1	0.1	-0.17	0.877

Comparisons of metagenome derived β diversity by ADONIS testing of Bray-Curtis distances yielded no significant effect of treatment (R^2=0.21, p = 0.188). β diversity takes both the identity and proportion of taxa into consideration. Metabarcode derived β diversity results yielded no obvious clustering by treatment (Appendix A i). No effect of treatment on the identity of taxa and their abundances was detected, (weighted UNIFRAC ADONIS R^2 = 0.11, p = 1.00). Unweighted UNIFRAC yielded no significant difference (R^2 = 0.18, p = 0.89), signifying no difference in the taxa present within the communities.

Results of Benjamini-Hochberg corrected STAMP analysis of metagenomic taxonomic data indicated no significant difference in abundance for any taxa at the level of phylum, class, order, family or genus (q=0.05). Similarly, STAMP analysis of metabarcoding data using the same methods yielded no significant differences (q=0.05). Therefore, as no effect of drought treatment was detected, there were no significant changes in the identities of taxa present, or their abundances.

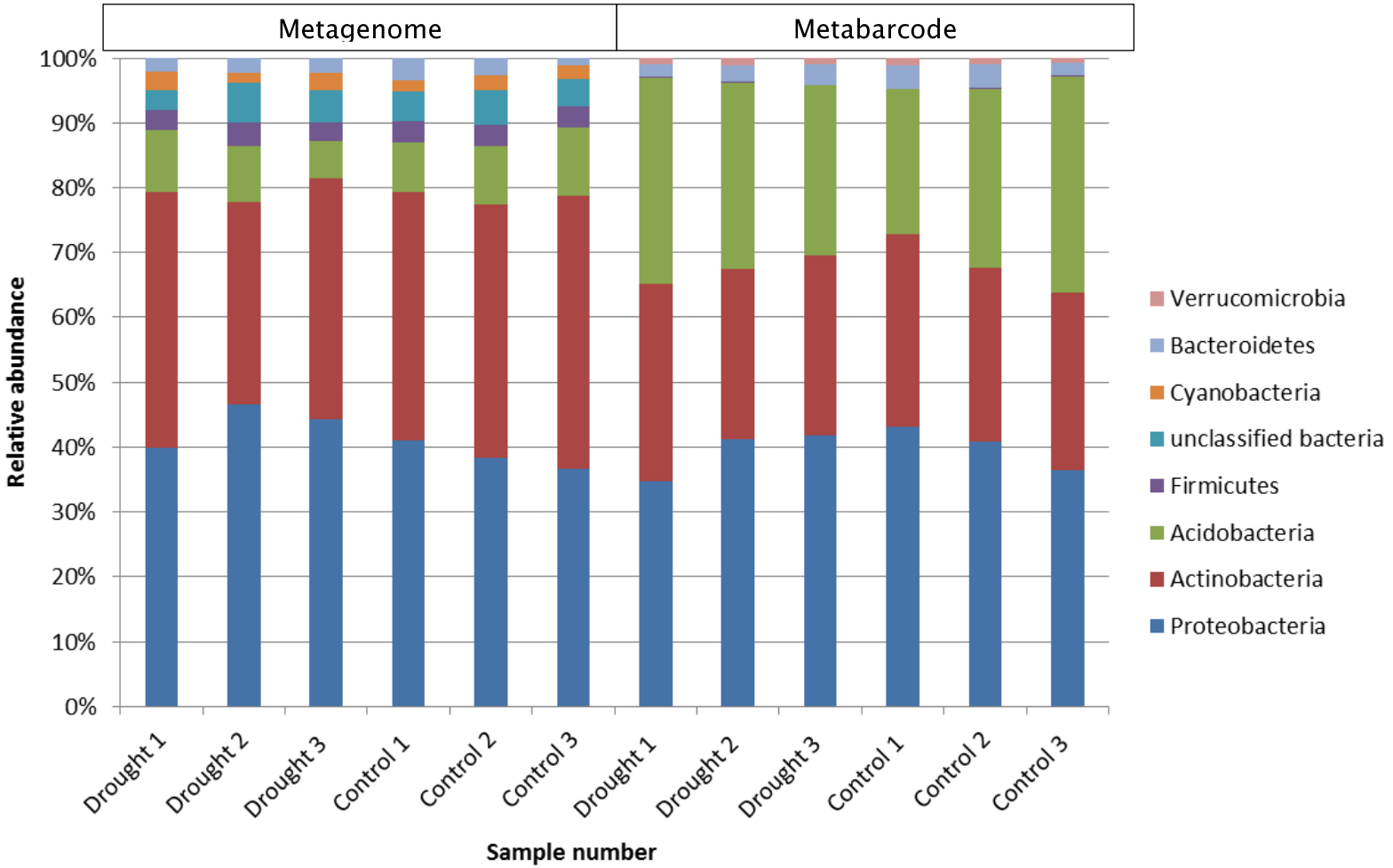


Figure 9 Relative abundance of taxa contributing >1% in Metagenomic or Metabarcoding datasets at the Tolfa site.

Table 10 Breakdown of proportions of SEED functional groups present in drought and control plots for both R1 and R2 reads.

SEED functional groups	Drought Sample 1 R1 %	Drought Sample 1 R2 %	Drought Sample 2 R1 %	Drought Sample 2 R2 %	Drought Sample 3 R1 %	Drought Sample 3 R2%	Control Sample 1 R1 %	Control Sample 1 R2 %	Control Sample 2 R1 %	Control Sample 2 R2 %	Control Sample 3 R1 %	Control Sample 3 R2 %
Clustering-based subsystems	9.08	9.08	9.03	9.03	9.04	9.03	9.10	9.11	9.05	9.03	9.00	8.99
Miscellaneous	8.86	8.86	8.90	8.89	8.87	8.87	8.87	8.85	8.87	8.87	8.86	8.88
Protein Metabolism	8.64	8.64	8.57	8.54	8.58	8.55	8.59	8.56	8.64	8.62	8.67	8.64
Carbohydrates	6.94	6.93	6.85	6.87	6.80	6.82	6.78	6.78	6.85	6.86	6.93	6.91
RNA Metabolism	6.68	6.67	6.67	6.67	6.67	6.68	6.71	6.70	6.70	6.70	6.68	6.66
Respiration	5.67	5.67	5.70	5.71	5.65	5.66	5.65	5.64	5.64	5.63	5.66	5.66
Membrane Transport	5.20	5.21	5.33	5.33	5.33	5.34	5.36	5.38	5.30	5.29	5.26	5.27
Amino Acids and Derivatives	5.35	5.36	5.34	5.33	5.38	5.39	5.36	5.35	5.39	5.41	5.38	5.37
Nucleosides and Nucleotides	4.10	4.09	4.11	4.11	4.07	4.05	4.08	4.07	4.08	4.08	4.08	4.06
Cofactors, Vitamins, Prosthetic Groups, Pigments	4.10	4.11	4.10	4.10	4.11	4.13	4.08	4.08	4.08	4.09	4.14	4.13
Stress Response	4.02	4.01	4.05	4.04	4.05	4.03	4.07	4.06	4.04	4.05	4.01	4.01
Virulence, Disease and Defence	3.95	3.94	3.97	3.97	3.92	3.91	3.88	3.90	3.92	3.92	3.95	3.96
Regulation and Cell signalling	3.65	3.66	3.66	3.67	3.67	3.68	3.70	3.70	3.67	3.68	3.66	3.67
Phages, Prophages, Transposable elements, Plasmids	3.56	3.56	3.47	3.47	3.48	3.48	3.52	3.52	3.51	3.51	3.48	3.49
Metabolism of Aromatic Compounds	3.26	3.26	3.30	3.29	3.30	3.30	3.27	3.27	3.28	3.27	3.25	3.25

Nitrogen Metabolism	2.35	2.36	2.34	2.34	2.35	2.35	2.38	2.39	2.34	2.34	2.32	2.33
DNA Metabolism	2.28	2.28	2.23	2.23	2.26	2.26	2.27	2.28	2.27	2.28	2.26	2.27
Cell Wall and Capsule	2.17	2.18	2.18	2.19	2.20	2.21	2.19	2.20	2.18	2.20	2.21	2.22
Fatty Acids, Lipids, and Isoprenoids	2.08	2.08	2.07	2.07	2.12	2.13	2.08	2.07	2.09	2.10	2.10	2.11
Sulphur Metabolism	1.51	1.52	1.52	1.51	1.53	1.53	1.53	1.53	1.51	1.52	1.51	1.51
Motility and Chemotaxis	1.42	1.41	1.45	1.44	1.46	1.46	1.45	1.45	1.46	1.45	1.44	1.44
Phosphorus Metabolism	1.38	1.37	1.41	1.42	1.35	1.35	1.33	1.34	1.35	1.35	1.38	1.39
Secondary Metabolism	1.04	1.04	1.03	1.03	1.05	1.04	1.05	1.05	1.04	1.03	1.03	1.04
Potassium metabolism	0.93	0.93	0.96	0.96	0.95	0.96	0.95	0.96	0.93	0.93	0.94	0.94
Cell Division and Cell Cycle	0.77	0.76	0.75	0.75	0.76	0.76	0.76	0.75	0.77	0.76	0.76	0.75
Photosynthesis	0.54	0.54	0.53	0.53	0.53	0.53	0.51	0.51	0.52	0.53	0.54	0.55
Iron acquisition and metabolism	0.35	0.35	0.38	0.38	0.39	0.38	0.37	0.38	0.39	0.39	0.36	0.37
Dormancy and Sporulation	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.13	0.12	0.12

2.3.2.2 Functional effects of drought

SEED classifications from R1 and R2 resulted in a perfect correlation of proportional abundances (R^2=1.0). Classifications were dominated by Clustering-based subsystems (9.0%), Protein metabolism (8.6%) and Miscellaneous (8.9%). Clustering-based subsystems consist of genes detected in association with other genes, across multiple taxa, implying a common role in a specific (although unknown) pathway. Precise breakdowns of these datasets can be seen in *Table 10*. Due to the similarity of results from R1 and R2, all subsequent results will discuss only R1 data.

KO functional α diversity was unaffected by drought, with no change in the number of different functions present. Graphs of the effect of rarefaction on all three metrics show a gradual levelling of the slope, indicating most of the functional diversity present within the sample was successfully captured. However, the fact the slope fails to plateau suggests that some rarer functions may remain unsampled (Appendix A ii). All results are shown in below (*Table 11*).

Table 11 Results from R1 functional α *diversity tests. μ = sample mean,* σ *= standard deviation.*

Sample	Chao1	OBS	Shannon	Rarefied to:
Metagenome α diversity	Drought: μ= 542.1, σ=27.4 Control: μ= 577.7, σ=32.2 P = 0.384	Drought: μ= 454.6, σ=11.1 Control: μ= 469.7, σ=8.3 P = 0.233	Drought: μ= 7.6, σ=0.1 Control: μ= 7.2, σ=0.5 P = 0.094	5576

PCoA of Bray-Curtis distances showed no effect of treatment confirmed by ADONIS testing. No significant difference in abundance or diversity of functional groups was present between treatments (R^2=0.16, p = 1.0) (Figure 10). To assess changes in functional groups in more detail, differential abundance of KO (KEGG ortholog) functions was undertaken through STAMP. No significant difference in differential abundance of any KO functions was detected after correction and filtering to a q-value of 0.05. Therefore there was

no significant difference between total number of functions, the functional groups themselves, or their abundances between drought or control plots, consistent with the lack of changes in taxonomic proportional abundance.

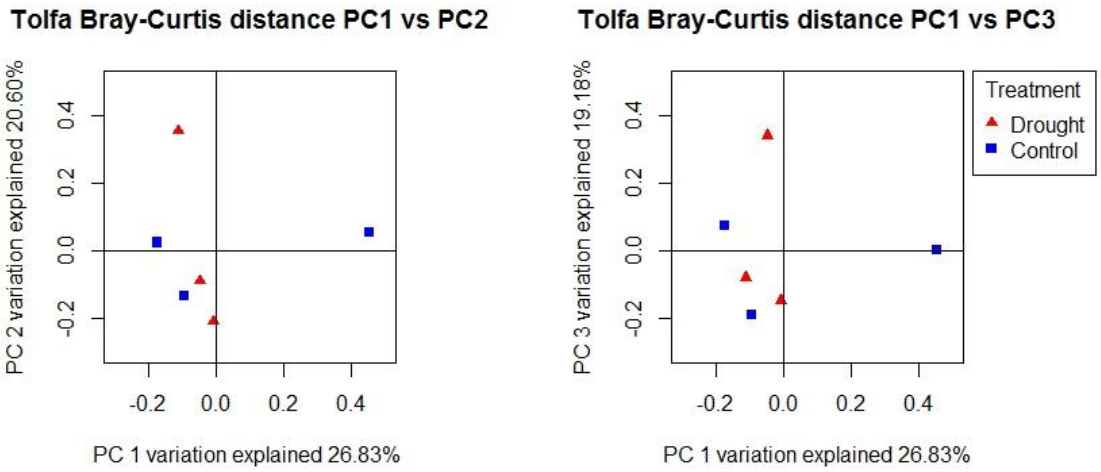


Figure 10 PCoA of Bray-Curtis for functional community for Tolfa.

2.3.3 Impacts of biochar application on microbial community structure, diversity and function

2.3.3.1 Biochar impacts on taxonomic and community composition

Correlation of R1 and R2 results was undertaken (Section 2.3.2), indicating taxonomic and metagenomic proportional abundances were almost identical for the two datasets (R^2=0.99). Therefore, all subsequent analysis will focus on R1 reads. Taxonomy of metagenomic samples from Beano were dominated by *Proteobacteria* (50-54%), with *Actinobacteria* (11.0-13.4%), *Bacteroidetes* (4.9-7.6%), *Firmicutes* (6.3-8.0%) and *Cyanobacteria* (5.0-7.6%). Unclassified bacterial reads comprise 5-18% of samples (Figure 12).

Results of metabarcoding indicated that communities were dominated by *Proteobacteria* (47.7-54.8%), *Actinobacteria* (15.6-23.1%), *Gemmatimonadetes* (9.7-12.8%), *Bacteroidetes* (5.9-8.9%) and *Acidobacteria* (4.1-5.3%). However, *Nitrospira* and were also detected in levels >1% whilst *Cyanobacteria* and unclassified bacteria were not detected. Similar to the results from Tolfa, the methods produce different taxa and proportional abundances, whilst agreeing on the dominant phyla present.

α rarefaction of metagenomic and metabarcoding of taxonomic diversity yielded no significant difference between treatments for any of the three metrics used (observed species, Shannon diversity and chao1). The observed species metric failed to plateau, indicative of the requirement for further sequencing depth is required to fully capture all diversity present, although the Shannon metric plateaus at approximately 1250 reads, suggesting that the majority of informative diversity is sampled at that depth (Figure 11).Therefore, there is no difference in species richness due to biochar treatment, the total number of taxa which are present remain approximately the same.

Table 12 Results of t-tests with Monte-Carlo permutations for α diversity in Beano (Biochar) for taxonomic results from metabarcoding and metagenomic methods.

	Biochar Mean	Control Mean	Biochar StDev	Control StDev	t-stat	p-value
Metabarcoding	Obs= 9462.40	9399.30	71.47	89.34	-0.78	0.407
	Chao1= 16975.00	16163.80	116.11	866.57	-1.31	0.166
	PD= 424.60	422.66	4.00	8.02	-0.31	0.826
Metagenome	Obs= 1217.20	1186.70	49.50	61.30	-0.55	0.592
	Chao1= 2695.80	2596.60	300.30	195.10	-0.39	0.756
	Shannon=9.20	9.20	0.10	0.10	0.08	0.973

PCoA of metagenomic taxonomic results showed no clustering confirmed by ADONIS analysis (R^2=0.195, p = 0.68). This suggests that the abundance and identities of taxa present in biochar treated samples are no different to those present in the controls. PCoA of metabarcoding also showed no obvious clustering (Appendix A iii). No significant effect was detected in weighted UNIFRAC (R^2 = 0.09, p = 0.92) or unweighted UNIFRAC (R^2 = 0.19, p = 0.91). There is therefore no significant shift in the identities of taxa present, nor their abundance in response to biochar treatment.

STAMP analysis of metagenomic taxonomic data showed no significant difference in any taxa by treatment, at any taxonomic level (q=0.05). This was also the case for metabarcoding data (q=0.05). Therefore, there was no difference detected in the abundance of reads for any taxonomic group due to treatment. Shifts in proportional abundance detected by previous studies (in Section 2.1.3) were not detected.

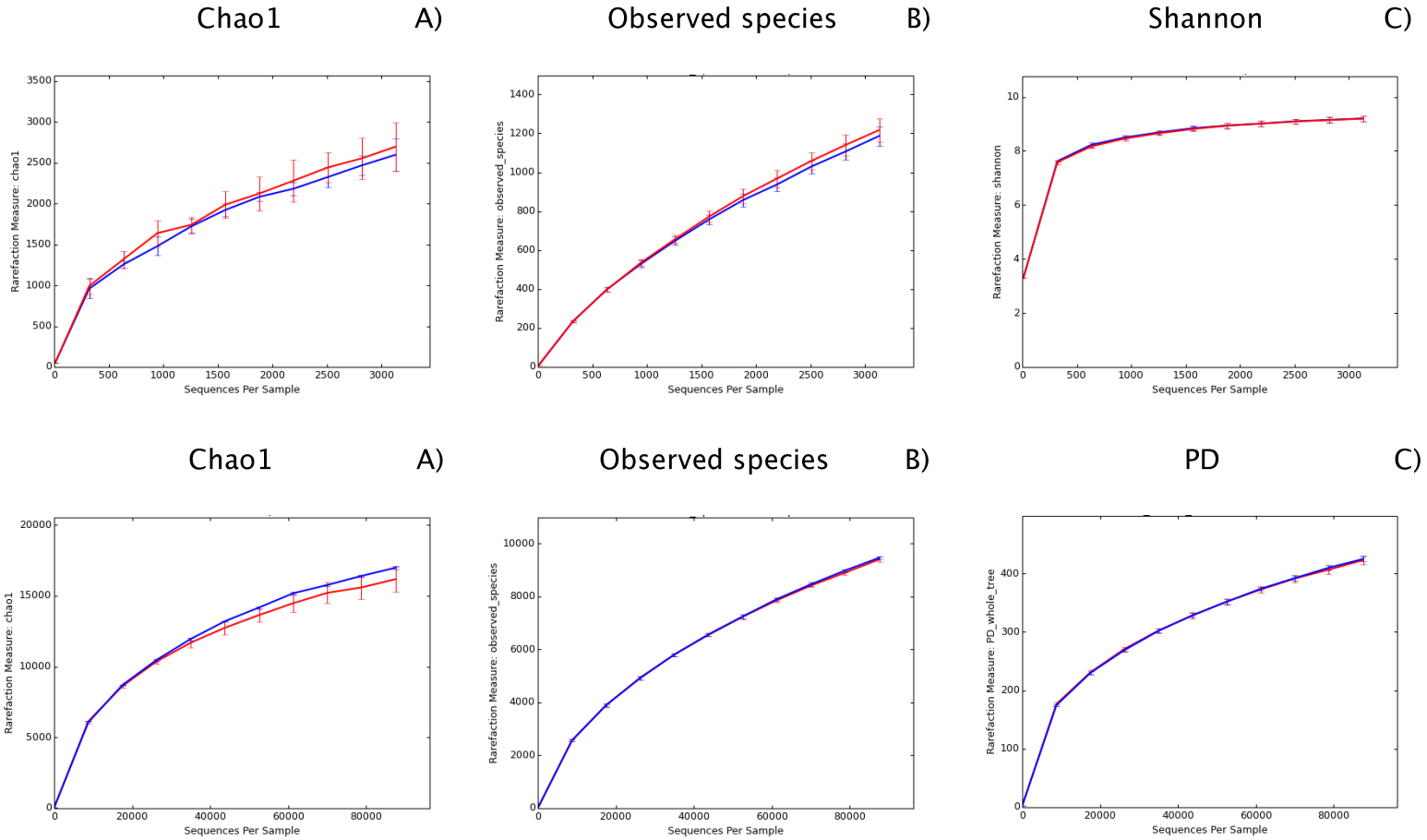


Figure 11 Rarefaction of metagenomic (top row) and metabarcoding (bottom row) taxonomic α diversity metrics at different sampling depths. A) Chao1 values; B) observed species values; C) Shannon (or for metabarcoding, PD) values. Red represents biochar samples, blue represents control samples. Error bars show the std dev of each rarefaction point.

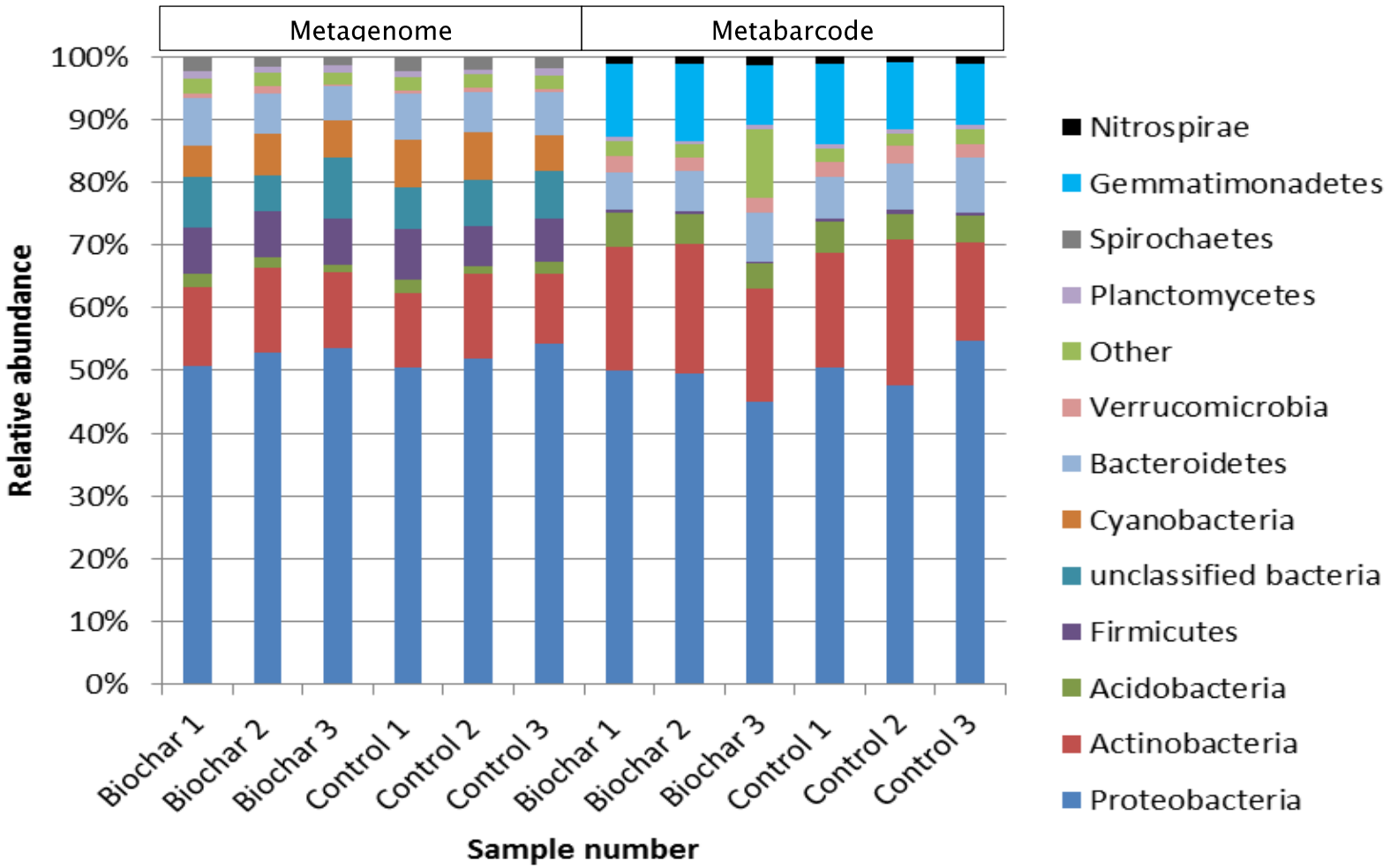


Figure 12 Relative abundance of taxa contributing >1% in Metagenomic or Metabarcoding datasets at the Beano site.

Table 13 Breakdown of proportions of SEED functional groups present in biochar and control plots for both R1 and R2 reads.

	Biochar sample 1 R1 %	Biochar sample 1 R2 %	Biochar sample 2 R1 %	Biochar sample 2 R2 %	Biochar sample 3 R1 %	Biochar sample 3 R2 %	Control sample 1 R1 %	Control sample 1 R2 %	Control sample 2 R1 %	Control sample 2 R2 %	Control sample 3 R1 %	Control sample 3 R2 %
Clustering-based subsystems	9.36	9.37	9.35	9.34	9.34	9.35	9.36	9.36	9.34	9.34	9.33	9.34
Protein Metabolism	9.00	9.00	9.01	9.00	8.97	8.94	8.97	8.96	8.85	8.85	9.05	9.00
Miscellaneous	8.86	8.85	8.88	8.88	8.87	8.88	8.87	8.89	8.91	8.91	8.84	8.85
RNA Metabolism	6.95	6.97	6.94	6.94	6.93	6.93	6.93	6.92	6.87	6.87	6.97	6.94
Carbohydrates	6.53	6.55	6.55	6.54	6.59	6.58	6.56	6.57	6.55	6.55	6.53	6.51
Membrane Transport	5.48	5.48	5.45	5.47	5.48	5.48	5.45	5.45	5.49	5.49	5.45	5.46
Amino Acids and Derivatives	5.45	5.44	5.46	5.46	5.51	5.46	5.42	5.42	5.47	5.47	5.48	5.45
Respiration	5.34	5.34	5.34	5.32	5.34	5.34	5.33	5.33	5.35	5.35	5.32	5.31
Stress Response	4.08	4.08	4.07	4.08	4.06	4.07	4.08	4.07	4.06	4.06	4.08	4.09
Nucleosides and Nucleotides	4.07	4.07	4.07	4.07	4.08	4.07	4.08	4.07	4.08	4.08	4.08	4.07
Cofactors, Vitamins, Prosthetic Groups, Pigments	4.04	4.04	4.05	4.05	4.06	4.06	4.05	4.05	4.07	4.07	4.04	4.04
Virulence, Disease and Defense	3.79	3.77	3.77	3.78	3.76	3.77	3.76	3.75	3.77	3.77	3.77	3.79
Regulation and Cell signalling	3.74	3.74	3.75	3.75	3.74	3.74	3.78	3.79	3.76	3.76	3.75	3.76
Phages, Prophages, Transposable elements, Plasmids	3.65	3.64	3.65	3.65	3.61	3.63	3.68	3.67	3.61	3.61	3.61	3.64
Metabolism of Aromatic Compounds	3.13	3.14	3.15	3.16	3.17	3.16	3.12	3.13	3.20	3.20	3.15	3.16
Nitrogen Metabolism	2.54	2.55	2.53	2.53	2.54	2.54	2.54	2.54	2.56	2.56	2.53	2.53
DNA Metabolism	2.33	2.33	2.34	2.34	2.32	2.32	2.35	2.36	2.32	2.32	2.34	2.34

Cell Wall and Capsule	2.20	2.20	2.19	2.20	2.18	2.19	2.20	2.20	2.19	2.19	2.19	2.21
Fatty Acids, Lipids, and Isoprenoids	1.90	1.89	1.89	1.89	1.90	1.90	1.88	1.89	1.92	1.92	1.89	1.88
Sulphur Metabolism	1.48	1.48	1.48	1.47	1.48	1.48	1.49	1.48	1.49	1.49	1.48	1.48
Motility and Chemotaxis	1.31	1.32	1.33	1.32	1.31	1.33	1.34	1.33	1.34	1.34	1.34	1.35
Phosphorus Metabolism	1.12	1.12	1.11	1.11	1.11	1.11	1.10	1.10	1.12	1.12	1.09	1.09
Secondary Metabolism	1.02	1.02	1.03	1.02	1.02	1.03	1.02	1.03	1.03	1.03	1.03	1.03
Potassium metabolism	0.85	0.84	0.84	0.84	0.84	0.85	0.85	0.85	0.86	0.86	0.84	0.84
Cell Division and Cell Cycle	0.83	0.83	0.82	0.82	0.81	0.82	0.82	0.82	0.80	0.80	0.83	0.82
Photosynthesis	0.42	0.42	0.42	0.42	0.42	0.43	0.43	0.43	0.43	0.43	0.41	0.42
Iron acquisition and metabolism	0.41	0.41	0.41	0.42	0.41	0.42	0.42	0.42	0.44	0.44	0.43	0.44
Dormancy and Sporulation	0.13	0.13	0.13	0.13	0.13	0.12	0.13	0.13	0.12	0.12	0.13	0.13

2.3.3.2 Functional effects of biochar

Correlation (described in Section 2.3.2.2) was used to determine similarity of R1 and R2 for SEED functional profiles. The R^2 value for these correlations was 1, and so all subsequent analysis is based upon R1 reads only. SEED functional profiles were primarily associated with Clustering-based subsystems (9.0-9.1%), Miscellaneous (8.6-8.9%) and Protein metabolism (8.5-8.6%) (*Table 13*).

α diversity of functional groups showed no significant difference, suggesting the number of different functions present within biochar and control samples were similar (*Table 14*). Rarefaction of observed species (i.e. functional categories) demonstrated a plateau effect occurs at around 1500 reads, indicating that the majority of functional diversity is captured at this depth. Shannon diversity on the other hand, shows that most information has been captured at a depth of 500 reads (Appendix A iv).

Table 14 Results from R1 functional α diversity tests. μ = sample mean, σ= standard deviation.

Sample	Chao1	OBS	Shannon	Rarefied to:
Metagenome α diversity	Biochar:μ= 261.1, σ=20.0 Control: μ= 238.2, σ=4.0 P = 0.29	Biochar:μ= 224.1, σ=8.9 Control: μ= 204.6, σ=3.7 P = 0.09	Biochar:μ= 6.9, σ=0.0 Control: μ= 6.6, σ=0.3 P = 0.101	2100

No clustering effect of samples by treatment was noted for functional datasets (Figure 13). ADONIS testing yielded no significant difference between functional KEGG groups by treatment (R^2=0.21, p = 0.41), signifying no impact of treatment on diversity and abundance of KEGG functions present within the dataset. STAMP analysis of differential abundance confirmed that no differences occurred by treatment, with no significant difference at the q=0.05 level.

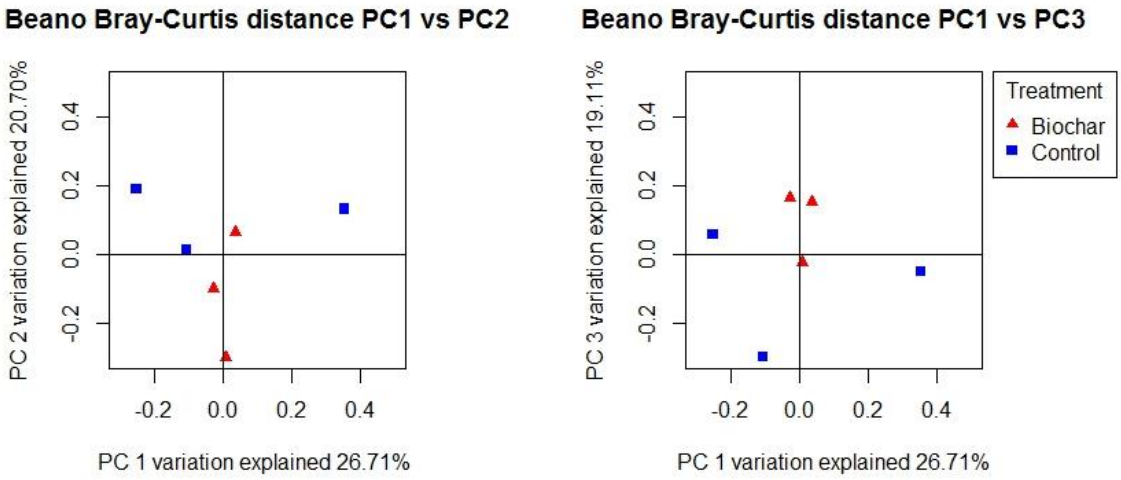


Figure 13 PCoA of Bray Curtis functional community for Beano.

2.4 Discussion

2.4.1 Does drought affect community structure and function?

The impact of long-term drought at the Tolfa site in a Mediterranean scrub forest on the soil microbiome was limited with no differences in taxonomic diversity between control and drought plots, in line with previous short-term (1-2 years) studies (Sheik *et al.* 2011; Cregger *et al.* 2012). The drought treatment in Tolfa was established 5 years prior to soil sampling, and as such we hoped to determine whether long term drought exposure would reduce diversity. No change in the proportional abundance was noted using either metagenomic or metabarcoding methods. Interestingly, whilst there was general agreement regarding the dominant phyla present, there was disagreement between methods regarding the proportional contributions of taxa. This is possibly a result of amplification bias on the part of metabarcoding data or due to differences in total sequencing depth, increasing the proportion of rare reads detected. Alternatively, bias may arise during pipeline processing, as each method is reliant upon different databases for OTUs identification. A similar effect was previously noted

in a study comparing targeted and shotgun methods in dryland soil communities in which the authors suggest the limited accuracy of annotations in shotgun databases may be the cause (Steven *et al.* 2012).

α diversity indicated no effect of drought on the richness of taxa present. The reduction of soil moisture is unlikely to select with sufficient stringency for the complete removal of specific taxa (Cregger *et al.* 2012), so a reduction was not expected. β diversity suggests that no change in the identities of genera nor their total abundance occurred in response to drought. Decreased water availability had been expected to change abundances of taxa within the samples, as oligotrophic bacteria which were better adapted to a low moisture environment began to dominate. However, this did not occur, as reflected in the β diversity results. Functional data also presented no shift in biogeochemical cycles. This is contrary to previous declines in alkaline phosphomonoesterase, urease and beta-glucosidase detected in similar Mediterranean studies (Hueso *et al.* 2012). Provided the lack of changes in taxonomic abundance, this is unsurprising, as in order for variation in functional groups to occur, abundance of genera with those features would need to vary. Thus a lack of drought induced taxonomic change may directly influence the detection of functional diversity within a sample.

Whilst both metagenomic and metabarcoding methods indicated no difference in community diversity, and metagenomic methods show no change in functional groups present, it is not possible to confirm whether the lack of an effect was due to the absence of a biological effect, or due to the limited number of replicates. Low resolution methods have previously detected shifts in droughted deserts, grasslands and Mediterranean communities, and as such it is unlikely that no effect took place (Clark *et al.* 2009; Yuste *et al.* 2011; Sheik *et al.* 2011). Therefore difficulties in detecting shifts in taxa may be a result of the limited replication and statistical power within the study. Alternatively, it may be that whilst the metagenomic profile remained unchanged, there were shifts in the regulation of genes expression. Metatranscriptomic shifts would result in increased or decreased levels of expression from genes within the metagenomic profile, potentially leading to substantial variation in soil function. However, metagenomic methods would not be able to detect such variation.

2.4.2 Does biochar application affect community structure and function?

No significant difference was detected in the taxa present in Beano. This is in line with one previous study (Rutigliano *et al.* 2014), although biochar has been previously demonstrated to cause shifts in microbial communities, and that this can be detected using lower resolution methods such as PLFA (Jindo *et al.* 2012; Anders *et al.* 2013; Gomez *et al.* 2014), TRFLP and DGGE (Chen *et al.* 2013; Ding *et al.* 2013). Changes in taxa have previously been detected in biochar treatments, resulting in elevated abundance of *Bradyrhizobiaceae*, *Hyphomicrobiaceae* and *Streptosporangineae*. A decline in *Streptomycetaceae* and *Micromonosporaceae* was also detected in the same study (Anderson *et al.* 2011). Similarly, *terra preta* soils contain proportions of several *Proteobacteria* classes in levels greater than those associated with surrounding soils (Kim *et al.* 2007; Grossman *et al.* 2010). No such difference was detected in this study, at any taxonomic level using STAMP analysis. No changes in total α diversity were detected, indicating that species richness was unaffected by treatment. β diversity also showed no effect of treatment, which suggests that not only did the number of taxa remain unchanged, but the identities of the taxa and their abundance remained unaffected by biochar treatment.

This may be a result of limited replication within the study. Although 5 samples were collected per plot, in this preliminary analysis, the DNA was pooled after extraction, making it impossible to differentiate any variation within each sample, reducing the power available for statistical testing. This was carried out in order to increase the number of reads produced per sample after sequencing. However, it has subsequently been discovered that additional sequence data is better utilised in sequencing additional samples rather than increasing sequencing depth (Shokralla *et al.* 2012; Caporaso *et al.* 2012).

Alternatively, it is possible that the single time-point for the biochar samples missed a short-term shift in taxonomic abundance. Of the studies listed above, most are short-term (Anders *et al.* 2013; Ding *et al.* 2013; Gomez *et al.* 2014), studying the impact of biochar in the weeks and months after its application. The

application at the Beano site occurred four years prior to the sampling effort. It is therefore possible that short-term shifts from *k* to *r* strategists may have already occurred, as labile portions of carbon became available (Lehmann *et al.* 2011). These effects may have subsided prior to the collection of the sample. However, this should be asserted with caution, due to the difficulty in determining whether the low sample size prevented detection of taxonomic variation between treatments. It is equally possible that biochar application causes no response of community, although this seems unlikely given the ability of methods with coarser resolution to detect a difference.

Functional diversity in the samples from Beano also showed no significant difference due to treatment. Again, this is probably a result of low sample size and a lack of power, although this cannot be confirmed. The previous detection of elevated abundance of genes related to denitrification were not detected (Xu *et al.* 2014a), nor were genes related to phosphate processing and accumulation (Fox *et al.* 2014) or carbohydrate degradation (Jones *et al.* 2011c). Biochar may have had a short-term impact on these pathways, but this would have been reflected in the metagenomic profile in the short-term after biochar application.

At the time that sequence data was produced (Winter of 2011), it was common practise to use 454 sequencing for metagenomic datasets (Mackelprang *et al.* 2011; Mason *et al.* 2012). Alternatively, Illumina paired end reads were used. These would be utilised after gel size selection of sequences approximately 150bp in length, with the aim of sequencing each read from the 5' and 3' ends, allowing for longer reads for analysis. However, in the sequence data inherited for this project, paired end reads had been used with data from a 400-500bp region. Whilst it is expected that each of the two reads would be from within the same protein coding region, this could not be guaranteed, and it was for this reason that R1 and R2 reads were processed separately. Preliminary comparison of the R1 and R2 sequence data indicated that whilst there were occasional deviations in proportional taxonomic and functional abundance detected between reads, there was a high level of correlation (see Section 2.3.2).

2.4.3 Conclusions

Through analysis of the data from this pilot study, it was determined that an increased minimum number of samples were required for future analysis in order to detect changes in community structure. Comparison of the taxonomic results from the QIIME pipeline with the MG-RAST pipeline indicated differences in the rate of detection of several phylogenetic groups. MG-RAST detected higher proportions of *Bacteroidetes* within the Tolfa dataset (1-3%) whilst it was detected only in levels below 1% through QIIME analysis. In order to successfully assess any changes in bacterial diversity or variation in functional gene abundance, a greater number of samples should have been collected, both for metabarcoding and metagenomics. Additionally data from MG-RAST detected *Acidobacteria* in proportions half of that found in the QIIME analysis. This may be a result of an amplification bias in the 16S primers used in the preparation of the QIIME dataset, although this is unlikely, as they were designed with primer bias in mind (Caporaso *et al.* 2012). Alternatively, the differing databases used in the metagenomic and metabarcoding pipeline may have introduced bias. Regardless of the method used, no difference in community or functional diversity was detected in either treatment type (drought or biochar). It was for this reason that it was opted to increase the minimum sample number in future studies, processing samples individually.

It is recommended that future studies increase sampling size. This could be done with relative ease, by keeping individual samples separate rather than pooling them. Additionally, metagenomics studies should use size selection protocols to produce reads of an appropriate size (this will vary by sequencing method). This would allow for each pair of reads to be overlapped prior to analysis, increasing the length of sequence data available for pipeline analysis. Future metabarcoding studies should also increase sample numbers, as three pooled samples proved insufficient to detect changes in microbial communities

Chapter 3: Shifts in soil bacterial communities in 3 European sites in response to biochar amendment

3.1 Introduction

Biochar (pyrolysed biomass) has been discussed as both a soil conditioner and a method for carbon sequestration (Lehmann *et al.* 2006; Major *et al.* 2010; Mao *et al.* 2012) (Chapter 1, Section 1.3). The interest arises from studies of *terra preta* soils, ancient anthrosols produced in South America by indigenous populations (Glaser *et al.* 2001; Lima *et al.* 2002; Kim *et al.* 2007; Grossman *et al.* 2010; Glaser & Birk 2012). Whilst the stability of biochar within the soil has been thoroughly studied (Vaccari *et al.* 2011; Singh *et al.* 2012; Gurwick *et al.* 2013; Bamminger *et al.* 2014a; Fang *et al.* 2014), there are few in-depth studies which identify the potential changes in the structure microbial communities. Previous experiments assessing these communities have been based on incubation studies assessing microbial biomass, respiration or activity (*Table 15*), or use low resolution methods. Whilst essential for assessing general trends in community changes, such methods cannot accurately detect changes in abundance or shifts in microbial ecology (*Table 16*).

Application of biochar to soil can increase plant growth (Baronti *et al.* 2010; Vaccari *et al.* 2011; Jones *et al.* 2012; Viger *et al.* 2015), with abiotic factors including increased pH, cation exchange capacity (CEC) and improved soil water content all proposed to have a role (Verheijen *et al.* 2010; Jeffery *et al.* 2011; Jones *et al.* 2012). Soil physicochemical changes induced by biochar may also play a pivotal role in determining soil bacterial biodiversity; pH has previously been suggested as the strongest variable influencing the biogeographical distribution of bacterial taxa (Fierer & Jackson 2006). Shifts in microbial communities may result from a wide range of biochar-mediated interactions, including variations in microbial signalling (through sorbtion of signalling molecules to biochar particles Masiello et al. 2013), increased transfer of electrons, resulting in augmentation of biological processes (Cayuela *et al.* 2013), shifts in microbial nitrogen cycling (Harter *et al.* 2014) and decreased abundance of fungi relative to bacteria (Gomez et al. 2013). For further discussion of the implications of these abiotic effects, refer to Chapter 1: Section 1.3.3.

Increased fertility associated with biochar amendment may be linked to these changes in the soil environment, and resulting variation in the microbiome. For example, addition of biochar has been found to increase populations of bacteria and archaea that oxidise ammonia to nitrates and nitrites (Prommer *et al.* 2014), augment *Bradyrhizobiaceae* and *Hyphomicrobiaceae* populations (Anderson *et al.* 2011) and increase *amoA, amoB, nifH, nirS, nirK* and *nosZ* gene abundance (Ducey *et al.* 2013). Pot experiments studying S and P mobilizing bacteria in *Lolium perenne* indicated increased abundance of *Rhizobacteria* associated with the mineralization of S and P soils limited in these nutrients (Fox *et al.* 2014). This illustrates the potential for shifts in taxa (and subsequent functional roles associated with those taxa), to affect nutrient cycling. However, previous studies have been undertaken over short time scales and in microcosm experiments, thus their relevance to mid to long-term field impacts in unknown.

One further limitation to existing research is the use of technologies such as Phospholipid Fatty Acids analysis (PLFA e.g. Ameloot et al. 2014) and Terminal Restriction fragment length polymorphism coupled with denaturing gradient gel electrophoresis (T-RFLP and DGGE respectively e.g. Dempster et al. 2011; Watzinger et al. 2014). Whilst useful for assessing general trends in changes in community structure, these techniques lack both the throughput and resolution to detect specific changes in proportional abundance in microbial taxa (Anderson & Cairney 2004; Frostegård *et al.* 2011; Neilson *et al.* 2013). Advances in high-throughput DNA sequencing have provided an opportunity to assess shifts in community abundance through use of gene amplicon surveys. In the past this methodology has been used to assess changes in the human gut microbiome (Shah *et al.* 2011), shifts in marine microbial communities after the Deepwater Horizon oil spill (Mason *et al.* 2012), the response of microbial communities to plant community succession (Blaalid *et al.* 2012), the interplay between plant genotype, rhizosphere and soil properties in *Arabidopsis* (Hirsch & Mauchline 2012) and nitrogen gradients (Fierer *et al.* 2012a) (see Chapter 1: Section 1.2).

Use of the 16S rRNA subunit gene and the ribosomal internal transcribed spacer region (ITS), enables surveys of the relative abundances of bacteria and fungi within a sample, with the possibility of linking changes to functional attributes of

the soil (further detailed discussion of these methods can be found in Chapter 1: Section 1.6.2).

Observed effects of biochar on edaphic microbial processes are often conflicting. Studies have observed soil respiration to be; increased (Kolb *et al.* 2009; Zavalloni *et al.* 2011; Belyaeva & Haynes 2011; Castaldi *et al.* 2011; Quilliam *et al.* 2012), decreased (Dempster *et al.* 2011; Paz-Ferreiro *et al.* 2011; Carlsson *et al.* 2012) or unchanged (Galvez *et al.* 2012; Bamminger *et al.* 2014a). Microbial biomass measurements have displayed a similar range of responses to biochar application, including increases (Kolb *et al.* 2009; Belyaeva & Haynes 2011; Paz-Ferreiro *et al.* 2011), decreases (Dempster *et al.* 2011) and no change (Castaldi *et al.* 2011; Galvez *et al.* 2012; Bamminger *et al.* 2014a).

Effects of biochar treatment have also been noted on microbial community structure, with decreases in *Betaproteobacteria*, *Bacteroidetes*, *Firmicutes*, *Proteobacteria* and *Planctomycetes* noted (Kolton *et al.* 2011; Ding *et al.* 2013; Hu *et al.* 2014) as have (sometimes contradictory) increases in *Bradyrhizobiaceae*, *Hyphomicrobiaceae*, *Actinomycetes*, *Chloroflexi*, *Nitrospiraceae*, *Proteobacteria*, *Trichoderma*, *Pseudomonas*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Gemmatimonadetes* (Graber *et al.* 2010; Khodadad *et al.* 2011; Kolton *et al.* 2011; Anderson *et al.* 2011; Chen *et al.* 2013, 2014; Ding *et al.* 2013; Hu *et al.* 2014).

Very few studies have determined the impact of biochar on fungal abundance and diversity but these communities have also displayed a range of responses, including; fluctuations in arbuscular mycorrhizal fungi (AMF) colonisation and abundance (Warnock *et al.* 2010; Elmer & Pignatello 2011), decreased diversity (Hu *et al.* 2014), increased fungal growth (Sun *et al.* 2013), decreased fungal growth (Quilliam *et al.* 2012) and decreases in the abundance of fungi (Ameloot *et al.* 2014). Studies of fungi have indicated a decline in α diversity due to the inability of fungal taxa to adapt to rapid variation in the soil environment (Hu *et al.* 2014), shifts in community composition (Chen *et al.* 2013) and increased rates of colonisation by AMF in host plants (Warnock *et al.* 2010; Elmer & Pignatello 2011). Increased abundance of *Trichoderma* and *Paecilomyces* in biochar samples has also been noted (Hu *et al.* 2014).

It therefore remains unclear how biochar application will effect bacterial and fungal populations within the soil. Furthermore, it is uncertain whether the disparate findings of previous studies is due to differences in the biochar used, the nature of biodiversity assessment, differences in environments/communities studied, or some combination of these factors. In this study, 16SrRNA and ITS short read amplicon sequencing were applied to assess detailed taxonomic changes in both bacterial and fungal microbiomes as a result of short to medium term field-scale treatment using a standardised biochar, applied at three contrasting sites across Europe. This aimed to determine whether biochar application changed the structure of microbial communities, and whether there were significant effects on specific taxonomic groups which could be detected across the sites. Furthermore, a time series experiment in the UK was used to assess whether these effects change communities temporally, and to determine potential interactions with annual variations.

Soil total C and N measurements were carried out by Dr Katja Weidner at the Martin Luther University, Halle-Wittenberg.

Table 15 Effects of biochar amendment experiments on soil respiration (SR) and microbial biomass/enzymatic activity

Study, pyrolysis method and feedstock	Impact on Soil respiration	Impact on microbial biomass/ enzyme activity
Bamminger (2014): Hydrochar & pyrochar (maize silage at 220°C and 600°C respectively)	Short-term increase after soil rewetting. Hydrochar 40% treatment showed at 24 hour lag. Hydrochar increased basal respiration, but pyrochar showed no significant difference.	Significantly increased microbial biomass in hydrochar treatment (2-3 times control). No significant effect of pyrochar. Hydrochar increased nitrogen cycling, phosphate uptake and proteolysis enzymes. Pyrochar little effect, increasing activity of some cell wall degradation enzymes whilst decreasing others.
Belyaeva and Haynes (2011): Poultry manure at 450°c added to coal fly ash	Biochar not significantly different from other treatments (manure, bio solids, compost) at the 50Mg ha-1 rate. Biochar shows significantly higher respiration than control samples.	Total biomass increased vs control
Carlsson et al (2012): Household charcoal, activated carbon and charcoal ash	Activated carbon respiration peaked after 25h, while charcoal reduced respiration compared to controls. Respiration was no different to controls after 8 days.	N/A
Case, McNamara et al (2012): Oak, Cherry and Ash at 400°c	No consistent effect on CO2 flux across biochar treatments. Increased at low application rates, decreased at higher application rates.	No obvious changes in microbial activity with increased biochar application
Castaldi et al (2011): Beech, hazel, oak and birch at 500°c	Respiration significantly increased compared to control. Respiration increased with proportion of char in lab experiments, although this was short-lived.	No change in microbial biomass, or rates of nitrification.

Dempster et al (2011): Eucalyptus at 600°c	Decrease in CO2 evolved at 5t/ha-1 rate, no difference in CO2 evolution at 25t/ha-1 and control.	Decreased biomass, SOM breakdown and N mineralisation by soil microbes in biochar treatment.
Galvez et al (2012): Green waste biochar at 550°c	No significant increase in CO2 when compared with control. No N2O emission increase detected in char or control	No increase in microbial biomass as a result on addition or increase in hydrolytic enzymes related to organic matter cycling in biochar treatments.
Kolb et al (2009): 2:1:1 bull manure, dairy manure and pine at 500°c	77% of increases in basal respiration due to charcoal addition.	Biomass and activity were increased with char addition. Increased extractable N and P with time in charcoal incubation experiments.
Liang et al (2014): Poultry manure at 400°c	N/A	Increased beta-glucosidase and phosphomonoesterase (involved in SOM and P cycling)
Paz-Ferreiro et al (2011): Sewage sludge at 600°c	4% amendment decreased respiration to 85% of the control. 8% reduced it to 49% of the control	Significant change in biomass in both treatments. Compared to control (+320 mg kg in 4%, +349 mg kg in 8%), increased dehydrogenase in 8% indicative of greater efficiency of microbes.
Prayogo et al (2013): Willow at 470°c	2% amendment lowered rate of CO2 release from soil	Decreased NH4+ levels, with significant increases in immobilisation in 2% biochar treatments with litter present. Biochar significantly reduced NO3- mineralisation
Quilliam et al (2012): Ash, Beech and Oak at 450 °c	CO2 evolution increased in biochar vs control with significantly increased rates in the higher application rates.	Reapplication slightly stimulated bacterial growth, but significantly decreased fungal growth. AMF colonisation doubled in reapplied plots compared with field aged plots
Wang et al (2013): Bamboo at 600°c	N/A	No change in microbial N emissions in biochar treated compost
Zavalloni et al(2011): Commercial from coppiced beech, hazel, oak and birch) at 500°c	Increased SR in biochar compared with control. Even greater increase in biochar with crop residues present, decreasing with time.	No significant effect on microbial C, although crop residues x biochar significantly increase it. Microbial N was significantly different after 3 days, but biochar alone showed similarity to control at 7 days.

Table 16 Shifts in microbial communities as a result of biochar amendment experiments

Study and biochar type	Change in microbial community	Taxa affected
Anderson et al (2011): Unweathered from Pine	Few significant changes in comparison with sintered glass control, possibly indicating biochar stabilises existing microbial communities. Method: TRFLP	*Bradyrhizobiaceae* and *Hyphomicrobiaceae* increased relative abundance of 11%. *Mycobacteriaceae* had the highest average relative abundance (16%) in treated bulk soil.
Anders (2013): Vineyard prunings at 400°C and 525°C, wheat straw at 525°C and woodchip mixture at 525°c	Gram-positive and Gram-negative PLFA's showed no change in the greenhouse experiment, whilst *Actinomycetes* increased at day 170 and 297 samples. Saprophytic fungi decreased with time. In field experiments, treatment had no significant effect on communities. Method:PLFA	*Actinomycetes* and saprophytic fungi
Chen (2013): Straw pyrolysed between 305-550°c	Shift to bacterially dominated community. Method: TRFLP/DGGE	Decreased *Betaproteobacteria* in biochar. Increased proportion of *Chloroflexi* and *Nitrospiraceae* in biochar. Impacts on Fungi included increased *Agaricomycetes* and *Sordariomycete* species in 20t/ha^{-1} treatment and a decrease in a *Glomeromycetes* and a *Sordariomycetes* species at 40t/ha^{-1} treatment.
Dempster et al (2011): Eucalyptus at 600°c	No change in ammonia oxidiser community in biochar additions alone. Method: TRFLP	N/A
Ding (2013): Charcoal (unspecified) added to artificial soil	Dramatic shift in several bacterial groups. Method: DGGE	Increased relative abundance of *Proteobacteria*, *Actinobacteria* and *Gemmatimonadetes*, decreased *Firmicutes* and *Bacteroidetes*. Significantly increased *Proteobacteria* include: *Phenylobacterium*, *Devosia*, *Rhizobium*, *Sphingomonas*, *Cupriavidus*, *Massilia*, *Luteimonas*, *Pseudoxanthomonas* and *Peredibacter*.

Gomez et al (2013): Oak pellet pyrolysed at 550°c	No significant effect of biochar addition on community at final time point. However, a shift was seen between 0% biochar and higher additions. Method: PLFA	Fungi decreased and Gram-negative bacteria increased.
Graber et al (2010): Citrus wood at unknown temp	*Trichoderma* increased dramatically in biochar treated plots. Biochar increased abundance of culturable microbes on root surfaces of pepper and tomato plants. Method: Culture of pot extract and partial 16S sequencing.	*Trichoderma* and *Pseudomonas* increased, both associated with improvements in plant growth and resistance.
Hu et al (2014): Forest litter at 400°c	Bacterial and fungal diversity increased in biochar samples. Method: Vector cloning of bacterial and fungal 16S prior to sequencing. Total of 169 bacterial and 145 fungal sequences studied.	*Bacteroidetes* and *Firmicutes* detected only in biochar. *Actinobacteria* was 5.6% higher in biochar, with decrease in proportion of *Proteobacteria* and *Planctomycetes*. Whilst total proportion of *Proteobacteria* decreased, more taxa within the phylum were detected. *Basidiomycota* increased slightly in the fungal survey, and proportion of *Trichoderma* was significantly increased in biochar. *Paecilomyces* however, was increased in the control.
Jindo et al (2012): Japanese oak at 400-600°c	Decreased Gram-positive and Gram-negative bacterial biomass in char treatments. Method: PLFA	N/A
Jones and Rousk et al (2012): Ash, Beech and Oak at 450 °c	Shift to bacterially dominated community Method: PLFA	Bacteria and fungi
Khodadad et al (2011): Laurel and oak at 650°c or 250 °c	Distinct clustering of populations into burned and unburned soils. Unburned soils treated with char showed the greatest similarity, with a decrease in species abundance. Method: Bacterial cultivations and ARISA	*Gemmatimonadetes* and *Actinobacteria* showed an enrichment in burned soils treated with char.

Kolton et al (2011): Citrus wood at unknown temp	Biochar caused a shift in the dominant bacterial group present within root communities of pepper plants grown in pots in a greenhouse. Method: 16S pyrosequencing.	*Bacteroidetes* elevated in biochar root communities, *Proteobacteria* higher in control root communities. No noted change in *Actinobacteria*. *Flavobacterium* genus showed an increase from 4.2% in control to 19.6% in root associated biochar treatments, explaining much of the increase in *Bacteroidetes*.
Pietikäinen et al (2000): Crowberry twigs at 450°c, forest humus at 450°c or activated charcoal	Distinct difference in PLFA analysis showing different taxa present in the communities of Crowberry and activated charcoal treated humus vs. forest humus char and pumice. Method: PLFA	N/A
Prayogo et al (2013): Willow at 470°c	Biochar and litter increased total, Gram-negative and *Actinobacteria* compared with litter alone. No increases detected compared with control soils. Fungal PLFAs significantly increased in short-term. Method: PLFA	*Actinobacteria*, Gram-negative bacteria, fungi
Quilliam et al (2012): Ash, Beech and Oak at 450 °c	No consistent effect of biochar on fungi or bacterial communities. Fungal root colonisation increased in reapplication plots. Method: ARISA	Arbuscular mycorrhizal fungi colonisation increased.
Rutigliano (2014): Beech, hazel, oak and birch at 500°c	No significant change in richness, or diversity. Method: DGGE	N/A
Sun (2013): Corncob (temperature and pyrolysis details not specified)	Majority of DGGE bands present in both biochar and adjacent soils, suggesting similarity of organisms present. Method: DGGE	N/A
Watzinger (2014): Wheat and willow biochars at 525°c	Promotion of Gram-negative bacterial groups over Gram-positive or fungi, reflective of the increased nutrient conditions available. Gram-negative bacteria are capable of rapidly adapting to the shift, whilst fungi and Gram-positive bacteria take longer to adjust. Method: PLFA	Gram-negative and *Actinomycetes* showed strong positive results in Planosol. Chernosem showed short-term increases in Gram-negative bacteria and fungi, but a significant reduction in Gram-positive PLFA's by the end of the experiment.

3.2 Method

3.2.1 Biochar characterisation

Biochar was produced by Advanced Gasification Technologies (AGT s.r.l, Cremona, Italy), using a fixed bed, open core, down draft gasifier for the EuroChar project (Ventura *et al.* 2015). AGT biochar was produced from *Zea mays* silage at 1200°C, held at atmospheric pressure for 40 minutes. Physico-chemical properties are shown in *Table 18*.

3.2.2 Metabarcoding sites

Three field sites were established across Europe. The sites were part of the EuroChar network (www.eurochar.eu), located in West Sussex (UK), Prato Sesia (IT) and Lusignan (FR) (Figure 7). Prato Sesia was established during 2010 by the IPLA institute, an agency associated with landscape and environmental management in Piedmont. The French site is part of a long-term environmental research network (Observatoires de Recharche en Environnement, ORE). The UK site was established as part of the EuroChar project, a project studying the environmental implications of biochar application. Site details are given in *Table 17*.

These sites were planned, implemented and managed as part of a wide consortium of several PIs and researchers that formed part of the EuroChar Project and this thesis contributed to that international scientific effort. Specifically, in relation to this PhD, UK, Italian and French sites were designed by G. Taylor, F. Miglietta, G. Alberti, C. Rumpel, B. Glaser and G. Tonon. The UK site was implemented by Dr Maud Viger and Dr Giorgio Alberti who designed the biochar site set up.

Plots differed in size due to differences in the agricultural practises at each site. UK plots were 4.3 m x 2.75 m, each containing approximately 20 pre-established *Salix spp.* trees, planted in double rows with spacing of 0.75 m between rows, and 0.55 m within rows. In IT plots were 5 m x 9 m, containing 5 *Populus x candadensis* trees in each plot planted in single rows 3 m apart. Spacing within the row was 0.5 m. Finally, plots in FR were 5 m x 4 m, and sown with *Festuca arundinaceae* and *Dactylis glomerata* seed.

At the UK site, biochar was applied by hand at a rate of 30 t/ha^{-1} (65 kg of biochar fresh weight, or 5.5 kg/m^2, 45 % water content) to four randomised experimental plots during June 2012. Control plots were treated with 29.5 l of water (equivalent to water added in the biochar treatment). Treatments were applied to the surface of each plot and incorporated by hand to a depth of 15 cm. Eight plots in total were treated (four control, four biochar), of which six were used for further study (additional plots were part of an separate experiment at the same site). IT and FR treatments were applied at the same rate as the UK site, but using rotary hoeing during June 2012. Treated and control plots were arranged in a completely randomised design, with four replicates per treatment. Only three replicates were sampled for microbial community composition due to financial constraints of sequencing.

Table 17 Site location and environmental properties for UK, IT and FR

Location and site name	Mean annual temperature and rainfall	Soil type	pH	Altitude	Crop
West Sussex (UK), 50°58'38"N, 0°27'33"W	10°C 742.3 mm	Permeable, seasonally wet, clay and loam	6.04	33m a.s.l.	*Salix sp.* SRC
Prato Sesia, Novara (IT), 45°39'32.27"N; 8°21'16.83"E	12°C 1200 mm	Sandy	5.4	279m a.s.l.	*Populus x candadensis Mönch*, clone "Oudemberg", SRC
Lusignan (FR), 46°25'12.91"N; 0°07'29.35"E	10.5°C 600 mm	Loamy cambisol	6.8	153m a.s.l.	*Festuca arundinacea* and *Dactylis glomerata* grassland

Table 18 Physicochemical properties of AGT biochar applied to all sites

Parameter	Value
Bulk Density	1.41 g cm^{-3}
pH (H_2O)	11.6
Salinity	758 mS m^{-1}
H	2.3 %
H/C	0.5
C	56.1 %
N	1.35 %
C:N	42.9
Ca	38.1 g/kg
K	32.3 g/kg
Mg	9.4 g/kg
Al	4.27 g/kg
Fe	3.12 g/kg
Mn	211.3 mg/kg
Zn	183.1 mg/kg
Sr	148.1 mg/kg
Cu	46.3 mg/kg
Si	25.45 mg/kg
P	8.56 mg/kg
S	1.32 mg/kg

3.2.3 Soil sample collection in UK, FR and IT

The microbial community was assessed at each site one year after biochar application (samples were collected in July 2013). An additional intensive time series experiment was carried out at the UK site, with samples collected pre-treatment (UKPT) during March 2012, one month after biochar amendment (UK1M) during July 2012 and as well as July 2013 (UK1Y, one year after UK1M). At all sites, biochar treated plots are referred to as BC and control denoted C. Thus a control sample at one month will be labelled UK1MC.

A total of 130 soil samples were collected from biochar amended plots and control plots using a systematic sampling design, with 30 samples from each of FR, IT UK1M and UK1Y (5 samples x 3 replicates x 2 treatments). A further 10 samples were collected prior to biochar addition from UKPT (10 samples). Samples were collected at a 1.5 m radius from the centre of each plot. Considerable effort was maintained throughout sampling to ensure clean, uncontaminated samples, including use of gloves during collection and decontamination of equipment prior to and during sampling. Collection was

carried out using a stainless steel soil corer (15 cm x 2.5 cm). Each soil corer had been sterilised, treated with RNaseZap and ethanol, sealed in foil and autoclaved.

Samples were sieved (mesh size 2 mm) to remove roots and a 50 ml sterile falcon tube filled with homogenised soil, prior to freezing in liquid nitrogen. Samples were transported to the lab at -80 °C by cryoshipper. Between sampling at each site, previously collected samples were stored at -80 °C.

3.2.4 Soil characteristics

Soil pH was measured for each sample using a 1:5 soil : water dilution (weight : volume) in distilled water. Samples were agitated and left to equilibrate for 1 hour, before measurement of pH using a Jenway 3510 pH meter. An additional soil core collected from the top 20cm of each plot during sampling was analysed for total C and N using a "vario EL" analyser by Elementar GmbH in the Soil Biogeochemistry lab at Martin Luther University, Halle-Wittenberg, by Dr Katja Wiedner (Wiedner *et al.* 2013). Differences between treatments were assessed using two-factor ANOVA, in which treatment and site were factors.

3.2.5 DNA extraction

DNA extraction was carried out using MoBio Powersoil Extraction kit (MO BIO Laboratories, Carlsbad, CA, USA). Briefly, 0.5 g (increased from the recommended 0.25 g, to improve DNA yield) of homogenized frozen soil was placed into a PowerSoil Bead Tube, before following manufacturer's specifications. DNA quality and concentration was assessed using NanoDrop 1000, (Thermo Scientific, Waltham, MA, USA) ensuring all samples had a minimum 260/280 ratio of 1.8. Extracted samples were stored at -80 °C until all extractions were complete, ready for transport to LGC Genomics (Berlin, Germany).

3.2.6 Amplification and sequencing methodology

Isolated DNA from each sample was amplified using the 16SrRNA gene primers 341F (5'-TCCTACGGGNGGCWGCAG-3') and 785R (5'-GAC TACHVGGGTATCTAAKCC-3') and the fITS7 (5'-TGTGARTCATCGAATCTTTG-3')

and ITS4 (5'-TTCCTCCGCTTATTGATATGC-3'). In the case of the 16S region, these were chosen as they provide approximately 470 bp of sequence and are suitable for a wide range of bacterial taxa, amplifying the hypervariable V3-V4 region (Klindworth *et al.* 2013). The ITS primers were chosen as they amplify the ITS2 region, and include a portion of the 5.8S region. This primer pair has been shown to increase the diversity of fungi identified, whilst decreasing misrepresentation in communities (Ihrmark *et al.* 2012).

Library construction utilised the Encore Rapid DR Multiplex System (NuGen, San Carlos, CA), each sample was tagged with a combination of one of 48 barcoded primers, and one of 8 pool specific 8 nucleotide barcode sequences. Therefore each sequence could be identified and demultiplexed by the combination of its 8 base index adapter in combination with its barcoded primer sequence. By barcoding sequences in this way, all samples (both 16S and ITS) could be sequenced in a single MiSeq run. Amplification was carried out using 15 pmol of each forward and reverse primer, added to 20 µl of MyTaq buffer, including 1.5 units of MyTaq DNA polymerase and 2 µl of BiostabII PCR Enhancer. 30 cycles of PCR were undertaken for 2 mins at 96 °C, followed by 96 °C for 15 seconds, 50 °C for 30 seconds and 72 °C for 60 seconds, gel-electrophoresis was utilised to assess concentration. Finally, approximately 20 ng of PCR product was pooled prior to purification using preparative gel-electrophoresis. Purified DNA was sequenced in a single lane, single flowcell run on an Illumina MiSeq, using V3 reagent chemistry, producing 2 x 300 bp paired end reads producing a total of 9,020,619 reads across 330 samples (165x16S samples, 165xITS samples, comprising of 30xUK1M, 30xUK1Y, 30xFR, 30xIT 30xUK grassland, 15xUK Prechar each), with a mean of 27,335 reads per sample. Reads were demultiplexed and separated *in silico* by their sample specific barcodes. These steps were undertaken at LGC Genomics (Gmbh), Berlin, Germany.

3.2.7 Analysis Pipeline

3.2.7.1 Analysis of Bacterial 16S rRNA

For each site, paired end reads were first quality controlled and combined using PandaSeq (Masella et al. 2012). The program combines paired end reads through areas of overlapping sequence, converting 2 x 300 bp reads into a

single read of approximately 500 bp in length prior to adapter and primer clipping, and renaming (see Chapter 2: Section 2.2.5.2). Formatted files were run through the Quantitative Insights into Microbial Ecology (QIIME v1.8) pipeline (Caporaso, Kuczynski, et al. 2010) as previously described (Section 2.2.5.2). Unless otherwise stated, named python scripts (*.py) are from the QIIME package. Reads were clustered into operational taxonomic units (OTUs) using the pick_denovo_otus.py workflow, clustering all reads at 97% identity using UCLUST (Edgar 2010), prior to alignment using PyNAST (Caporaso, Bittinger, et al. 2010). Classification of sequences was carried out using the RDP Classifier (Wang et al. 2007), trained by the GreenGenes 13.5 database (DeSantis et al. 2006). Phylogenetic trees were produced using the make_phylogeny.py command using FastTree2 (Price et al. 2010). The OTU table was then filtered to remove singletons using filter_otus_from_otu_table.py, before sorting samples by treatment utilising sort_otu_table.py.

Taxonomic summaries were generated using the summarize_taxa_through_plots.py script, generating bar charts showing the raw relative abundance output of the pipeline, and mean values by treatment. α diversity was calculated through use of multiple rarefied OTU tables (each sample rarefied to 90 % of the total reads in the smallest sample), producing normalised mean metrics for observed species (OBS), Chao1 and Faiths Phylogenetic diversity (PD) (Table 2). Chao1 estimates a value of the true diversity of a sample based on the number of rare species detected. Observed species is a direct measure of the number of OTU's detected, whilst Phylogenetic Diversity (PD) computes the similarity of samples phylogenetic trees by calculating the total sum length of the branches. Therefore trees containing closely related OTU's will have a more similar value than those containing distantly related OTU's.

β diversity was assessed through pairwise weighted UNIFRAC distances (Lozupone & Knight 2005). UNIFRAC compares the shared portions of the phylogenetic tree produced for each sample, and is a metric accounting for the abundance of each OTU (weighted) and the phylogenetic similarities alone (unweighted). These were displayed using Principle Coordinates Analysis (PCoA), whereby samples with similar communities cluster together in 3D space. Again, samples used for assessment of β diversity were rarefied to

90% of the total of the smallest sample in each data set. All output data from these steps can be accessed on Dryad: Doi:10.5061/dryad.pj08b.

3.2.7.2 Bacterial 16S statistical analyses

Monte-Carlo adjusted nonparametric two-sample t-tests were utilised using the compare_alpha_diversity.py script within QIIME to assess differences between α diversities. β diversities in treated and control samples were analysed ADONIS statistical testing (999 permutations) (Oksanen J, et al 2012). To compensate for multiple ADONIS tests, a Benjamini-Hochberg correction was applied, where q = <0.05 (Benjamini & Hochberg 1995). Comparisons of taxonomic differential abundance utilised STAMP (Parks & Beiko 2010), using a two sided White's non-parametric t-test (White *et al.* 2009), with Benjamini-Hochberg FDR correction for multiple testing (Benjamini & Hochberg 1995). Results were then filtered to exclude taxa which included <5 sequences, and where the difference between proportions was <0.5 % or the ratio of proportions was less than 2. A q-value filter of 0.05 was used, representative of a 95 % confidence that a statistically significant result was not a false discovery.

3.2.7.3 Analysis of Fungal ITS rRNA

Initial QC and read combination of ITS reads was carried out identically to the methods outlined for 16S (Section 3.2.7.1). OTU's were picked using the open reference pipeline, clustering reads against a reference sequence database (UNITE ITS database Kõljalg et al. 2013). Reads failing to match the database are grouped with their closest matching cluster. Due to difficulties in aligning the ITS sequences, non-phylogenetic measures were used to analyse α and β diversity. Subsequent analyses were undertaken as described in the 16S method, but utilising non-phylogenetic measures. β diversity analysis for ITS data utilised Bray-Curtis dissimilarity, the sum of total differences in two samples counts, divided by total abundance between the two samples. A value of 0 indicates identical taxa with identical abundances, whilst a value of 1 advocates no shared taxa.

3.2.7.4 Fungal ITS statistical analysis

Statistical analysis of fungal datasets used identical methods to those described in Section 3.2.7.2.

3.3 Results

3.3.1 Biochar effects on soil characteristics

No significant differences between soil total C was detected due to treatment at UK or IT but organic C was significantly higher in FR BC samples (p=0.0013), (UK1MC mean = 13.57±0.93g Org C kg^{-1}, UK1MBC mean = 24.84±4.89g Org C kg^{-1}, UK1YC mean = 12.48±0.988g Org C kg^{-1} , UK1YBC = 10.01g Org C kg^{-1} . ITC mean = 12.9±1.36g Org C kg^{-1}, ITBC mean = 17.2±5.4g Org C kg^{-1}, FRC mean = 11.29±0.87g Org C kg^{-1}, FRBC mean = 21.88±2.10g Org C kg^{-1}). Significant differences in UK total C emerged when assessed by Year of sampling, but no interaction between Year and treatment was detected. Similarly, there was no significant interaction between site and treatment. Total soil N also exhibited no significant difference between treatments, sites or interactions between treatment and site (UK1YC mean = 0.14±0.009% Total N, UK1YBC mean = 0.17±0.021% Total N, ITC mean = 0.11±0.0001% Total N. ITBC mean = 0.12±0.011% Total N. FRC mean = 0.12±0.005% Total N, FRBC mean = 0.14±0.004% Total N). Significant differences were detected in pH between treatment and control (UK1MC mean pH = 6.2±0.19, UK1MBC mean pH = 7.37±0.12 p = <0.001, UK1YC mean pH = 6.22±0.04, UK1YBC mean pH = 7.37±0.11 p= <0.001, ITC mean pH = 6.62±0.09, ITBC mean pH = 7.25±0.07 p= <0.001, FRC mean pH = 6.77±0.04, FRBC mean pH = 7.08±0.04, p=<0.001) although no interaction of site, treatment or sample were detected.

3.3.2 Biochar effects on bacterial richness

No significant difference in any of the three metrics was detected in UK1Y, IT or FR samples (*Table 19*). However, a significant difference in all three metrics was noted between UK1M samples (regardless of treatment) and UK1Y, with higher species richness in UK1M samples. Temporal shift in α diversity for the UK site between the 1-month and 1-year sample also showed significant differences, with UK1MBC having significantly higher richness than UK1YBC and UK1YC. Similarly, UK1MC had a higher richness when compared with UK1YC which may be a result of climatic and annual variability between the two sampling dates (UK1MBC vs UK1YBC: p = 0.015, UK1MC vs UK1YC: p = 0.015).

Therefore, one year after biochar application, there was no major change in the number of taxa present between control and biochar plots, although there appears to be an interaction between annual variation and treatment in the time series samples.

Table 19 Results of t-tests for difference in 16S α diversity between treatment (BC, biochar, or C, biochar) at each of three sites. Results of sampling in the UK at 1 month (UK1M) and 1 year (UK1Y) are shown μ= sample mean, σ = standard deviation.

Site	Chao1	OBS	PD	Rarefied to:
FR	BC: μ= 4217.2, σ=297.2 C: μ= 4163.2, σ=211.2, p=0.584	BC: μ= 1560.1, σ=85.6 C: μ= 1503.3, σ=110.2, p=0.124	BC: μ= 115.2, σ= 5.7 C: μ= 109.9, σ=7.9, p=0.053	3354
IT	BC: μ= 3928.4, σ=348.5 C: μ= 4123.2, σ=213.4 p=0.093	BC: μ= 1397.1, σ=113.8 C: μ= 1466.6, σ=47.8 p=0.168	BC: μ= 107.5, σ=7.4 C: μ= 111.2, σ=4.2, P=0.121	2940
UK1M	BC: μ= 5838.1, σ=282.7 C: μ= 5960.8, σ=265.4, p=0.550	BC: μ= 2686.3 σ=129.4 C: μ= 2744.2, σ=124.9, p=0.618	BC: μ= 155.3 σ=6.6 C: μ= 160.0, σ=6.6, p=0.137	7619
UK1Y	BC: μ= 2325.7, σ=175.5 C: μ= 2364.7, σ=162.1 p=0.243	BC: μ= 879.1 σ=21.2 C: μ= 885.5, σ= 39.3 p=0.235	BC: μ= 67.3 σ=2.7 C: μ= 69.1, σ=3.4 p=0.069	1827

3.3.3 Changes in bacterial community structure due to biochar application

Although the number of taxa present remained unchanged one year after treatment, it is possible that the identities and abundances of the taxa might change. This is measured through β diversity metrics, such as UNIFRAC (Lozupone & Knight 2005). β diversity results for community data from UK1Y show a significant difference in weighted UNIFRAC (Figure 15 A) between treatments (weighted UNIFRAC ADONIS: R^2=0.12, q = 0.004). Unweighted UNIFRAC also showed a significant change (Figure 15 B) (unweighted UNIFRAC ADONIS: R^2=0.06, q= 0.004). This demonstrates that the changes in community detected were a result of shifts in both the identities and the abundance taxa present.

Results from UK1M indicated no significant effect of treatment for weighted UNIFRAC metrics, although unweighted β diversity (Figure 16) was significantly

different (unweighted UNIFRAC ADONIS: R^2= 0.05, q= 0.04). Therefore, whilst no significant change in abundance occurred due to treatment, there was a small shift in which taxa contributed to the community. When all UK samples were run collectively, weighted UNIFRAC showed a distinct effect of the date of sample collection on the community present, with all samples collected during 2012 (both UKPT and UK1M) clustering closely together (Figure 17). UK1Y samples dislocated to the right of the X axis and showed a differentiation in clustering into control and biochar samples. Thus, whilst there is a short-term change in the taxa present in biochar samples, this is the beginning of a gradual shift in communities with time since biochar application. Based upon the PCoA components results, year of sampling appears to explain around 36 % of the variation (splitting samples by left, 1 month, and right, 1 year), whilst treatment splits samples along the PC2 axis, explaining up to 10 % of the variation described.

β diversity results for FR showed no significant effect for weighted UNIFRAC analyses, signifying no abundance shifts in the taxa present within the community due to treatment. However, unweighted UNIFRAC results were significant (unweighted UNIFRAC ADONIS: R^2= 0.04, q= 0.004, Figure 18). This suggests a slight variation in the identity of taxa present between treatments, but that shifts are primarily in rare taxa.

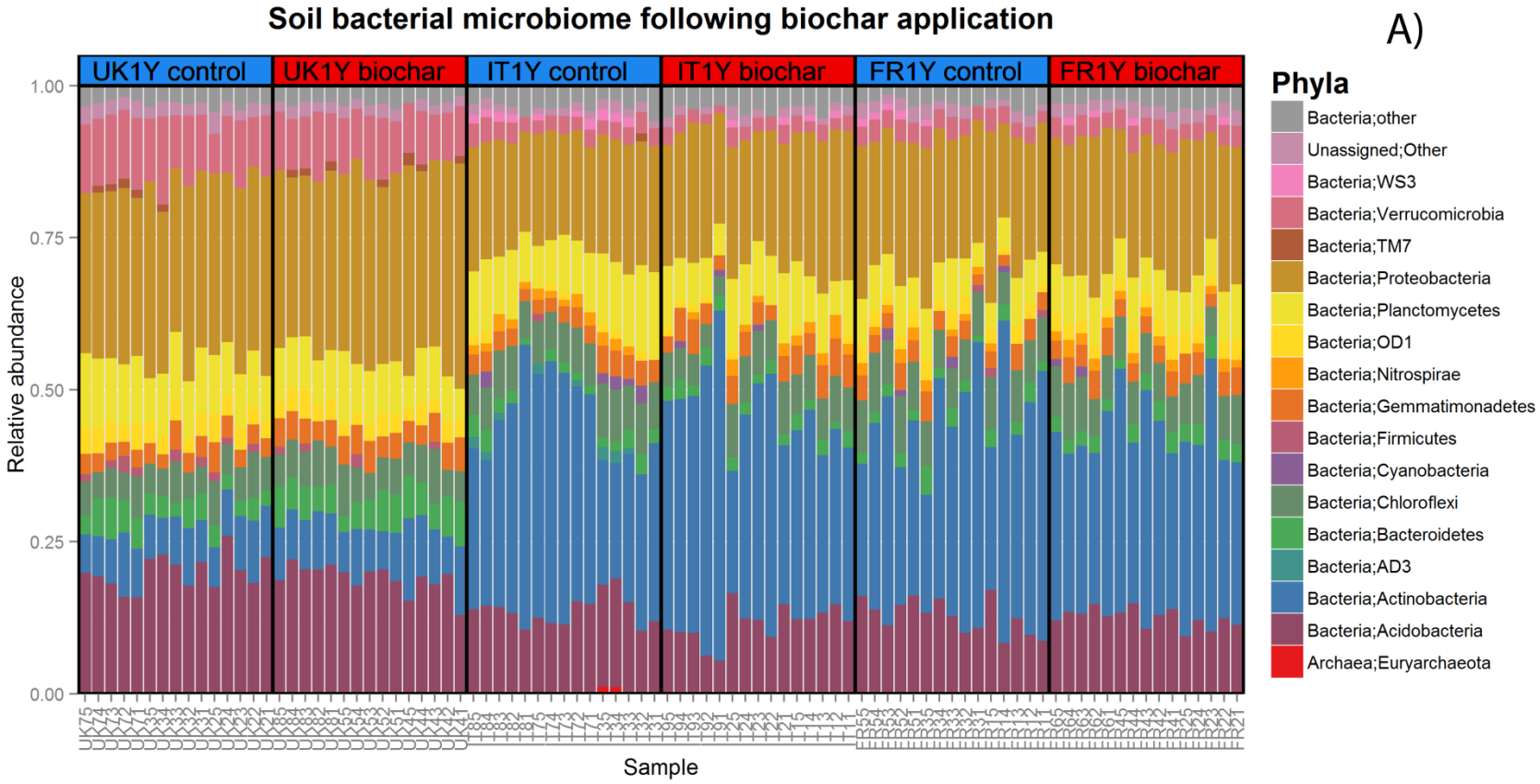


Figure 14 Taxonomic breakdown of proportional change in bacteria. Relative abundance of each of the core phyla (above 1%) is represented. Blocks at the top of the figure indicate the treatment type associated with each "block" of replicated samples. Red represents biochar treated samples, whilst blue indicates a control treatment. Labels within each block indicate the sample site, UK, IT or FR. All samples shown are from samples collected 1 year after biochar application.

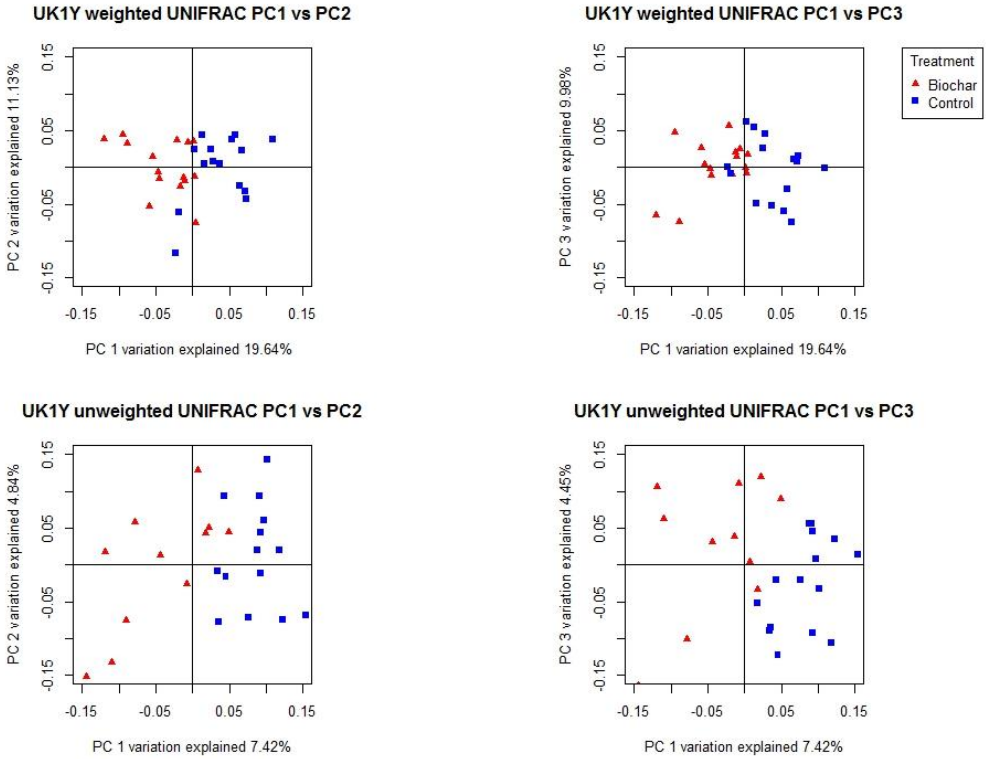


Figure 15 PCoA of weighted and unweighted UNIFRAC distances for UK1Y.

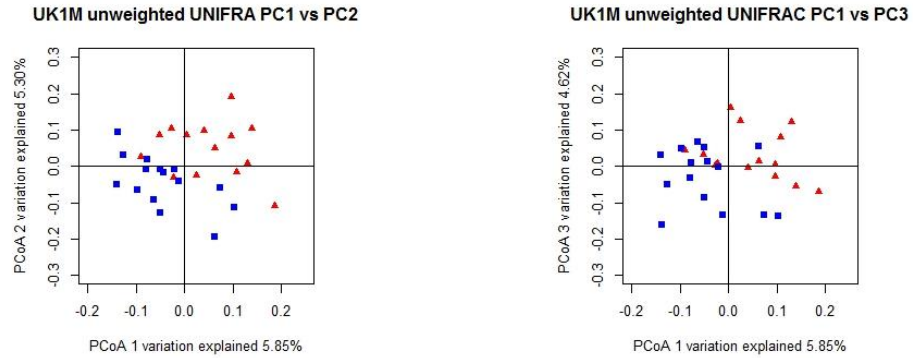


Figure 16 PCoA of unweighted UNIFRAC distances for UK1M samples.

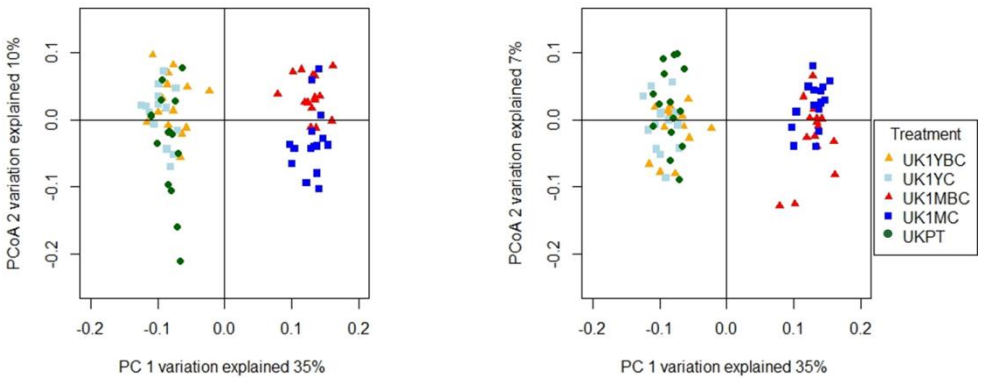


Figure 17 PCoA of weighted UNIFRAC distances for all UK time series samples.

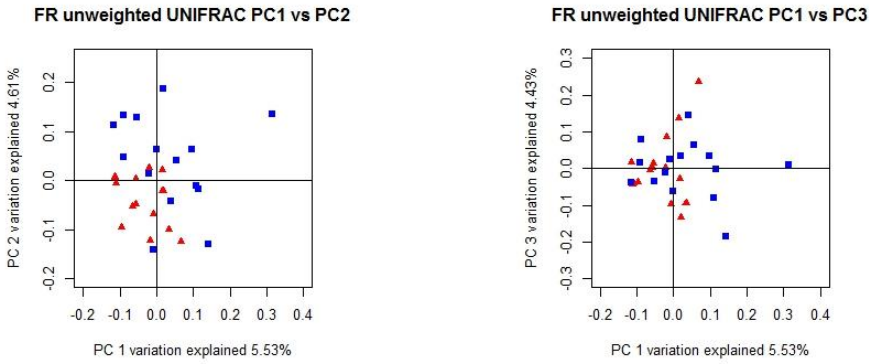


Figure 18 PCoA of unweighted UNIFRAC distances for FR samples. Control samples (blue) and biochar samples (red)

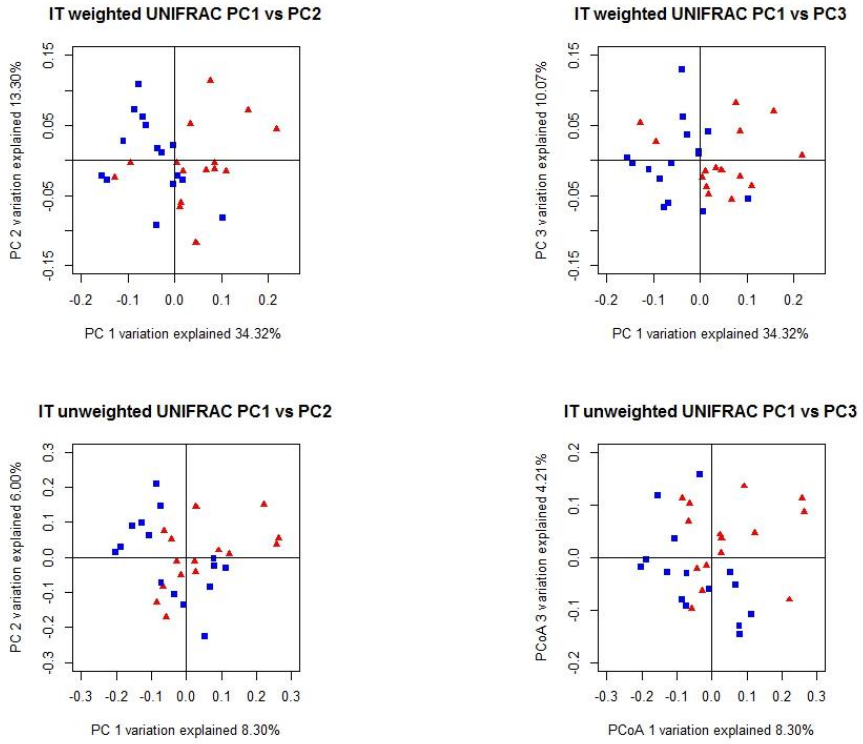


Figure 19 PCoA of weighted and unweighted UNIFRAC distances for IT samples. Control samples (blue) and biochar samples (red)

β diversity at the IT site highlights a significant effect of treatment on community structure in both weighted (weighted UNIFRAC ADONIS: R^2= 0.08, q = 0.013) and unweighted UNIFRAC results (unweighted UNIFRAC ADONIS: R^2= 0.06, q = 0.004) (A and B respectively). Therefore both the taxa present and their abundances vary as a result of biochar application at this site, although the shifts in community are likely to be subtle due to the small proportion of variance explained by the treatment.

3.3.4 Changes in bacterial taxonomic abundance due to biochar application

Sites differed in their dominant bacterial phyla (Figure 14). UK1Y samples were dominated by *Proteobacteria* and *Acidobacteria*, whilst IT was dominated by *Actinobacteria*, *Proteobacteria* and *Actinobacteria*. Communities from FR were similar to those present in IT (Figure 14).

Results from the time series collected in the UK indicated a difference in community structure over time. UKPT samples were dominated by *Proteobacteria*, *Actinobacteria* and *Acidobacteria* with a range of prominent phyla including *Planctomycetes*, *Verrucomicrobia*, *Chloroflexi* and *Nitrospirae*. UK1M samples (Figure 20 A) were still dominated by *Proteobacteria*, *Actinobacteria* and *Acidobacteria* although both the latter phyla had declined when compared with the UKPT abundances. Abundances of the other dominant UKPT phyla showed small adjustments (±1%). This may indicate an annual component in community turnover.

To determine where the differences in community structure between treatments may have occurred, the mean difference in proportions of taxa was calculated using $= Cn - Tn$, where D was the mean total difference in taxon proportions, Cn was the mean proportion of taxon n in control samples populations and Tn was mean proportional abundance each taxon in treated samples.

Using the above method, it was possible to see which taxa exhibited the largest mean differences, which are likely to drive differences in β diversity between treatments. UK1YBC samples exhibited increased proportions of *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Gemmatimonadetes* and *Proteobacteria*, and corresponding declines in the proportion of *Acidobacteria*, *Planctomycetes* and *Verrucomicrobia (*Figure 20 B*),* potentially explaining the driving taxa behind the corresponding changes in β diversity. Likewise IT β diversity shifts were probably due to increased proportions of *Actinobacteria*, and declines in *Acidobacteria*. However, all other phyla were minimally affected (Figure 20 C). Examination of higher resolution phylogenetic proportions suggests shifts may be due to a 4.15% increase in the family *Microbacteriaceae* and a 1.63% decline in abundance of the family *Proprionibacteriaceae*.

Samples from FR (for which β diversity indicated no significant change) showed a decline in *Actinobacteria* (due to decreased prevalence of the genus *Mycobacterium*), and small increases in most other phyla (Figure 20 D).

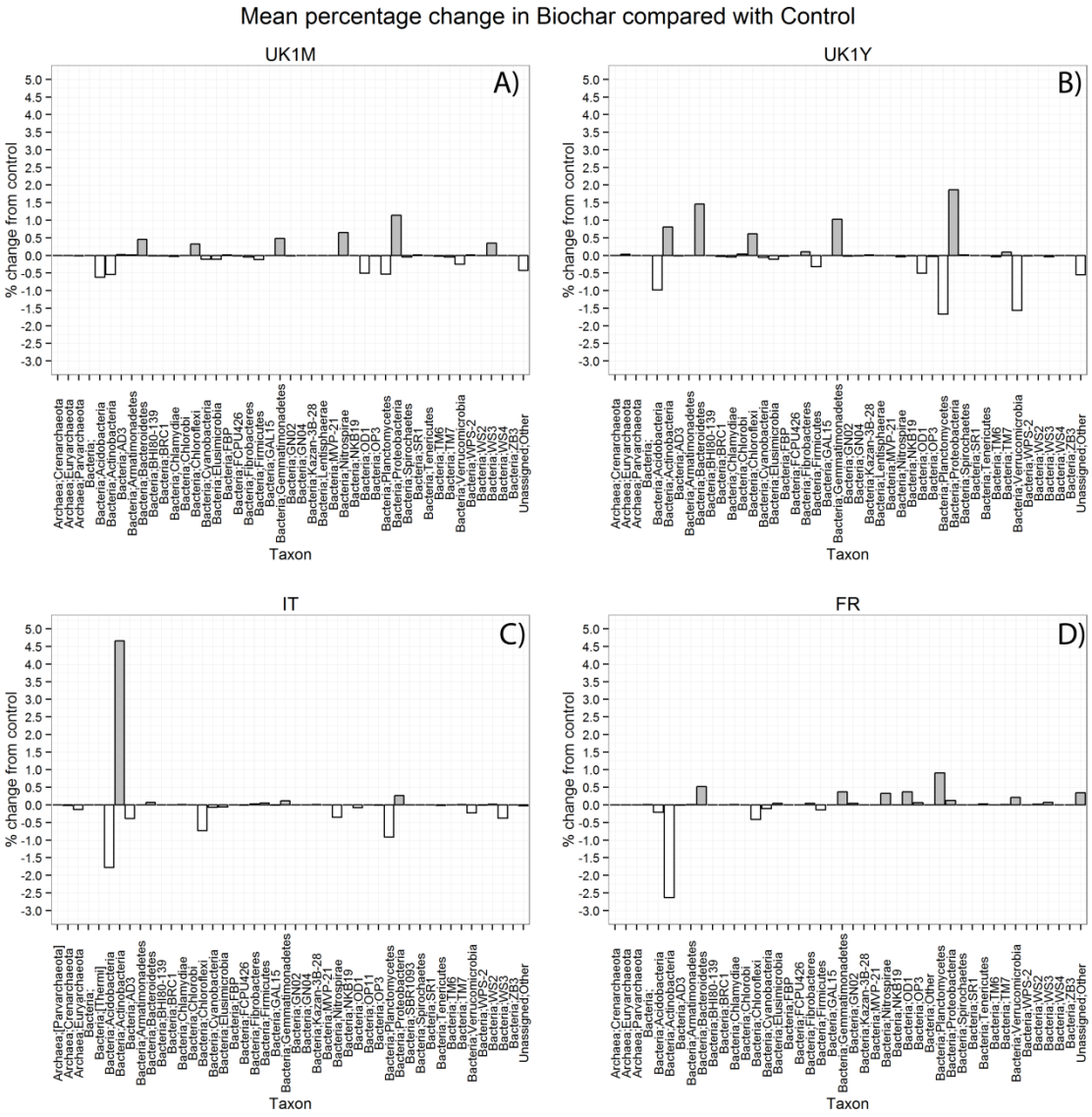


Figure 20 Mean percentage change in biochar plots from control for each of the bacterial phyla detected. Plots represent A) UK1M, B) UK1Y, C) IT and D) FR. Grey bars show an increase in percentage abundance, whilst white bars represent a decline.

To statistically determine whether differential abundance of taxa were different, STAMP was used, with the aim of determining significant differences in abundance to the level of genus. STAMP models an expected null distribution through repeated subsampling with replacement from the total dataset. Specialised t-tests (Fishers exact, Whites non-parametric or Welches)

compare the abundances of categories in treated and untreated samples with the null distribution, to detect significant differences. The software automatically adjusts for multiple test corrections, using the Benjamini–Hochberg FDR method (Benjamini & Hochberg 1995) and plots the effect size and confidence intervals for each feature, allowing for biological significance to be assessed more thoroughly (Parks & Beiko 2010). Once tests have been completed, filtering based on significance, number of reads and effect size can occur. Significance values reported in the section below are already corrected and filtered to represent only those results which contain at least 5 sequences and exhibit a minimum two-fold change (as described in Section 3.2.7.1). All changes in abundance are reported in terms of percentage of total sequences attributed to a given taxa.

Results from UK1Y at the level of phylum showed increased abundance of *Gemmatimonadetes* in biochar samples, with abundances approximately 1% greater than expected. A significant 1.5% overrepresentation in abundance of the class *Acidobacteriia* was detected in control samples, whilst the class *Acidobacteria-6* showed a mean 1.5% overrepresentation in biochar treatments. Candidate class *Ellin6529* also increased in biochar by 0.9% (Figure 21 B). Control samples were enriched in the *Plantomycete* order *Gemmatales*, and the *Spartobacteria* and the *Acidobacteria* order *Acidobacteriales* (Figure 21 C).

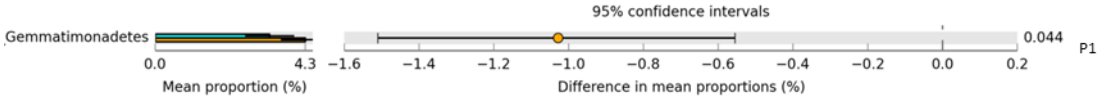
The families *Gemmataceae, Isosphaeraceae* and *Koribacteraceae* displayed elevated mean proportional abundances in control samples. Conversely, candidate family *RB40,* showed increases in biochar plots (Figure 21 D).

When analysed at the level of genus, *RB40* (increased by 0.6%) was overrepresented in biochar samples. Mean proportions of unknown *Gemmataceae, Koribacteraceae* and *Isosphaeraceae* genera were detected in elevated levels in control samples (Figure 21 E). Therefore it appears that community shifts detected in UK1Y communities were due to a range of small shifts in taxa, rather than a single large shift in the dominant bacteria present due to biochar treatment.

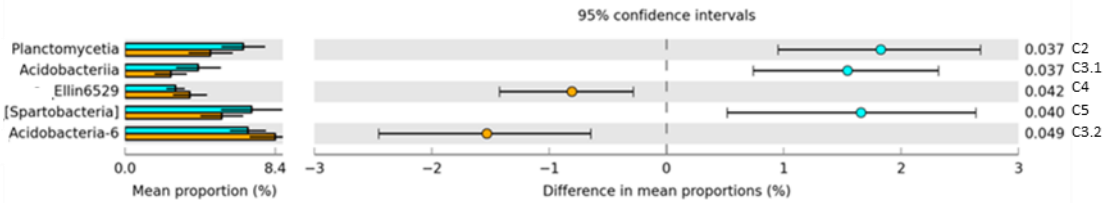
Results for UK1M exhibit a significant difference in the abundance of the phyla *Gemmatimonadetes*, approximately 0.6% more abundant in biochar samples (Figure 22). No significant differences in proportional abundance in class, order, family or genus were detected. This corroborates the picture of the

community gained from β diversity analysis, which suggested no change in the abundance of taxa within the community.

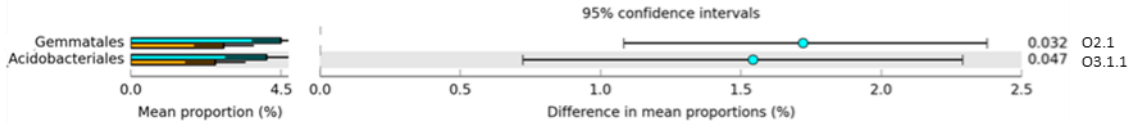
A)



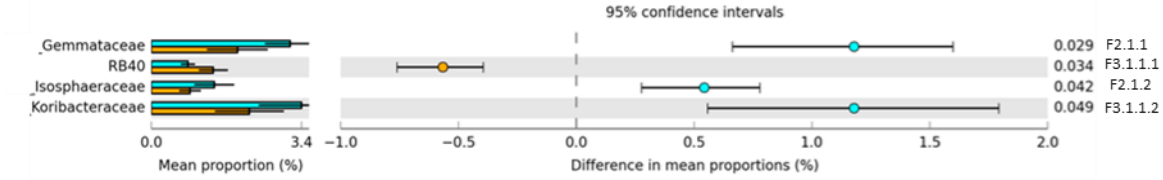
B)



C)



D)



E)

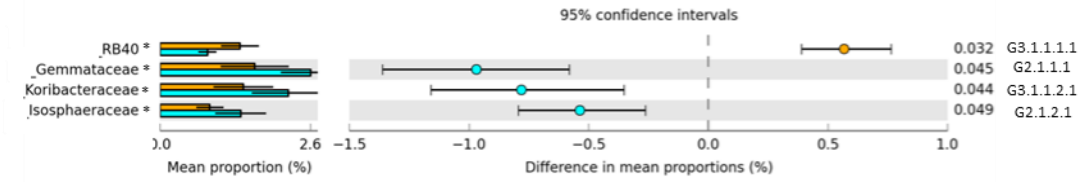


Figure 21 Extended error bar plot showing bacterial a) phyla, b) classes, c) orders, d) families and e) genera in UK1Y with significant q-values (<0.05). Light blue = control, orange = biochar. * = unidentified taxa within the taxon denoted.

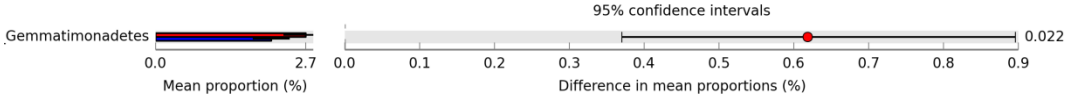
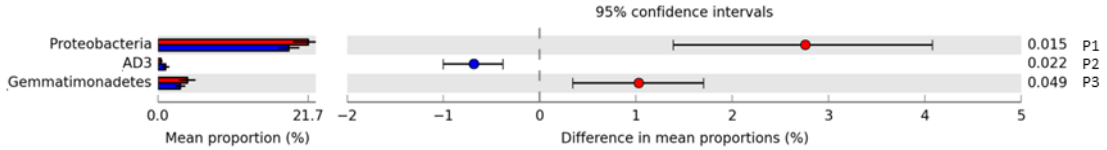


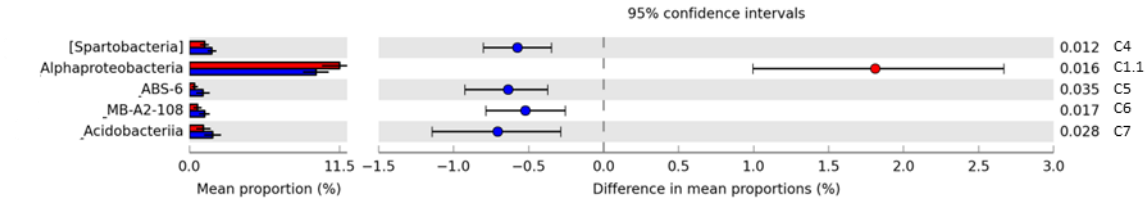
Figure 22 Extended error bar plot showing bacterial phyla in UK1M with significant q-values (<0.05). Blue = control, red = biochar

Results from FR showed no significant differences in abundance at the level of phylum, class, order or genus. Therefore biochar application had no major impact on the proportional abundance of bacterial taxa present.

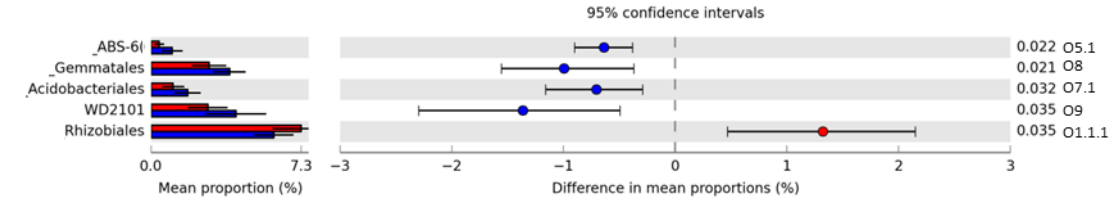
A)



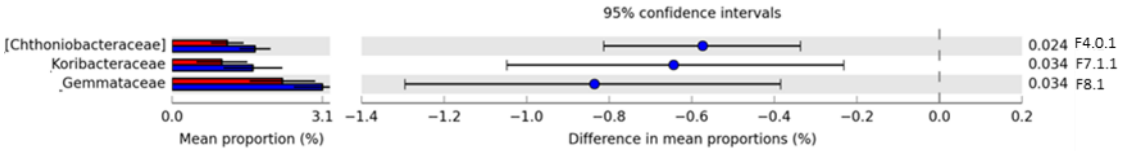
B)



C)



D)



E)

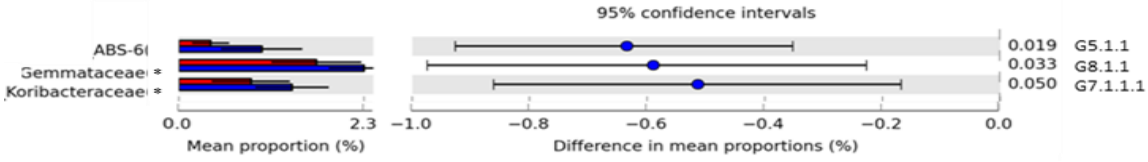


Figure 23 Extended error bar plot showing bacterial a) phyla, b) classes, c) orders, d) families and e) genera in IT with significant q-values (<0.05). blue = control, red = biochar, * = unidentified taxa within the taxon denoted

Samples from IT indicated an increase in *Proteobacteria* and *Gemmatimonadetes* in biochar samples (Figure 23 A). This appears to be a result of an approximate 2% increase in *Alphaproteobacteria* classes (Figure 23 B). Additionally, the order *Rhizobiales* showed a mean increase of approximately 1.25% in treated samples. At the level of family, treatment did not lead to the over-representation of any taxa, although control samples

contained elevated *Chthoniobacteraceae, Gemmataceae* and *Koribacteraceae* (Figure 23 D).

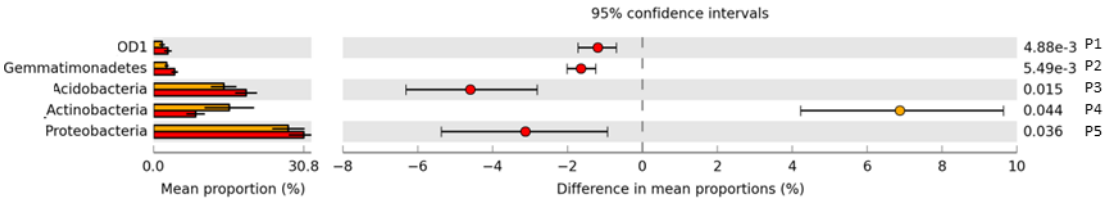
In conclusion, in a similar vein to the results from the UK, whilst a significant effect of biochar was detected on community diversity, this was a result of multiple small shifts in taxonomic abundance. This points towards small adjustments on the part of the ecology of the environment after biochar application rather than large scale shifts in taxa present.

3.3.5 Temporal changes in bacterial community structure

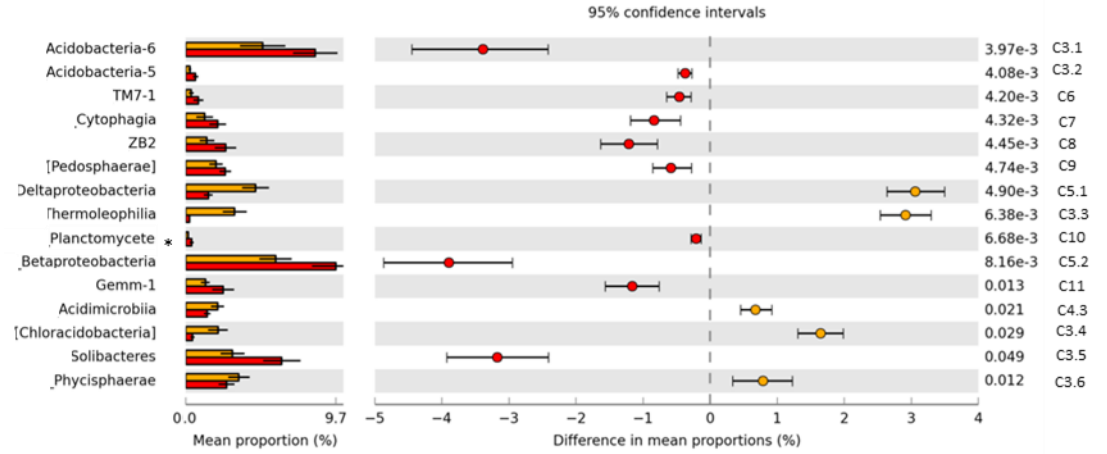
Time series data collected in the UK enabled detection of annual variations in community diversity. These data indicated a greater effect of time of sampling than treatment on bacterial community structure. Differential abundance analysis of these taxa enabled us to determine which taxa contributed to these shifts.

Temporal changes in biochar treated plots were a result of enriched *Acidobacteria* in UK1YBC samples, and elevated proportions of *Actinobacteria* in UK1MBC samples (4 % and 7% respectively) (Figure 24 A). The classes *Acidobacteria-6* (3 %) and *Solibacteres* (3 %), contributed to the UK1YBC *Acidobacteria* enrichment, whilst the UK1MBC *Actinobacteria* enrichment resulted from a single large increase in the *Thermoleophilia* (2 %) and several small genus level shifts (Figure 24 B). UK1MBC also exhibited increases in the unassigned genus of the candidate order *iii1-15* (3%), within the *Proteobacteria*. Shifts were detected at lower taxonomic levels which were not detected at higher levels, due to their being masked by shifts in subtaxa which counteract one another, the largest being a 2.5% enrichment in the *Alphaproteobacteria* genus *Rhodoplanes*, first detected as an increased abundance of *Hyphomicrobiaceae*, a family within the *Alphaproteobacteria*.

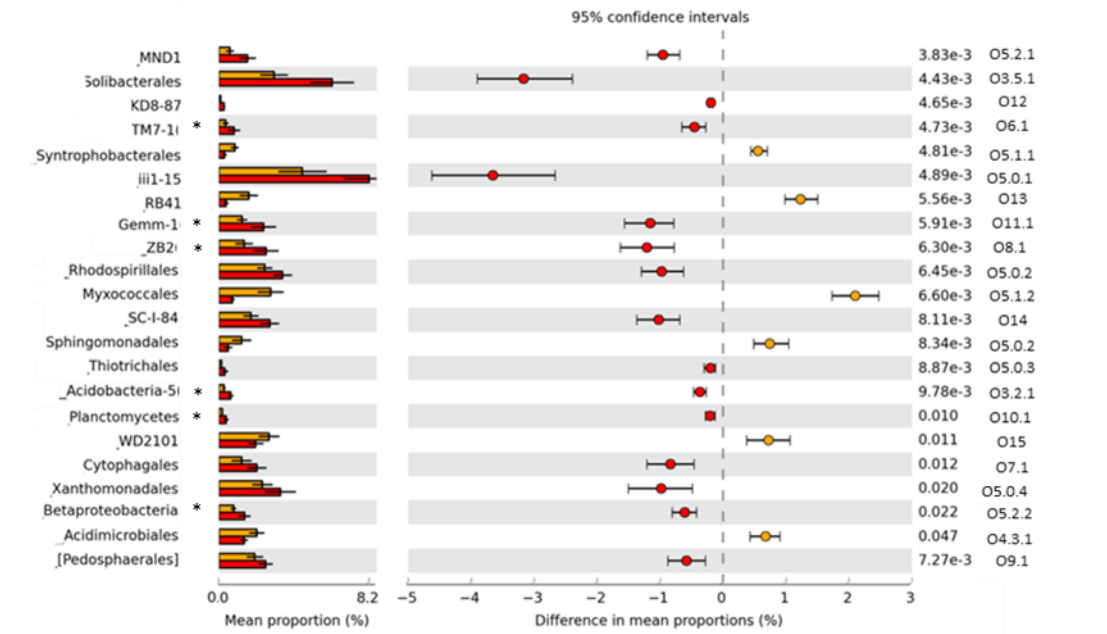
A)


95% confidence intervals
OD1
Gemmatimonadetes
Acidobacteria
Actinobacteria
Proteobacteria
4.88e-3 P1
5.49e-3 P2
0.015 P3
0.044 P4
0.036 P5
0.0
30.8
Mean proportion (%)
−8 −6 −4 −2 0 2 4 6 8 10
Difference in mean proportions (%)

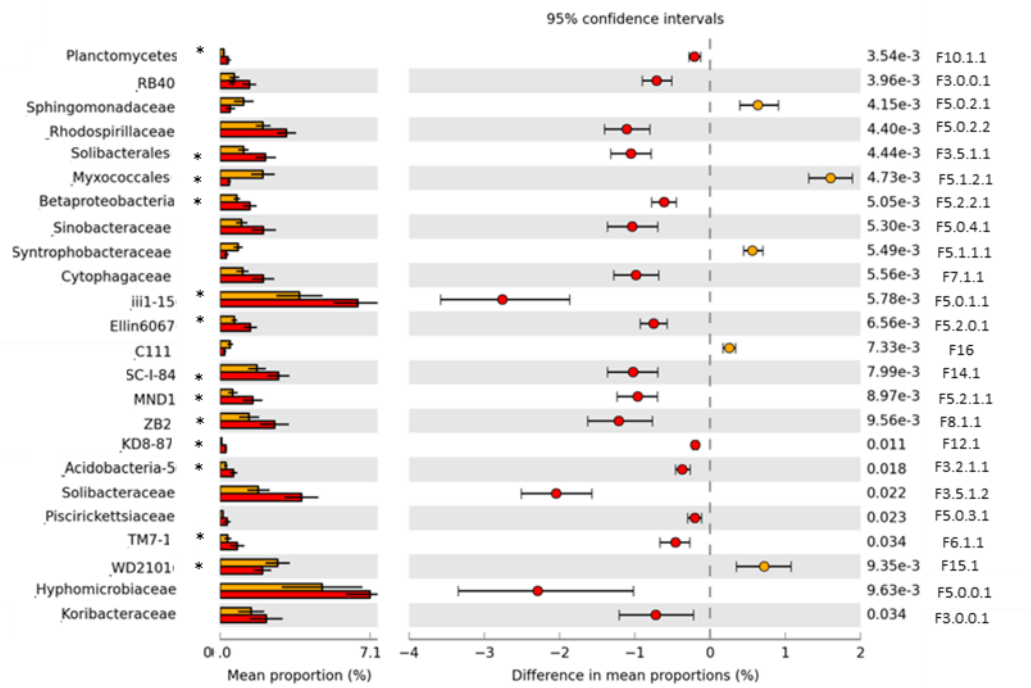
B)


95% confidence intervals
Acidobacteria-6
Acidobacteria-5
TM7-1
Cytophagia
ZB2
[Pedosphaerae]
Deltaproteobacteria
Thermoleophilia
Planctomycete *
Betaproteobacteria
Gemm-1
Acidimicrobiia
[Chloracidobacteria]
Solibacteres
Phycisphaerae
3.97e-3 C3.1
4.08e-3 C3.2
4.20e-3 C6
4.32e-3 C7
4.45e-3 C8
4.74e-3 C9
4.90e-3 C5.1
6.38e-3 C3.3
6.68e-3 C10
8.16e-3 C5.2
0.013 C11
0.021 C4.3
0.029 C3.4
0.049 C3.5
0.012 C3.6
0.0
9.7
Mean proportion (%)
−5 −4 −3 −2 −1 0 1 2 3 4
Difference in mean proportions (%)

C)


95% confidence intervals
MND1
Solibacterales
KD8-87
TM7-1
Syntrophobacterales
iii1-15
RB41
Gemm-1 *
ZB2 *
Rhodospirillales
Myxococcales
SC-I-84
Sphingomonadales
Thiotrichales
Acidobacteria-5 *
Planctomycetes *
WD2101
Cytophagales
Xanthomonadales
Betaproteobacteria *
Acidimicrobiales
[Pedosphaerales]
3.83e-3 O5.2.1
4.43e-3 O3.5.1
4.65e-3 O12
4.73e-3 O6.1
4.81e-3 O5.1.1
4.89e-3 O5.0.1
5.56e-3 O13
5.91e-3 O11.1
6.30e-3 O8.1
6.45e-3 O5.0.2
6.60e-3 O5.1.2
8.11e-3 O14
8.34e-3 O5.0.2
8.87e-3 O5.0.3
9.78e-3 O3.2.1
0.010 O10.1
0.011 O15
0.012 O7.1
0.020 O5.0.4
0.022 O5.2.2
0.047 O4.3.1
7.27e-3 O9.1
0.0
8.2
Mean proportion (%)
−5 −4 −3 −2 −1 0 1 2 3
Difference in mean proportions (%)

D)



E)

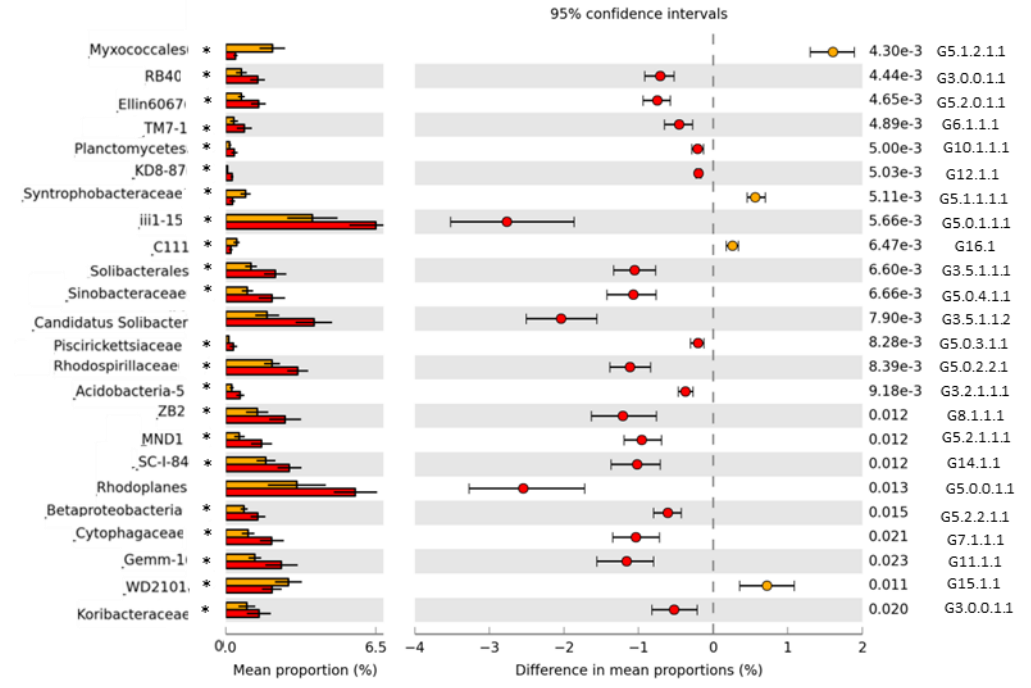


Figure 24 Extended error bar plot showing bacterial a) phyla, b) classes, c) orders, d) families and e) genera with significant q-values (<0.05) UK1M (Red) with UK1Y (Yellow), * = unidentified taxa within the taxon denoted

UK1YC samples showed similar results to biochar samples (Figure 25 A). However, these results also revealed an increase in the abundance of *Verrucomicrobia* in UK1YC samples not detected in biochar samples of the same time period. Therefore, it is possible that biochar actively selects against the phylum *Verrucomicrobia*. Similarly, *Nitrospirae* were significantly enriched in UK1MC samples only. There is an additional enrichment of classes *Alphaproteobacteria* and *Spartobacteria* in UK1YC, and *Actinobacteria* in UK1MC samples compared with their biochar equivalents (Figure 25 B). Again, this may reflect taxa which are affected by interactions between annual variation and treatment.

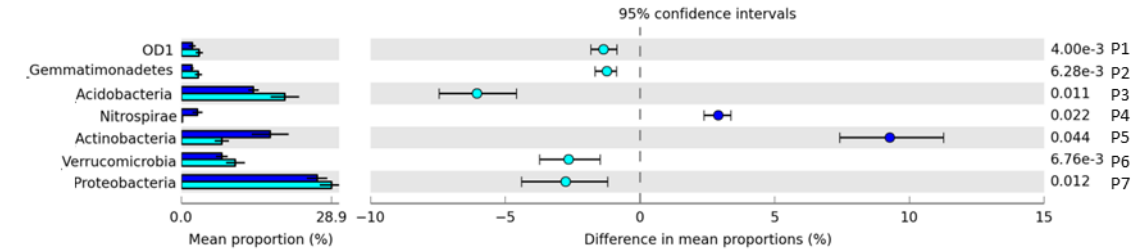
Mean abundance decreased in several orders in UK1MC and UK1YC samples by >1%, matching the shifts which occurred in UK1MBC and UK1YBC samples (Figure 24, Figure 25). However, a greater number of significant shifts in differential abundances were detected in control samples compared with biochar treated samples.

The bulk of abundance shifts in family level data were a result of increases of <1%, although the *Solibacteraceae, Hyphomicrobiaceae, Chthoniobacteraceae,* unassigned *iii1-15* and *Koribacteraceae* represented taxa enriched by >1% in UK1MC samples. This is in contrast to the *Gaiellaceae,* unassigned *Myxococcales* and candidate family *0319-6A21* which increased by >1% in the UK1MC samples (Figure 25 D). Genera which contributed to these shifts in UK1YC at >1% included *DA101, Rhodoplanes, Candidatus Solibacter* and a genus within candidate order *iii1-15*, whilst at UK1Y, candidate genera of the families *0319-6A21* and *Gaiellaceae* were increased (Figure 25 E). Again, these are very similar to the responses noted in the biochar treated samples.

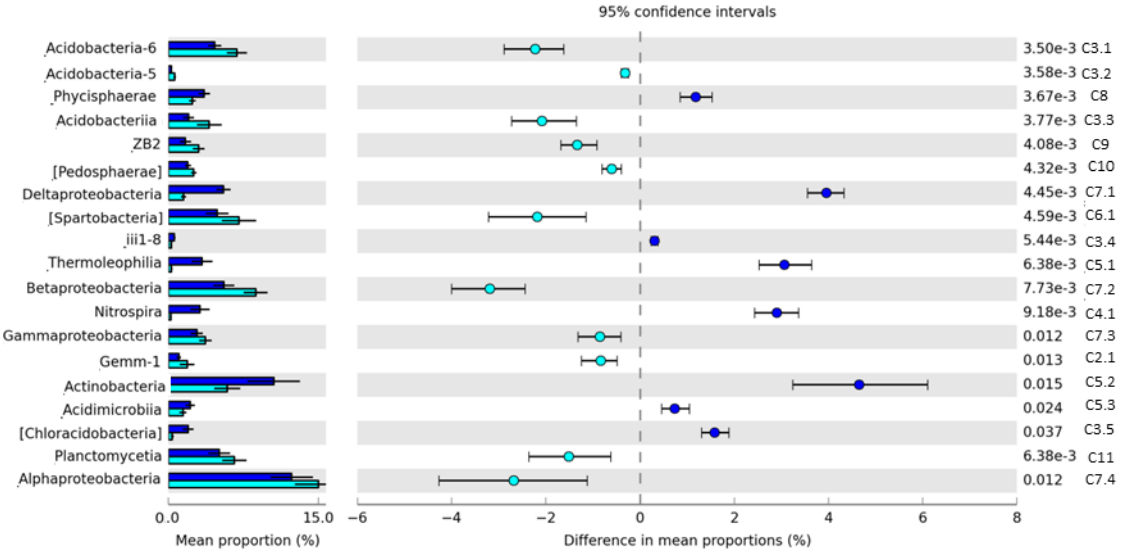
Taken together with the β diversity analysis, the divergence of clusters by time appears to be due to the phyla *Acidobacteria* and *Actinobacteria*, which show shifts of several percent due to time of sampling. However, the phylum *Proteobacteria* was also significantly affected, but only in control samples. In a similar vein to the *Verrucomicrobia*, this may indicate that the enrichment of *Proteobacteria* abundance may be prevented by biochar application. The *Proteobacteria* classes responsible for the shift are the *Alphaproteobacteria* and the *Gammaproteobacteria*, taxa which show no significant shift in biochar samples. Therefore it is these classes which may be affected by biochar treatment over time. However, it is difficult to determine with certainty any

further resolution of the taxa affected, as no substantial significant shifts occurred in the subordinate taxa of the *Alphaproteobacteria* and *Gammaproteobacteria*. Again, this points toward a range of small shifts within these taxa, which collectively caused the community shift noted in Section 3.3.3, but were not significant individually.

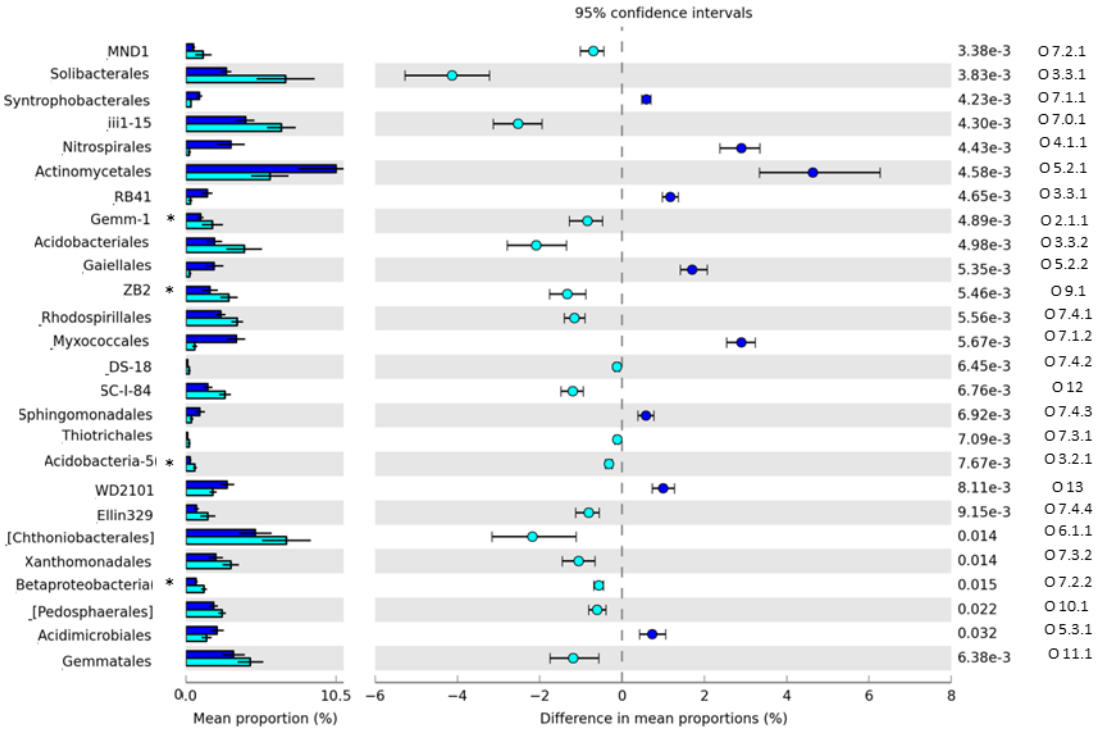
A)



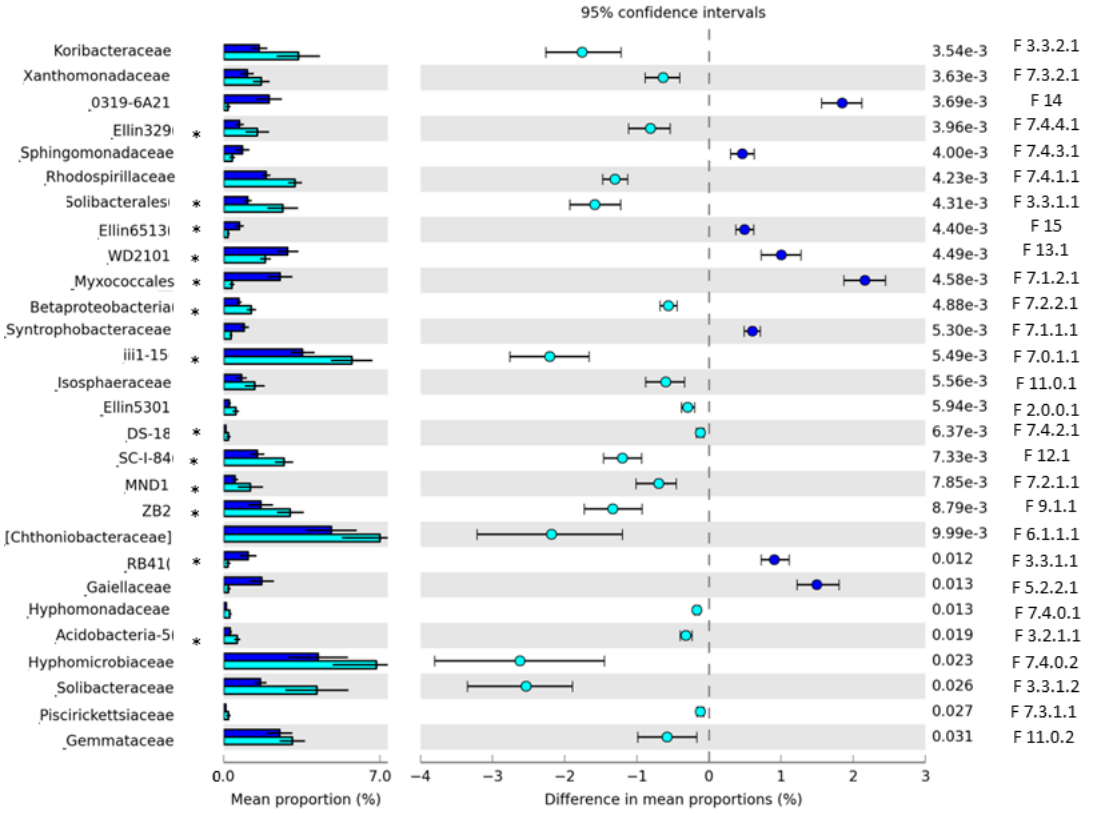
B)



C)


95% confidence intervals
MND1
Solibacterales
Syntrophobacterales
iii1-15
Nitrospirales
Actinomycetales
RB41
Gemm-1
Acidobacteriales
Gaiellales
ZB2
Rhodospirillales
Myxococcales
DS-18
SC-I-84
Sphingomonadales
Thiotrichales
Acidobacteria-5
WD2101
Ellin329
[Chthoniobacterales]
Xanthomonadales
Betaproteobacteria
[Pedosphaerales]
Acidimicrobiales
Gemmatales
3.38e-3 O 7.2.1
3.83e-3 O 3.3.1
4.23e-3 O 7.1.1
4.30e-3 O 7.0.1
4.43e-3 O 4.1.1
4.58e-3 O 5.2.1
4.65e-3 O 3.3.1
4.89e-3 O 2.1.1
4.98e-3 O 3.3.2
5.35e-3 O 5.2.2
5.46e-3 O 9.1
5.56e-3 O 7.4.1
5.67e-3 O 7.1.2
6.45e-3 O 7.4.2
6.76e-3 O 12
6.92e-3 O 7.4.3
7.09e-3 O 7.3.1
7.67e-3 O 3.2.1
8.11e-3 O 13
9.15e-3 O 7.4.4
0.014 O 6.1.1
0.014 O 7.3.2
0.015 O 7.2.2
0.022 O 10.1
0.032 O 5.3.1
6.38e-3 O 11.1
0.0
10.5
Mean proportion (%)
−6
−4
−2
0
2
4
6
8
Difference in mean proportions (%)

D)


95% confidence intervals
Koribacteraceae
Xanthomonadaceae
0319-6A21
Ellin329
Sphingomonadaceae
Rhodospirillaceae
Solibacterales
Ellin6513
WD2101
Myxococcales
Betaproteobacteria
Syntrophobacteraceae
iii1-15
Isosphaeraceae
Ellin5301
DS-18
SC-I-84
MND1
ZB2
[Chthoniobacteraceae]
RB41
Gaiellaceae
Hyphomonadaceae
Acidobacteria-5
Hyphomicrobiaceae
Solibacteraceae
Piscirickettsiaceae
Gemmataceae
3.54e-3 F 3.3.2.1
3.63e-3 F 7.3.2.1
3.69e-3 F 14
3.96e-3 F 7.4.4.1
4.00e-3 F 7.4.3.1
4.23e-3 F 7.4.1.1
4.31e-3 F 3.3.1.1
4.40e-3 F 15
4.49e-3 F 13.1
4.58e-3 F 7.1.2.1
4.88e-3 F 7.2.2.1
5.30e-3 F 7.1.1.1
5.49e-3 F 7.0.1.1
5.56e-3 F 11.0.1
5.94e-3 F 2.0.0.1
6.37e-3 F 7.4.2.1
7.33e-3 F 12.1
7.85e-3 F 7.2.1.1
8.79e-3 F 9.1.1
9.99e-3 F 6.1.1.1
0.012 F 3.3.1.1
0.013 F 5.2.2.1
0.013 F 7.4.0.1
0.019 F 3.2.1.1
0.023 F 7.4.0.2
0.026 F 3.3.1.2
0.027 F 7.3.1.1
0.031 F 11.0.2
0.0
7.0
Mean proportion (%)
−4
−3
−2
−1
0
1
2
3
Difference in mean proportions (%)

E)

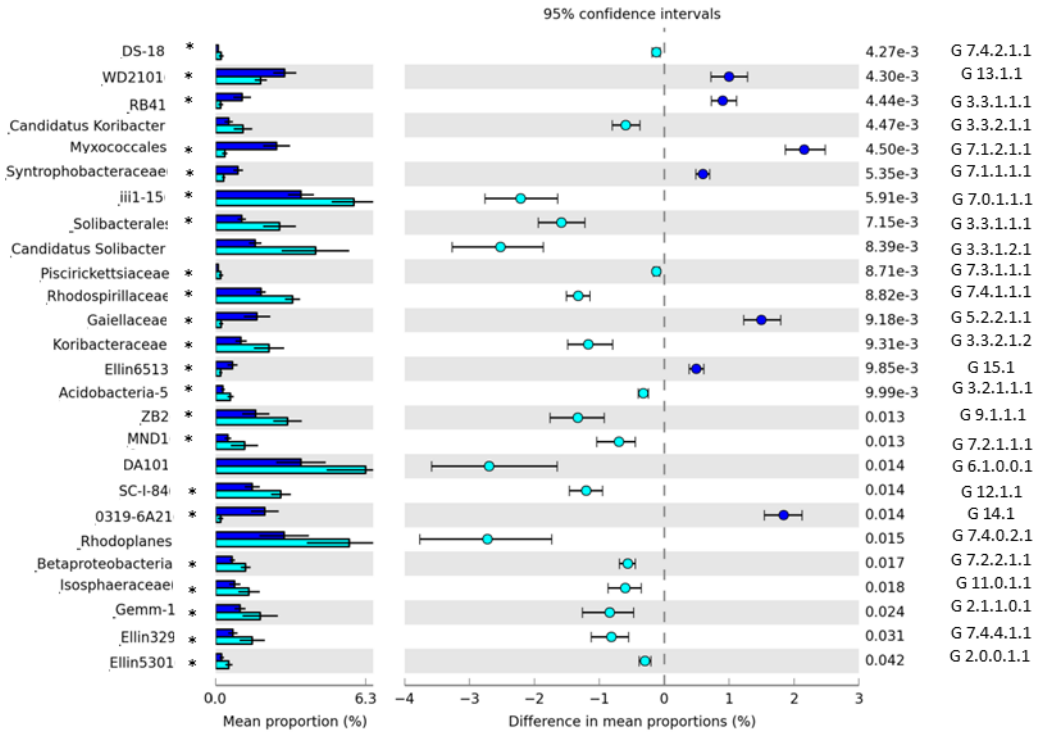


Figure 25 Extended error bar plot showing bacterial a) phyla, b) classes, c) orders, d) families and e) genera with significant q-values (<0.05) UK1M (Blue) with UK1Y (Light blue), * = unidentified taxa within the taxon denoted

3.3.6 Biochar effects on fungal richness

Comparison of the Observed Species metric 1 year after treatment (UK1Y) yielded a significant difference (p = 0.032), and Chao1 noted a similar increase, although not significant (*Table 20*). However, no significant shift in fungal richness was noted in either FR or IT. It therefore appears that application of biochar leads to increased numbers of fungal taxa being present within the soil environment in the UK site, although this is not noted in the short-term, one month after treatment. As sites differ in their abiotic conditions, and baseline fungal communities, this may indicate the ability of fungal taxa in the UK to utilise newly available niches in the biochar environment, which cannot be accessed by taxa in continental Europe.

Table 20 Results of t-tests for difference in ITS α diversity between treatment (BC, biochar, or C, biochar) at each of three sites. Results of sampling in the UK at 1 month (UK1M) and 1 year (UK1Y) are shown. μ = sample mean, σ = standard deviation.

Site	Chao1	OBS	Shannon	Rarefied to:
FR	BC: μ=672.3, σ=57.5, C: μ=666.1, σ=62.3, p= 0.79	BC: μ=416.3, σ=32.1, C: μ=408.5, σ=39.3, p= 0.56	BC: μ=6.6, σ=0.3, C: μ=6.6, σ=0.5, p= 0.855	3580
IT	BC: μ=770.4, σ=75.2, C: μ=764.6 , σ=53.7, p= 0.79	BC: μ=450.4, σ=46.6, C: μ=450.4, σ=43.0, p= 1.0	BC: μ=6.0, σ=0.5, C: μ=5.7, σ=0.4, p= 0.075	5061
UK1M	BC: μ=907.5, σ=173.8, C: μ=941.8, σ=98.6, p=0.53	BC: μ=582.1, σ=83.6, C: μ=560.8, σ=122.4, p=0.59	BC: μ=5.8, σ=1.1, C: μ=6.0, σ=0.7, p= 0.63	10562
UK1Y	BC: μ=360.8, σ=64.1, C: μ=309.4, σ=50.1, p= 0.032	BC: μ=627.4, σ=85.7, C: μ=562.7, σ=85.7, p= 0.051	BC: μ=5.1, σ=0.8, C: μ=4.7, σ=0.8, p= 0.119	4271

3.3.7 Changes in fungal community structure due to biochar application

The UK1Y dataset showed no significant difference in fungal β diversity, with no distinct clustering occurring due to treatment (Appendix B ii). Time series data showed that whilst no difference was noted between treatments in UK1Y samples, a significant difference was detected in UK1MBC and UK1MC samples (Bray–Curtis, ADONIS: R^2 = 0.06, q = 0.017) (Figure 26). Thus short-term changes in community occurred due to biochar treatment, caused by changes in the abundances of taxa present. When combined with the α diversity results for this time-point, this suggests that the shift is either a result of variations in abundance alone, or due to succession effects as some taxa are replaced by others.

Running UK samples collectively yielded no visible clustering effect by year or treatment (Appendix B i), suggesting that fungi were more adapted to deal with annual variation than bacterial communities. Combined with the PCoA plots, UK1Y dataset divides from the UK1M dataset although there is considerable overlap between the groups. Therefore a shift in the communities by year of

sampling occurs, as UK1Y communities diverge from their UK1M counterparts. Pretreatment samples cluster, suggesting that UK1M samples diverge from them due to temporal effects, although again, there is considerable overlap between the pre-treatment communities and their UK1M counterparts. Therefore a gradual shift in community occurs with time, but not as a result of treatment, and may be related to other edaphic variables.

Results from IT indicated a significant difference between biochar and control plots (Bray-Curtis, ADONIS: $R^2 = 0.09$, $q = 0.008$ Figure 28). There was also a significant effect of biochar on β diversity for the FR samples (Bray-Curtis, ADONIS: $R^2 = 0.05$, $q = 0.021$). Again, this was seen as a separation of biochar and control sample (Figure 27).

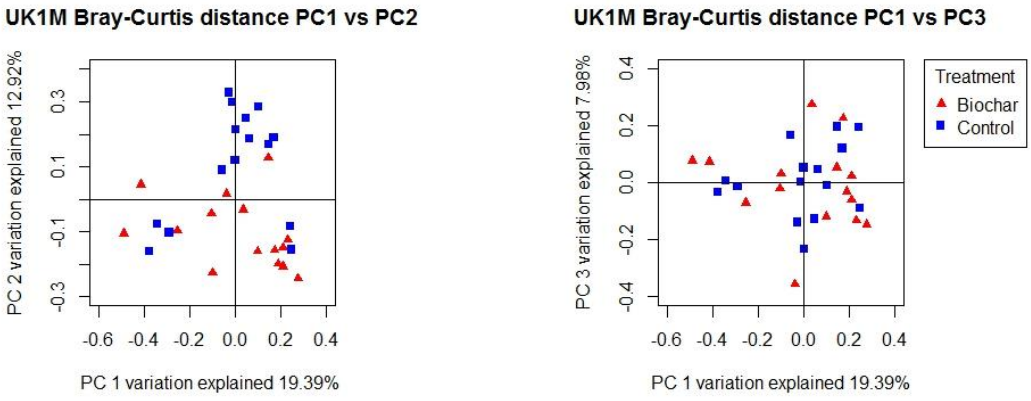


Figure 26 PCoA of Bray-Curtis dissimilarity for UK1M

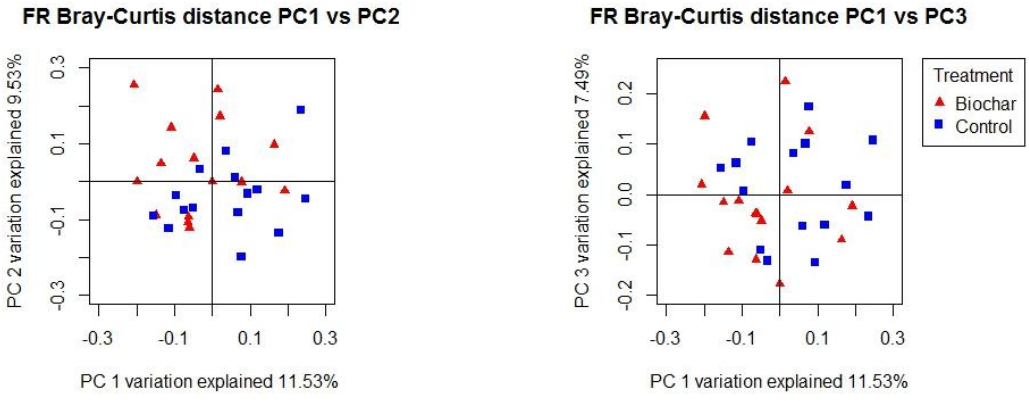


Figure 27 PCoA of Bray-Curtis dissimilarity for FR

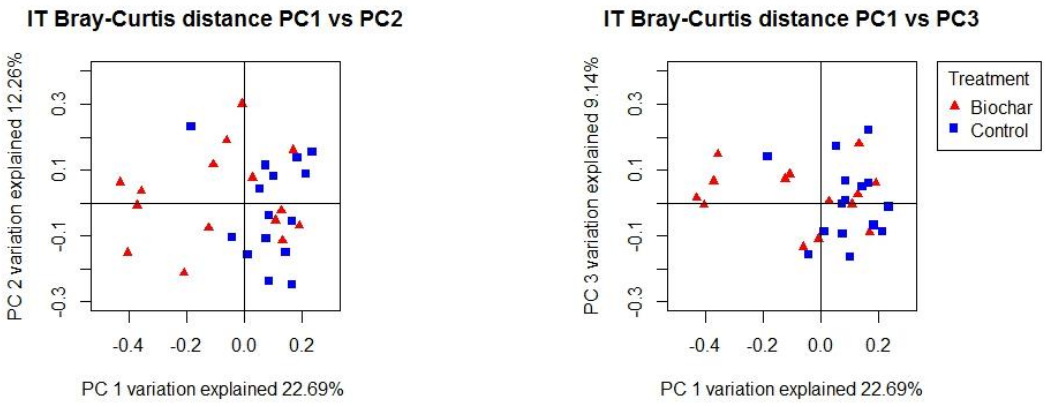


Figure 28 PCoA of Bray-Curtis dissimilarity for IT

3.3.8 Changes in fungal taxonomic abundance due to biochar application

Results of fungal analysis also showed variation in community composition by site (Figure 30). UK1Y samples were dominated by *Basidiomycota*, unidentified fungi and *Ascomycota* (35-37%, 42-42.5% and 15-16% respectively). IT samples were prevalently *Basidiomycota*, with similar proportions of *Ascomycota*, whilst FR samples consisted of *Ascomycota* and unidentified fungi, with small numbers of *Basidiomycota*. It should be noted that the phyla of *Glomeromycota* and *Zygomycota* were only detected in FR. Mean differences between treatments (as described in Section 3.3.4) suggest the significant β diversity change noted in IT was due to a decline in *Basidiomycota* and an increase in unidentified fungi and *Ascomycota* (Figure 31 C).

Whilst a difference in β diversity was noted in the samples from FR, probably due to a range of small variations in proportional abundance (Figure 31 D). The changes in β diversity detected in UK1M appear to be driven by increased *Basidiomycota*, decreased *Ascomycota* and unidentified fungi in biochar plots (Figure 31 A).

Results of STAMP analysis using the same cut-offs described in Section 3.3.4 indicated a significant increase in the family *Chaetothyriaceae* ($q = 0.034$) with a mean increased effect size of 0.65% in biochar treated samples (Figure 29). No other significant differences in abundance were detected at any taxonomic level in any of the other samples. Therefore, significant differences in β diversity detected in IT, FR and UK1M are representative of many small, but

individually non-significant changes in fungal taxon abundance, which collectively cause a detectable difference in total community structure.

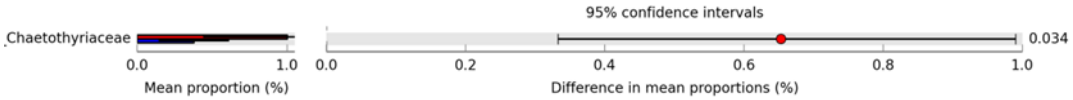


Figure 29 Extended error bar plot showing genera in IT with significant q-values (<0.05). Blue = control, Red = biochar

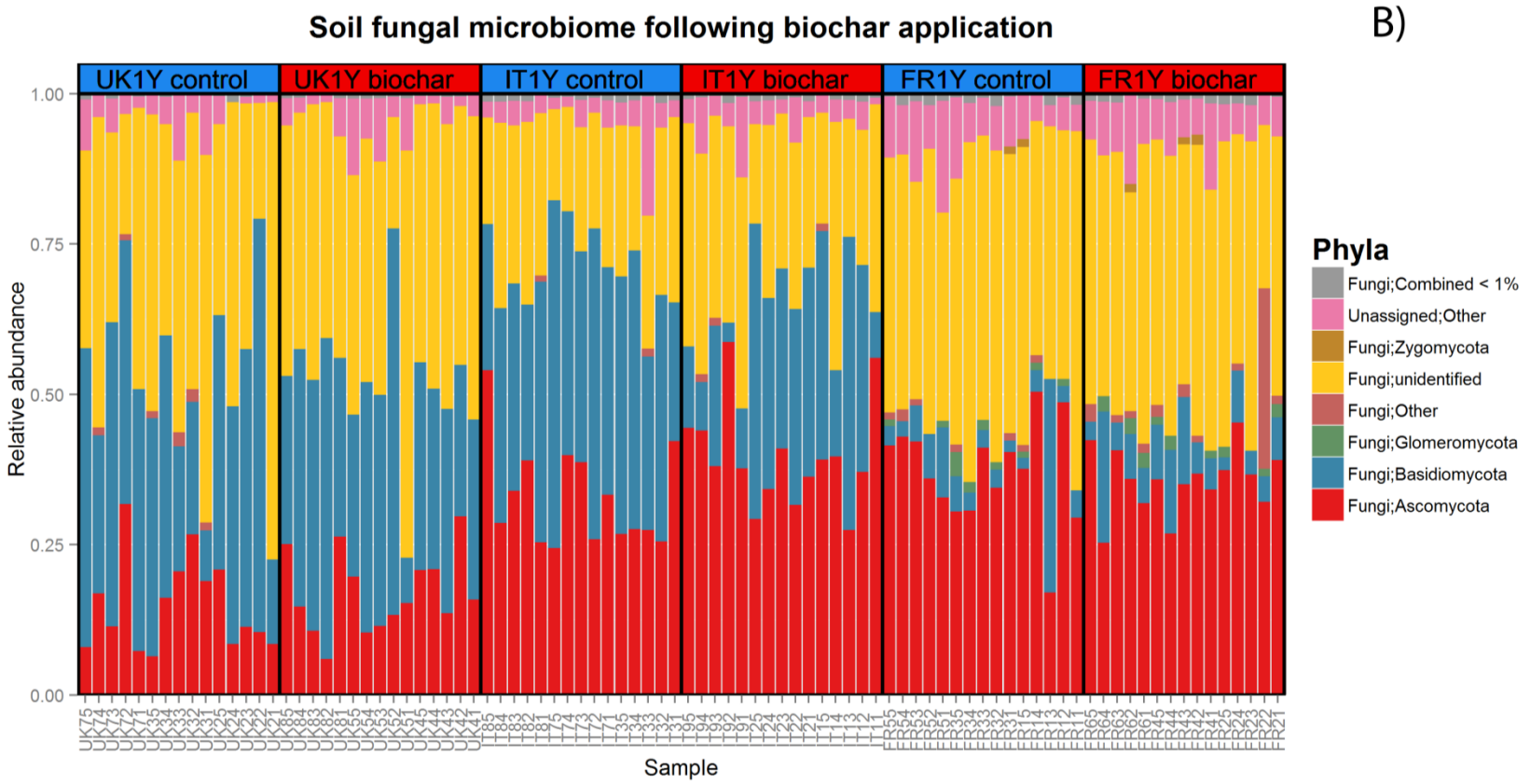


Figure 30 Taxonomic breakdown of proportional change in fungi. Relative abundance of each of the core phyla (above 1%) is represented. Blocks at the top of the figure indicate the treatment type associated with each "block" of replicated samples. Red represents biochar treated samples, whilst blue indicates a control treatment. Labels within each block indicate the sample site, UK, IT or FR. All samples shown are from samples collected 1 year after biochar application.

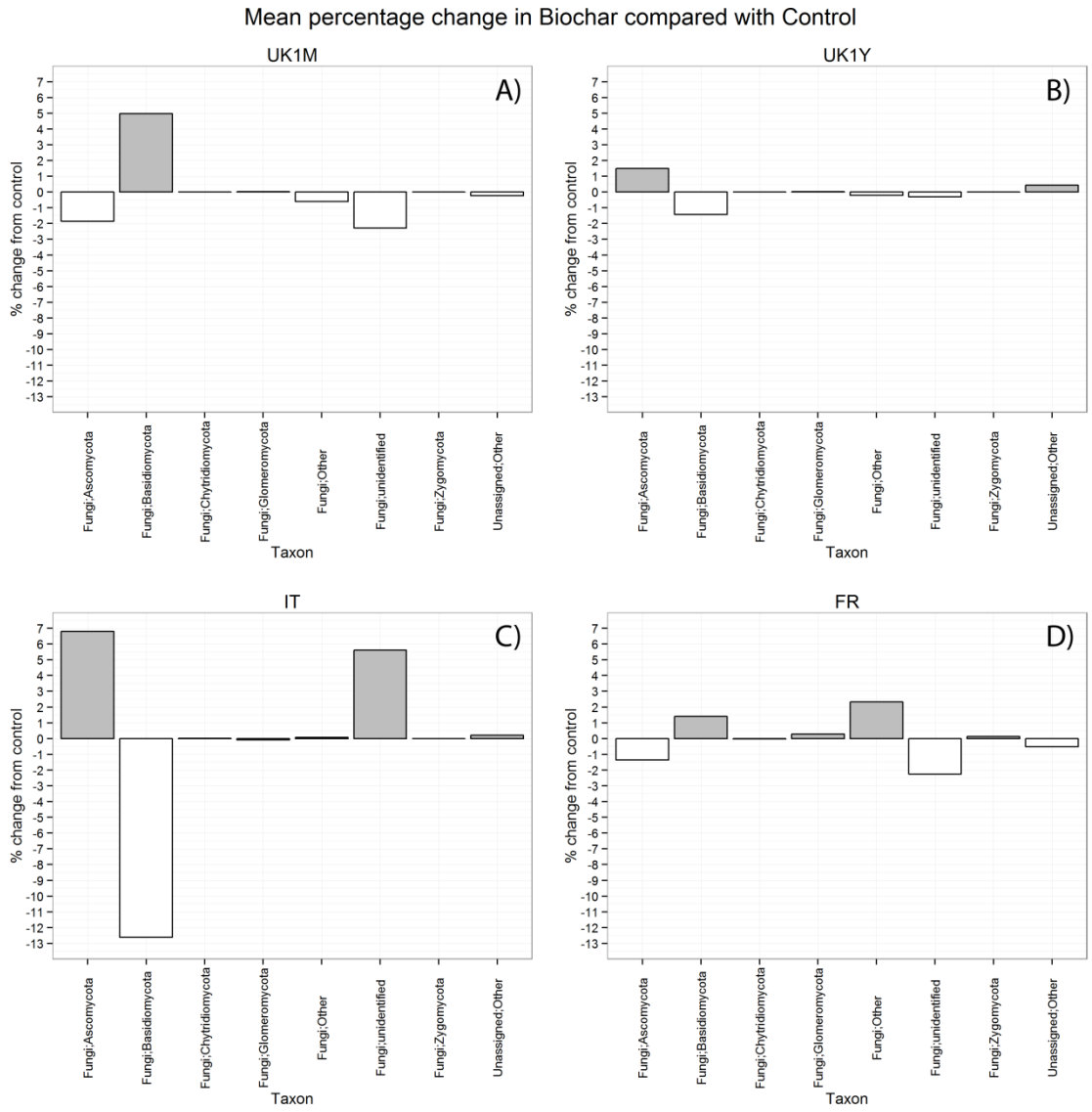


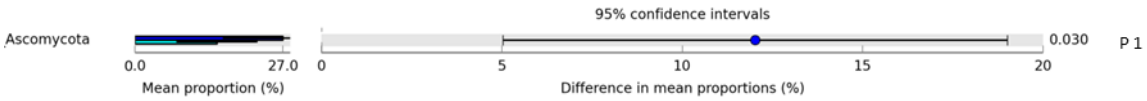
Figure 31 Mean percentage change in biochar plots from control for each of the fungal phyla detected. Plots represent A) UK1M, B) UK1Y, C) IT and D) FR. Grey bars show an increase in percentage abundance (total), whilst white bars represent a decline.

3.3.9 **Temporal changes in fungal community structure**

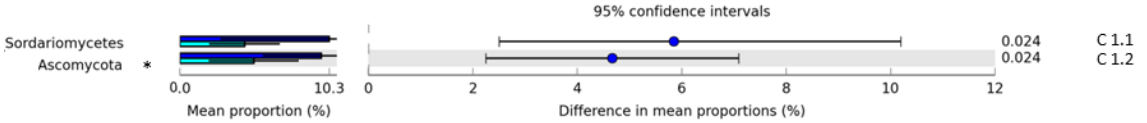
STAMP analysis of temporal variation revealed no significant differences in UK1YBC compared to UK1MBC at any taxonomic level, suggesting annual shifts detected were due to small adjustments in the abundances of many taxa. This indicated fungal communities are more stable over time, and show less variability than their bacterial counterparts. UK1YC and UK1MC displayed a significant mean increase (12%) in the phylum *Ascomycota* in UK1M samples

(Figure 32) as a result of a 6% increase in the *Sordariomycetes* and a 4.5% increase in unidentified *Ascomycota* classes (Figure 32). At the level of order, *Sordariales* and an unassigned *Sordariomycetes* were increased by approximately 1.5%, whilst unassigned *Ascomycota* still contributed 4.5% (Figure 32). This is further reflected at family and genus levels, with unassigned subgroups of each of the aforementioned orders contributing significantly to the differences noted. Therefore, although no significant differences in taxonomic abundance in biochar treated samples occur by year of treatment, there is annual variation on the abundance of unidentified *Ascomycota* and *Sordariomycetes* in control samples. Biochar therefore appears to prevent annual variation occurring in the aforementioned fungal taxa.

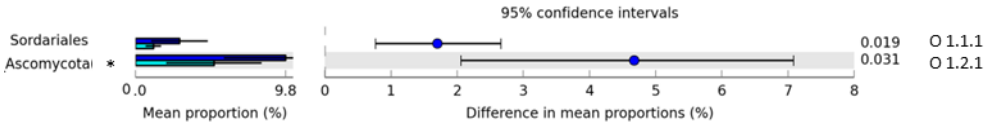
A)



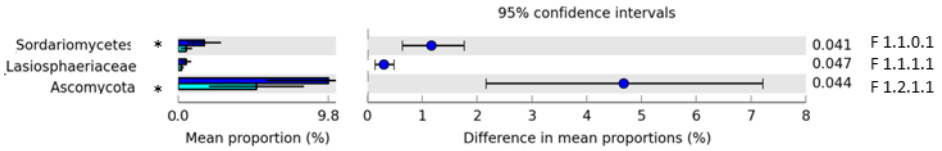
B)



C)



D)



E)

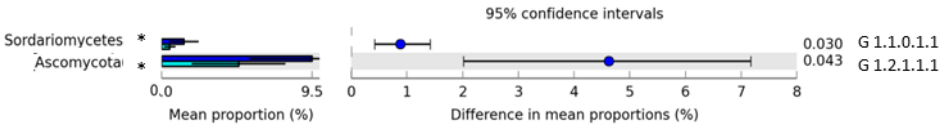


Figure 32 Extended error bar plot showing fungal a) phyla, b) classes, c) orders, d) families and e) genera with significant q-values (<0.05) Blue = UK1MC, light blue = UK1YC, * = unidentified taxa within the taxon denoted

3.4 Discussion

3.4.1 Context and summary of findings

Many previous studies have relied on incubation experiments in microcosms, followed by assessment of community change (Anderson *et al.* 2011; Ding *et al.* 2013; Xu *et al.* 2014a; Hu *et al.* 2014). Whilst providing valuable information, these studies do not assess the changes caused by biochar in the field. Continuity of microbial response was assessed by applying consistent methods and the same biochar to diverse long-term field sites, and determining whether similar modifications in community structure occurred at all three sites. However, the most striking finding is that annual variation and site are considerably more important in influencing the soil microbiome than relatively large-scale applications of biochar.

Significant changes in bacterial and fungal community composition were identified following biochar application, consistent with previous findings, (Khodadad *et al.* 2011; Kolton *et al.* 2011; Prayogo *et al.* 2013; Xu *et al.* 2014a; Hu *et al.* 2014). Bacterial community change appears to result from several small changes in abundance across phyla in the UK. However, the UK time series analysis revealed temporal shifts in bacterial community after one year of treatment that were not detectable after one month. For fungi, the inverse was apparent, with community shifts revealed at one month, but not one year. These shifts across field sites and time suggest that the inconsistency of previous findings reflect true diversity of response, which were not artefacts of differences between methodologies and/or the biochar used. In addition, whilst community change due to biochar was noted at each site, the UK time series experiment indicated that annual variation in bacterial soil community diversity is greater than that of the treatment.

3.4.2 The effect of biochar on community structure, and possible ecological implications

3.4.2.1 Bacteria

No change in the number of taxa present was noted, regardless of treatment although a temporal shift between sampling dates was detected. Whilst the

identities of taxa within the community may have changed, the total number of taxa remained the same. This scenario would arise if either there is no change in the taxa within the community, through a succession effect, or through a combination thereof. This contrasts with data obtained in laboratory experiments in which biochar significantly increased taxonomic diversity (Hu et al. 2014; Xu et al. 2014). However, both studies used soils with low pH (4.5 and 3.7 respectively), and the biochars applied had significant liming effects. Neutral pH soils are known to support greater diversity of taxa than acid soils (Fierer & Jackson 2006). Thus, liming effects in the aforementioned studies are likely to be the driver of increased diversity. However, it has been suggested that liming effects caused by pH may have minimal effects on microbial community structure, and that other biochar associated variables may have greater impact (Prayogo *et al.* 2013). Presently, pH at each site was near neutral prior in control samples, and although a significant increase in pH occurred at each site, the soil environment was not strongly acid initially, limiting associated increases in α diversity.

Significant changes in bacterial community structure were detected. In the UK and IT, weighted and unweighted β diversity responded, suggesting a change in both the taxa present and their abundance. However in FR, only unweighted diversity was significantly different. This indicated different taxa were present between treatments, but that shared taxa across treatments dominated the community and were similar in proportion.

Whilst mean differences by treatment provided an indication of which phyla were likely to respond to biochar treatment, STAMP analysis of differential abundance of individual taxa found no significant difference at the level of phylum. This confirmed that the response was a result of a multitude of small but significant changes in taxa, the largest of which being decreased abundance of the *Acidobacteria* class in UK1YBC samples. *Acidobacteria* are often unaffected by the presence of charcoal in *terra preta* soils (Grossman *et al.* 2010; Taketani *et al.* 2013), although the results in this section contradict this. However, *terra preta* soils contain ancient sources of pyrolysed carbon, which may induce a short-term effect after application, which passes with time.

IT samples indicated an enrichment of *Acidobacteria* in control soils, in line with the results found in UK1YC samples, although the effect was not as great

(0.75 in ITC, compared with 1.5% in UK1YC. An increase in *Proteobacteria* (ITBC 3%) was detected driven predominately by *Alphaproteobacteria* (a 1.5% increase in ITBC) a class known to be able to metabolise arabinose, a component of vegetable derived hemicelluloses and bacterial membranes, possibly indicating an increased role for this taxa in decomposition (Verastegui *et al.* 2014). Approximately 1.25% of this increase was in the order *Rhizobiales,* a taxa associated with nitrogen fixation, plant growth promotion and increase SOM turnover (Spain *et al.* 2009; Bruto *et al.* 2014; Tkacz & Poole 2015).

Comparison across European sites shows very few consistent changes at the level of phylum. *Acidobacteria* was enriched in control samples at all sites and time points, although the extent of this varied by site. This may be due to an enrichment in control samples, or due to a decline in *Acidobacteria* in biochar plots. *Acidobacteria* dominate oligotrophic soils, so a decline in this phylum in biochar-treated soil implies an increasingly copiotrophic environment (Verastegui et al. 2014). Soil microbiome studies of Central Park have previously noted the driving effect of pH gradients, and also noted its particular effect on *Acidobacteria* proportional abundance (Ramirez *et al.* 2014).Therefore, the influx of high pH biochar and the subsequent associated increase in pH at the sites may further lead to declines in *Acidobacteria* abundance.

As the rate and production method of biochar applied at each site was identical, it is interesting to note the range of responses. Incubation and pot experiments with biochar have noted changes in community (C. R. Anderson et al., 2011; Fox et al., 2014; Kolton et al., 2011). This indicates the potential of biochar to cause shifts in bacterial biodiversity, but these changes differ with environment. Interactions between biochar and environmental parameters such as soil chemistry, meteorological events and vegetation type are therefore likely to impact on community structure.

PLFA based methods suggest that bacterial diversity is strongly related to mean annual precipitation, indicating that biogeographical variables can trigger change in soil microbial communities (de Vries *et al.* 2012). Annual shifts in community has been associated with temperature, although variation in functional groups remained low (Delmont *et al.* 2012). However, the variations in weighted β diversity in the UK and IT, and unweighted shifts in FR

indicate that whilst the nature of the change varied by site, biochar treatment consistently led to variation in bacterial biodiversity.

3.4.2.2 **Fungi**

For fungi, β diversity in IT indicated a significant change in OTUs present compared with controls, which mean differences suggest were due to a large (12%) decline in *Basidiomycota*, resulting from *Agaricomycetes*, a class containing a range of saprotrophic, pathogenic and mutualistic organisms (Hibbett *et al.* 2014). However, detailed STAMP analysis did not indicate any significant differential abundances, with the exception of an increase in the pathogenic fungus *Chaetothyriaceae* in IT biochar samples (0.6 %). These are known leaf mould forming fungi (Chomnunti *et al.* 2012), and as such biochar application either increases their abundance within the soil directly, or elevates their rate of infection on fallen leaves. This may indirectly suggest an increase in the infection rate of the fungus, linking with previous research indicating biochar application may result in the down-regulation of immune response genes (Viger *et al.* 2015). However, the small effect size should be considered, and therefore this interpretation should be taken with caution.

Fungal diversity and abundance in FR varied due to multiple small shifts in OTUs, the largest of which was an increase in other unidentified fungi (2%). Whilst the community was impacted by biochar, there was no single driving taxon. STAMP analysis was in line with the results obtained by comparing proportional changes, showing no significant difference in any taxonomic groups. This corroborates the FR bacterial results, indicating that biochar application had little impact on *Dactylis* grassland populations. Similarly, UK1Y showed no single shift in any one taxon, and no change in fungal β diversity was noted. Lack of significant changes in differential abundance indicates that whilst short-term shifts occurred in fungal taxa in the UK, this was not translated to a large long-term change in community structure. It is unlikely that biochar application significantly affects fungal community structure or function in the long-term.

A range of responses were found across the sites, with substantial shifts occurring in IT due to single taxa, whilst the UK and FR exhibited small variations in many taxa. This may indicate that the variability in previous fungal research is a result of a genuine effect of biochar, in combination with

soil parameters and the original community composition. Fungal community composition is known to be driven by climatic variables, as these can determine the distribution of fungal host organisms soil moisture (Tedersoo *et al.* 2014). Drought experiments investigating heathland fungal diversity note a greater effect of seasonality than the treatment itself, with gradual declines in diversity during summer months. These result from declines in SOM reaching the soil for decomposition and decreased moisture availability (Toberman *et al.* 2008). Therefore, the range of climatic variables present at the sites may explain the variability of response.

3.4.3 Year of sampling has a greater effect on fungal and bacterial community structure than biochar treatment

UK bacterial time series data showed a strong effect of time of sampling, along PC1. Biochar caused grouping along PC2, although only in UK1Y samples. This indicated a change in communities due to an interaction between biochar and time, possibly as recalcitrant portions of the biochar became available (Watzinger *et al.* 2014). Gradual changes in soil nutrient profiles may select for copiotrophic organisms, as *r*-strategist bacteria outcompete more specialised oligotrophic *k*-strategists. Furthermore, differences in α diversity between UK1M and UK1Y indicate annual variations in diversity, potentially due to meteorological events or annual variation which may affect edaphic variables differently in the presence of biochar. For example, biochar is known to increase pore space and decrease bulk density in soils, which in turn can elevate soil water content (Baronti *et al.* 2014) resulting in selection pressures for bacterial communities (Manzoni *et al.* 2012).

The UK fungal time series displayed the inverse of the UK bacterial time series, with significant treatment effects on UK1M samples, but no change in UK1Y. Mean differences indicated elevated proportions of an unknown *Agaricales* family, *Thelephoraceae*, *Cortinariaceae* and *Bolbitaceae*. Genera within both the *Thelephoraceae* and *Cortinariaceae* have previously been demonstrated to be ECM (Horton & Bruns 2001). However, STAMP analysis showed no significant difference in the abundance of these taxa after correction, due to the high variability present between samples. Whilst the mean difference method may

be useful for determining the taxa responsible for the drift in β diversity measurements, they are not a viable method for assessing changes in differential abundance of individual taxa.

Fungal time series data yielded no obvious clustering effect by year, although a small significant effect was detected. Subsequent STAMP analysis of these datasets showed that whilst biochar samples remained unchanged with time, control samples displayed elevated abundance of *Ascomycota*. This effect is likely to be a stabilisation effect of biochar over time. This may be linked to biochars porosity, and accompanying water holding capacity increases. Biochar has been advocated as a suppressant of this taxa in paddy fields, through use of low resolution molecular methods (Chen *et al.* 2013). The lack of a stimulation effect by biochar application has been suggested as evidence that biochar is not an adequate substrate for decomposition by *Ascomycota*, and as such is likely to remain recalcitrant within the soil (Chen *et al.* 2013).

Whilst high resolution effects of treatment upon individual genera were detected, the study is limited in its ability to fully interpret these data. The first limitation of the method is the large number of tests which were required to be carried out. For example, STAMP statistical tests for each site were carried out independently. Whilst multiple test corrections were carried out for each individual method where appropriate (α diversity t-tests, ADONIS and STAMP analysis), there was no correction for the fact that a separate analysis was undertaken for each dataset. Therefore, it may be argued that a meta-p score would be more appropriate to decide which results were significant. However, if this were to be carried out, an adjusted p would be in the range of 0.05/30, a value which is likely to be too stringent. Given that each site is independent of each other, this type of adjustment was not considered appropriate.

The second limitation of the study is the difficulty in determining biological meaning from the results. Whilst detailed shifts in the abundance of genera present between treatments can be captured, the lack of information about uncultured taxa, and the ecological roles of those identifiable genera make it difficult to draw conclusions about functional implications. It is for this reason that these methods, whilst valuable, may become more useful in future when further information about fungal and biological taxa is known. Additionally, many bacterial taxa are almost ubiquitous, being able to adapt to survive across a range of environments due to their ability to transfer genes

horizontally. This may result in difficulties resolving the limiting factors in bacterial biogeography taxonomically as organisms of the same taxa may have varying functional attributes.

These methods also cannot detect changes in the rate of gene expression. It is possible that whilst some taxa show no change in abundance, the rate of expression of specific functional genes may change, leading to a variation in the organisms functional role.

Finally, it should be noted that it may be argued that the experimental design used in this chapter contains pseudoreplication, as multiple samples were collected from each plot. This was undertaken due to limitations in the number of plots available, and the variability in bacterial biogeography at small scales. However, fungal community variation is likely to be considerably lower at these lower geographical resolutions. Taken together, it may be argued that the statistical power is inflated, and not appropriate for fungal taxa.

3.4.4 Conclusions

In conclusion, metabarcoding is a powerful technique that can be used to detect changes in the soil microbiome-between contrasting sites and with time. Following biochar treatment and in contrast to earlier studies using mesocosms, metabarcoding of soil following field exposure to biochar revealed change occurring consistently in the proportional abundance of the microbiome. However, and most strikingly, the nature of the shift varied, presumably due to a combination of edaphic variables. Since soil fungal and bacterial communities provide essential biogeochemical cycling, and a range of ecosystems services, it is possible that biochar application may have implications for microbial nutrient cycling. However, at present, given the highly variable responses, no conclusions can be drawn between shifts in microbial communities, changes in the differential abundance of individual taxa and their ecological roles within the biochar environment.

Chapter 4: The effect of Biochar amendment on soil respiration and soil nutrient content in a Short Rotation Coppice (SRC) Willow crop in Southeast England

4.1 Introduction

Application of biochar to soil is suggested as a method to sequester carbon (Lehmann *et al.* 2006; Lehmann 2007; Woolf *et al.* 2010; Cayuela *et al.* 2013). Its application causes physico-chemical changes in the soil environment (Section 1.3.3), including changes in nutrient availability, nitrogen and phosphate cycling (Section 1.3.3.1). By combining biochar application and production with SRC biomass and bioenergy production (discussed in Section 1.5), it may be possible to sequester carbon whilst producing dedicated 2nd generation biofuel crops, such as Poplar, Willow and Miscanthus.

The implications of biochar addition for soil respiration are not fully understood, with potential impacts for plant growth rates (Jones *et al.* 2012; Schulz & Glaser 2012) and root/microbe relations (Kolton *et al.* 2011) (Section 1.3.3.3). Studies suggest microbial activity increases within soils after biochar application (Steinbeiss *et al.* 2009; Castaldi *et al.* 2011; Quilliam *et al.* 2012) (Section 1.3.3.3). Increased microbial activity may indicate elevated rates of SOM or biochar decomposition, reducing its suitability as a C sequestration method. However, studies rely upon incubation experiments within a lab environment (see *Table 3*) which are not representative of amendment in-situ. Therefore field studies are required.

Although biochar is demonstrably recalcitrant in soil (Lehmann 2007; Vaccari *et al.* 2011; Bai *et al.* 2013), there appear to be a range of potential residence times, depending upon the pyrolysis method, edaphic variables, and weathering effects (Mao *et al.* 2012; Fang *et al.* 2014; Kuzyakov *et al.* 2014). Furthermore, application of biochar can lead to priming effects. These include both positive and negative priming. Positive priming causes elevated rates of soil respiration, whilst negative priming is a decrease in associated respiration (Whitman *et al.* 2014). Priming effects further complicate respiration studies, as biochar application can increase soil respiration in the short-term (as labile fractions are decomposed), whilst simultaneously decreasing mineralisation of extant SOM in the long-term (Farrell *et al.* 2013). Thus, carbon balances are a combination of carbon applied as biochar, minus labile fractions respired, plus any change in mineralisation of C from SOM due to priming effects. Therefore, studies partitioning sources of soil C are essential to determine how biochar influences C cycling.

This Chapter aimed to measure changes in soil respiration in plots treated with biochar. Comparison of total respiration with heterotrophic respiration enabled us to partition sources of soil respiration, and their responses to biochar application. Simultaneously, assessment of changes in nitrogen and phosphorous leachate, and analysis of enzymatic activity were undertaken to better understand changes in biogeochemical cycling.

All work in this Chapter was undertaken with the collaboration of Dr M. Ventura, who advised on the installation of equipment, carried out extraction of nutrient leachate and nutrient analysis, Dr G. Alberti who advised on installation of the soil respiration systems, the troubleshooting and statistical analyses of soil respiration data and Dr F. Fornasier, who carried out enzyme activity protocols and analysis. Plot design and layout were implemented by Dr Maud Viger and Dr G. Alberti as part of the EuroChar project, and also helped to collect data. Some of the soil respiration data in this study (data from June 19th – December 1st 2012) has been published in (Ventura et al. 2015)

4.2 Method

4.2.1 Site

The site was a well-established short rotation coppice (SRC) willow crop in Pulborough, West Sussex (described in Chapter 3, Section 3.2.2, Figure 7). It was coppiced on a three year rotation, and was in its third year during biochar incorporation.

Eight plots 2.75 m by 4.3 m plots containing 20 trees were established, with four biochar and four control treatment plots, of which 6 plots were fitted with automated respiration units (Figure 33). System interfaces could accept a maximum of 12 units. Automated respiration units were fitted in February 2012 and measurements initiated in early April 2012. In each plot, one respiration unit was fitted with a root exclusion collar on 24th May 2012, consisting of a steel cylinder 40 cm deep and 32 cm in diameter. Collars were hammered into the soil to minimise disturbance to soil. Plants (including *Dactylis glomerata, Schedonorus* spp., *Lolium perenne, Taraxacum officinale* and *Plantago lanceolata)* were removed from heterotrophic chambers to ensure roots were not present, allowing for measurement of heterotrophic

respiration. Removal of plants was undertaken through scouring of the topsoil, and hand removal of the resulting biomass. Heterotrophic and total respiration was compared, with the difference in the two values representing respiration associated with roots. Comparison of heterotrophic and total respiration in control and biochar plots, aimed to determine whether roots (and root associated microbes) interacted with biochar treatment. However, it should be noted that plant roots were not removed from the soil environment, and so their short term decomposition could act as a confounding variable within the chambers. Additionally, chambers were designed for a short term experiment, and therefore it is possible that by the end of the two year study period, root growth from surrounding trees may have resulted in the introduction of roots within the exclusion chambers from below.

Biochar was applied on June 19th 2012 to four of the eight plots. Biochar was produced by advanced gasification technology, from a feedstock of maize silage by A.G.T Italy (see Chapter 3, Section 3.2.1 for detailed application method and *Table 18* for biochar details). This was selected due to the distinct ^{13}C signature found in maize, which varied from native ^{12}C SOC and could be measured by an associated isotopic study, aiming to assess the rate of biochar mineralisation (Ventura *et al.* 2015).

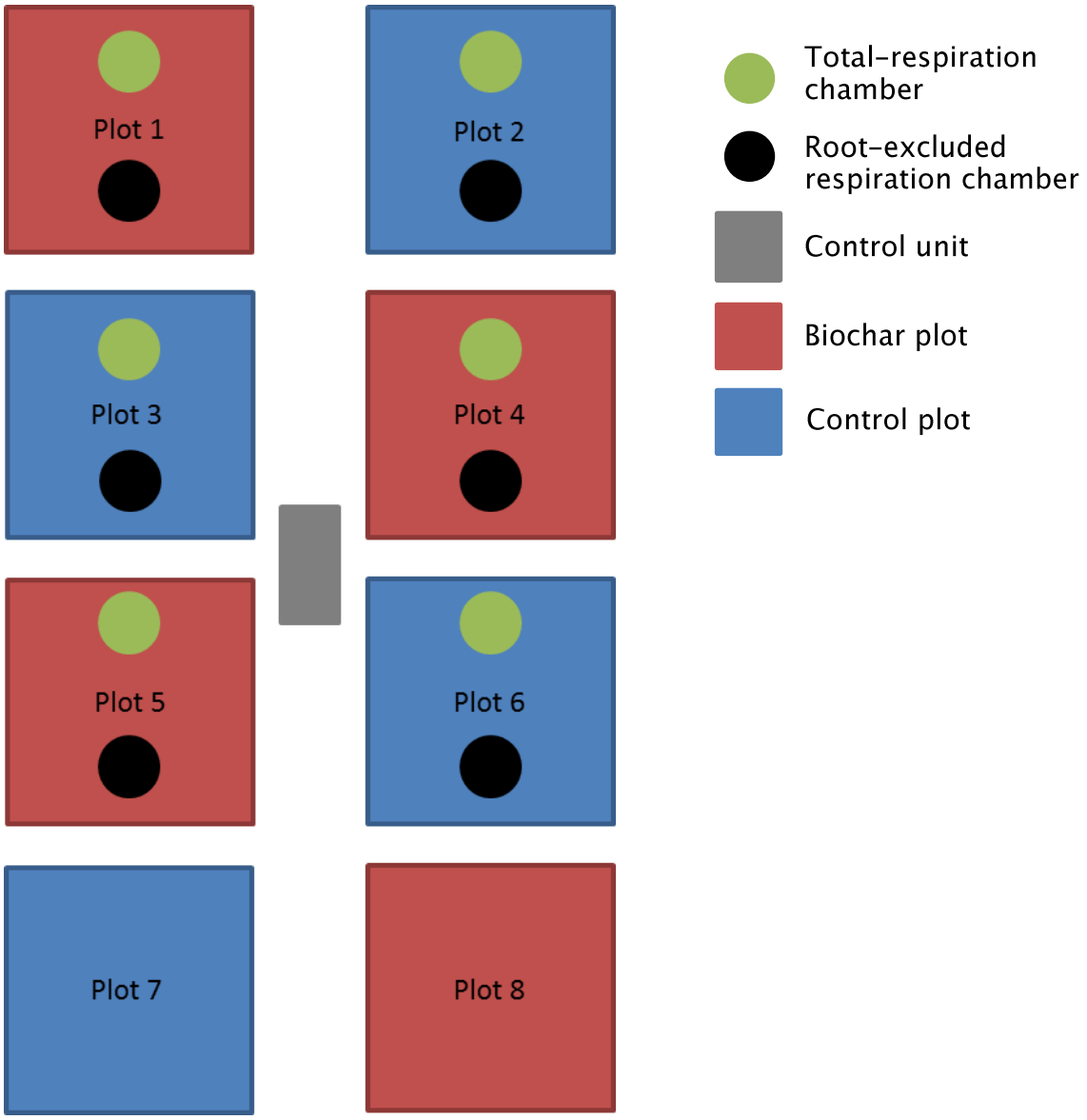


Figure 33 Plot layout and treatments for UK sites.

4.2.2 Soil respiration techniques

4.2.2.1 Sampling protocol

Soil respiration was measured automatically using the Uniud-SR system (Delle Vedove *et al.* 2007) every four hours, at 0:50, 4:00, 8:00, 12:00, 16:00 and 20:00 to encompass daily variation. Each chamber comprised of a steel cylinder with an automated lid. Chambers were connected to the soil respiration system by two pipes, allowing the air within each chamber to be cycled into the IRGA SBA-4 CO_2 analyser. Power was supplied to the motor on each chamber by a 10m cable attached to a solar and wind power unit.

A pressure vent was installed on each chamber so that static pressure within the chamber would remain in equilibrium after lid closure.

Soil moisture and temperature were measured automatically using six Campbell CS-616 soil moisture probes (one per plot) and twelve Campbell 107 thermocouples (2 per plot, at a depth of 2.5 and 5 cm) installed on 10th July 2012. Meteorological data on site was collected by an associated weather station consisting of a tipping bucket rainfall measure, incoming solar radiation monitor, air temperature thermometer, wind speed and direction (*Table 21*). Data was automatically collected and stored on a CR1000 data logger, retrieved manually on the 20th of each month.

Soil respiration was measured through increase of CO_2 within the chamber over time, from a baseline measurement. The rate of CO_2 accumulation provided an analogue for the rate of CO_2 evolution and dissipation in soils surrounding the chamber.

To estimate baseline concentrations, an average of 10 measurements of CO_2 concentration were produced during lid closure. This is C_0, concentration in µmol CO_2 mol^{-1} dry air at time point 0. Air samples from the chamber were pumped through the IRGA SBA-4, and CO_2 (µmol CO_2 mol^{-1} dry air during sampling, or *C*), water vapour mole fraction (*W,* mmol mol^{-1}), air temperature and pressure (*T*, °C and *P,* kPa respectively) measured (A. Peressotti, 2012).

Table 21 Meteorological equipment used on site

Equipment	**Function**
NRLITE net radiometer	Total incoming and outgoing solar radiation.
HMP155A probe	Temperature and relative humidity measure
Campbell 52203 rain gauge	Assessment of rainfall
Campbell 05103-5 wind monitor	Assessment of wind direction and speed

Increasing CO_2 concentration within the chamber headspace of the chamber gradually decreases the rate of diffusion from the soil, leading to inaccurate estimations of CO_2 evolution when using linear models. For this reason, a

nonlinear regression was used to predict a rate of CO_2 evolution over time (Delle Vedove *et al.* 2007). The method outlined in the paper uses the equation:

$$C(t) = C_x - (C_x - C_0)\, e^{-a\,(t\,-t0)}$$

where C(t) is CO_2 concentration, including water mole fraction correction, C_x is the asymptote, t_0 is the intercept, the point at which $C_0 = C(\,t\,)$, a is the curve of the line, and C_0 is the concentration of CO_2 within the chamber at the point the chamber is closed.

Rate of CO_2 evolution is determined through the equation:

$$dC/dt = a(C_x - C_0)e^{-a(t=t0)}$$

whereby t= t_0 and dC/dt is rate change of CO_2 over time. Finally, soil respiration (SR) is calculated through use of the equation:

$$SR = \frac{V}{S} * \frac{dC}{dt} * \frac{P_0}{R * (T_0 + 273.15)}$$

where V is the volume of the respiration chamber, S is the area of the chamber, P_0 is the average air pressure (kPa) of the 10 measurements during baseline determination, T_0 is average air temperature (°C) of the 10 measurements during baseline determination and R is the universal gas constant (8.31 J $mol^{-1}K^{-1}$).

4.2.2.2 Data processing and statistical analysis

Raw data collected by the data logger included both efflux measured and efflux modelled from soil temperature and water content data. QC was undertaken automatically. Samples with a difference of <3 ppm between initial and final CO_2 concentrations or those samples which, when plotted, produced concave gradients were removed from the dataset. These criteria indicated an issue with lid closure, or a leak. When CO_2 fluxes were linear, they were modelled using a linear model, otherwise, non-linear models were used. Linear fluxes were discarded when regressions were lower than R^2=0.90, and gapfilled (Ventura *et al.* 2015).

Daily averages were calculated for days in which 50% of time points successfully passed QC, and were replicated by plot and subtreatment (heterotrophic vs total respiration). Where data was removed by QC, or was

unavailable due to equipment malfunction, data was gapfilled using a model based upon temperature and soil water content (Qi & Xu 2001; Ventura *et al.* 2015). This takes the form of:

$$R = aT^b * SWC^c$$

In which *R* is total CO_2, *T* is soil temperature in degrees Celsius (measured by soil temperature probes) and SWC is soil water content in percent (measured by SWC probes).

Results for the time period of the 19th June 2012 (the day of biochar application) to the 1th of December 2012 (the first day of low power mode) were analysed and published as part of Ventura *et al.* 2015. The remaining portion of the data (December 1st 2012 to the 18th June 2014 was analysed independently using identical methods. Due to limited hours of sunlight during the winter, the system had to be switched to power saving mode, collecting only soil temperature and moisture data. Therefore data during the winter months is gapfilled.

Some intermittent gapfilling continued throughout Spring of 2014 until 10th June. This was due to the need to remove the equipment to allow for a commercial harvest of the field. Gapfilling during this time period utilised data from an adjacent meteorological station, which included SWC and soil temperature sensors.

Daily averages were analysed using ANOVA, with the factors of Biochar (Treated vs Control) and Root presence (heterotrophic respiration vs total respiration) and their interaction. Statistical analysis was carried out using IBM SPSS Statistics for Windows Version 23.0 (IBM Corp, Released 2011. Armonk, NY).

4.2.3 Nutrient leaching

4.2.3.1 Sample protocol

Resin lysimeters were installed in each plot on 10th July 2012, to assess ammonium, nitrate and phosphates present within leachate after treatment. Lysimeters consisted of a mixed ion-exchange resin (16.2g, Amberlite MB-150, Sigma-Aldrich) held within PVC pipe sections 3 cm high and with a diameter of

5 cm. To prevent resin directly contacting the soil, a section of glass beading (2 mm in diameter) was placed at either end and held in place using 125 µm nylon mesh (Scubla s.n.c, Remanzacco, UD, Italy), as previously described (Ventura *et al.* 2013). Lysimeters were buried vertically at 20 cm, with three placed within each plot (within the row of trees, at the edge of the row, and between two rows). Lysimeters were collected after one year during July 2013, and shipped to the University of Bolzano for extraction and analysis.

Extraction of leachate was undertaken through washing of resin with 100mL of 2 molar MKCl solution within 500 mL Erlenmeyer flasks shaken at 100 rpm for 1 h, using an orbital shaker. The elutant was filtered through Whatman no. 42 filters, and NO_3- and NH_4+ concentration detected via continuous flow automatic analyser (AxFlow AA3, Bran+Luebbe, Norderstedt, Germany). Ammonium detection used a combination of salicylate and dichloro-isocyanuric acid (ISO_11732:2005), whilst nitrate was detected with sulphanilamide-NEDD [N-(1-Napthyhyl)ethylenediamine] (ISO 13395:2006). Analysis of phosphate within the extracted solution utilised an inductively coupled plasma optical emission spectrometer (ICP-OES, Spectro Arcos, Ametek, Germany), as described previously (Ventura *et al.* 2013).

4.2.3.2 Data processing and statistical analysis

Differences between treatments were assessed for NH_4, NO_3 and PO_4 , using a two-way analysis of variance (ANOVA), with treatment (biochar vs control) and position of the lysimeter (within tree row, between rows and edge of row) being independent variables. This analysis was carried out using IBM SPSS Statistics for Windows Version 23.0 (IBM Corp, Released 2011. Armonk, NY).

4.2.4 Enzyme activity

4.2.4.1 Sample protocol

Enzyme activity was assessed through analysis of soil samples collected during the sampling July 2013 (the same date as the UK1Y samples in Chapter 3: 3.2.3). These were transported to the Agricultural Research Council (CRA) Trieste, where dsDNA was extracted using 0.12 M, pH 8 Na_2HPO_4 buffer and bead beating, prior to quantification using PicoGreen reagent (Fornasier *et al.* 2014). Enzyme activities of arylsulfatase, β glucosidase, acid and alkaline

phosphatase, phosphodiesterase, esterase, and leucine aminopeptidase were assessed. The enzyme activity of substrates was determined by an extraction/desorption procedure (Fornasier & Margon 2007). 400 mg of each soil sample and 1.2 mL of 3% lysozyme were added to 2 mL Eppendorf tubes with 0.4 mL of 1mm diameter ceramic beads and 0.4 mL of 100 micron glass beads. Each tube was shaken for 3 minutes at 3 strokes s^{-1} with a Retsch 400 beating mill prior to centrifugation at 15,000 g for 3 minutes. Supernatant was then added to a 384 well microplate for enzyme activity analysis using of fluorescent 4-methyl-umbelliferyl substrates.

4.2.4.2 Data processing and statistical analysis

Differences in dsDNA and enzyme activity were assessed through a mixed models ANOVA, with treatment as a fixed factor, using SAS 9.1 (SAS Institute INC., Cary, NC, USA). Samples were checked for normality using the Shapiro-Wilk test.

4.3 Results

4.3.1 Data quality

Gapfilling was required in 58.1% of the initial dataset (June 19th–December 1st 2012), whilst 72.5% of the extended dataset (December 2nd 2012 – June 18th 2014) was gapfilled. Power saving mode was enabled during winter due to limited availability of solar power. Several power failures also occurred due to animal damage. After gapfilling, 100% of the initial dataset was accounted for, and 98.8% of the extended dataset was present. Remaining missing data resulted from days in which soil temperature or moisture data was unavailable due to system malfunction or total power loss. Pearson's correlation was conducted to examine the association between raw measurements and the modelled counterparts. This revealed a strong positive correlation (r = 0.77, $p<0.001$, $r^2= 0.59$) between modelled and measured data, suggesting that the gapfilling protocol appropriately estimates the rate of efflux.

4.3.2 The effect of biochar application on soil respiration

During the period of June–December 2012, biochar significantly increased total respiration (Biochar mean= 3.0886 g C m^2, Control mean = 2.506 g C m^2, $p = 0.017$), whilst no change in respiration was detected in heterotrophic plots (Figure 34). Across treatments, total respiration measurements were significantly higher than heterotrophic respiration ($p = 0.016$).

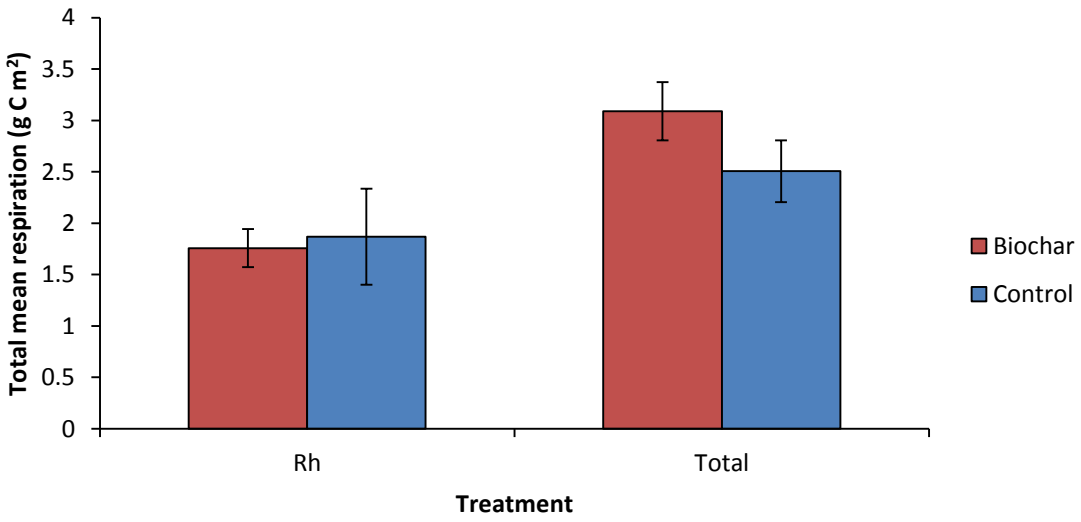


Figure 34 Total mean respiration during June–December 2012, comparing Heterotrophic (Rh) and Total respiration under biochar and control treatments. Error bars = ±SEM

Data from the period of December 2012 – June 2014 displayed a similar trend (Figure 35). Biochar treated total respiration plots had significantly higher respiration than heterotrophic plots (Biochar mean= 2.0204 g C m^2, Control mean=1.5214 g C m^2, $p = 0.003$), whilst no difference was detected between total and heterotrophic respiration in control plots. Again, across treatments, total respiration was significantly higher than heterotrophic respiration ($p = 0.010$). It should be noted that rerunning the analysis, omitting gapfilled data, returns the same pattern of significance. Total respiration remained significantly higher than heterotrophic respiration during the June–December 2012 period (p=0.009) and the December 2012 – June 2014 period (p=0.023). Furthermore, non-modelled data also confirmed the significant increase in biochar treated total respiration (p=0.02) during the initial 2012 period, and the December 2012–June 2014 period (p=0.023). No differences were detected at either dataset between total and heterotrophic respiration in control plots.

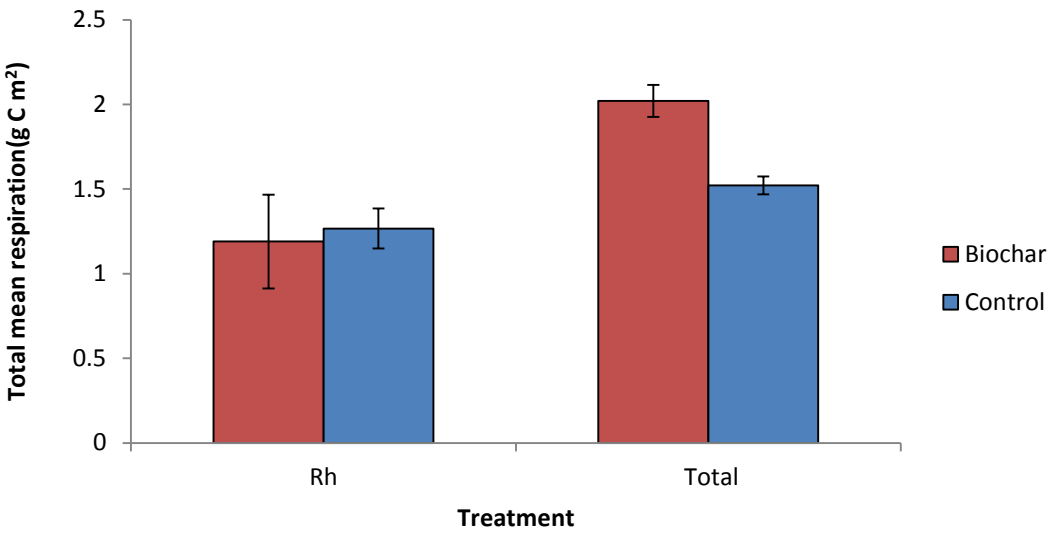


Figure 35 Total mean respiration during December 2012 to June 2014, comparing Heterotrophic (Rh) and Total respiration under biochar and control treatments. Error bars = ±SEM

Graphs displaying respiration over time for each of the treatments show seasonal variation, with higher rates occurring during spring and summer (2 – 6 g C m^{-2} d^{-1}) (Figure 36, Figure 37). Fluxes decrease to between 1 – 2 g C $m^{-2}d^{-1}$ during the winter. Seasonal trends displayed were similar regardless of treatment (either biochar vs control or heterotrophic vs total respiration), with peak respiration during July.

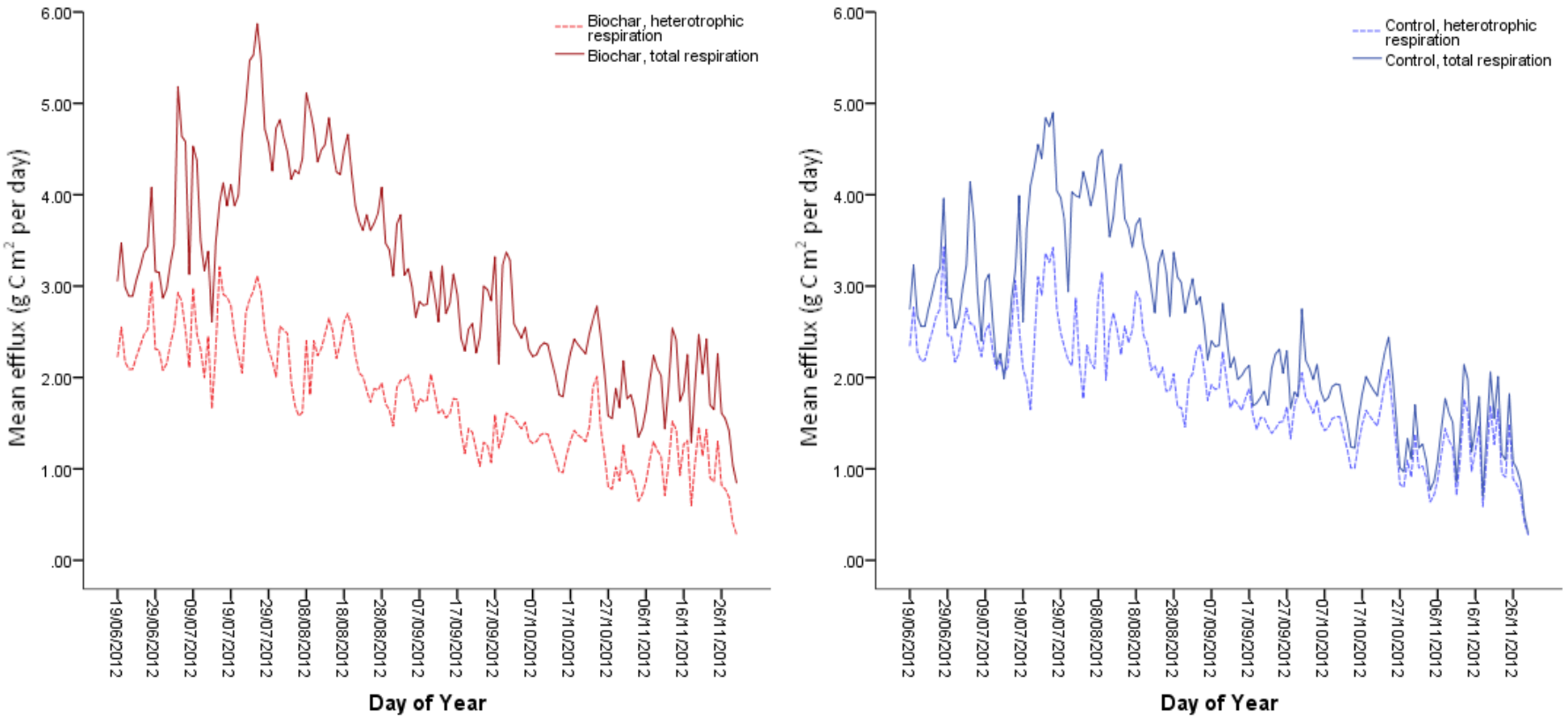


Figure 36 Mean daily heterotrophic and total respiration rates in biochar and control plots throughout the initial 165 day measurement

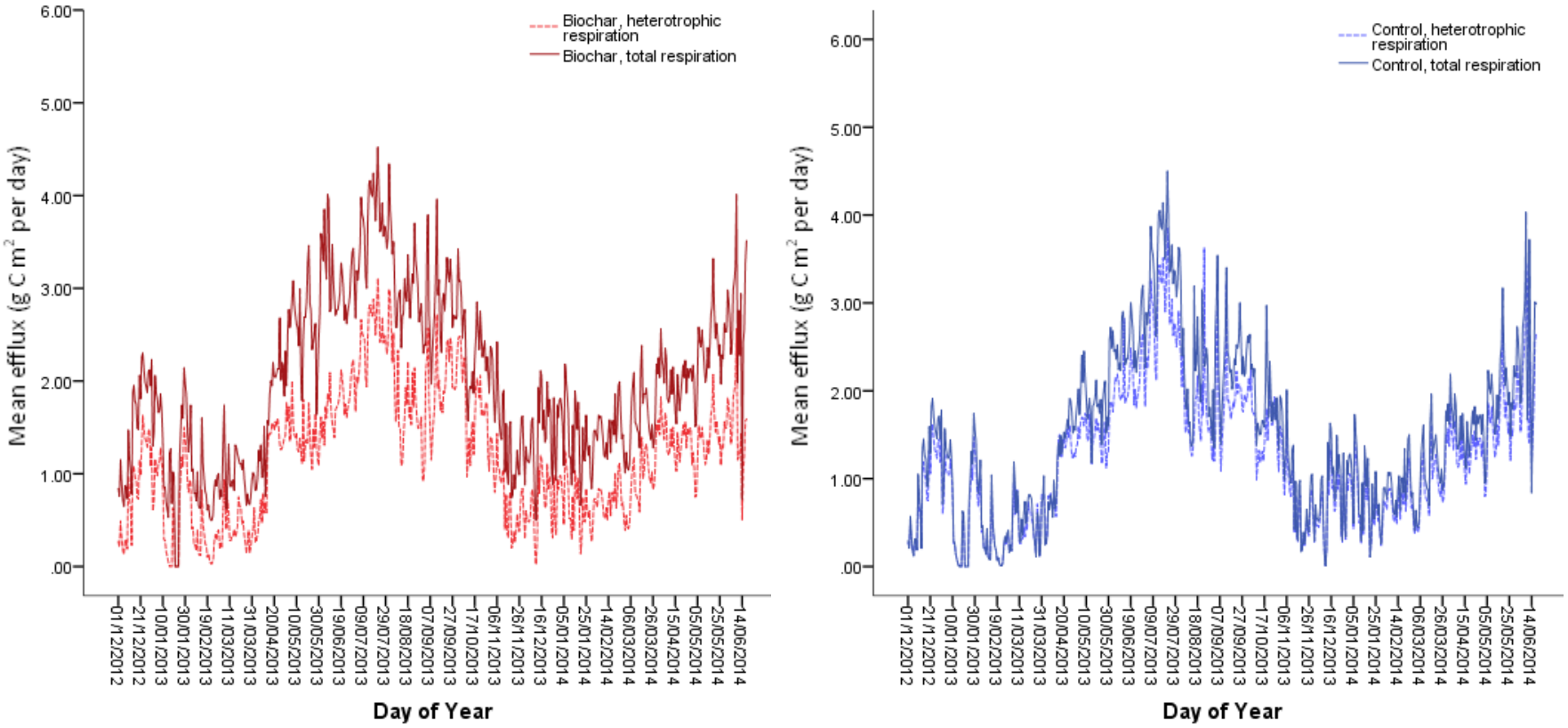


Figure 37 Mean daily heterotrophic and total respiration rates in biochar and control plots throughout the extended dataset

4.3.3 The effect of biochar application on nutrient leaching

Results from resin lysimeters indicated a significant effect of treatment on ammonium and phosphate, F (1,18)= 4.78, p = .042; F (1,17) = 7.53, p= .014 respectively (Figure 38). Differences in the degrees of freedom were due to the loss of one lysimeter, which was damaged during retrieval. Ammonium leachate was significantly increased in biochar plots (mean = 4.30 kg ha^{-1}, standard deviation = 1.83 kg ha^{-1}) compared with control plots (mean = 2.79 kg ha^{-1}, standard deviation = 1.35 kg ha^{-1}). Similarly, phosphate leachate was elevated in biochar plots (mean = 2.77 kg ha^{-1}, standard deviation = 1.26 kg ha^{-1}) compared with controls (mean = 1.43 kg ha^{-1}, standard deviation = 0.97 kg ha^{-1}) (Figure 38). No effect of biochar was detected on nitrate leachate, nor was there an interaction with position of lysimeter within the plots and treatment.

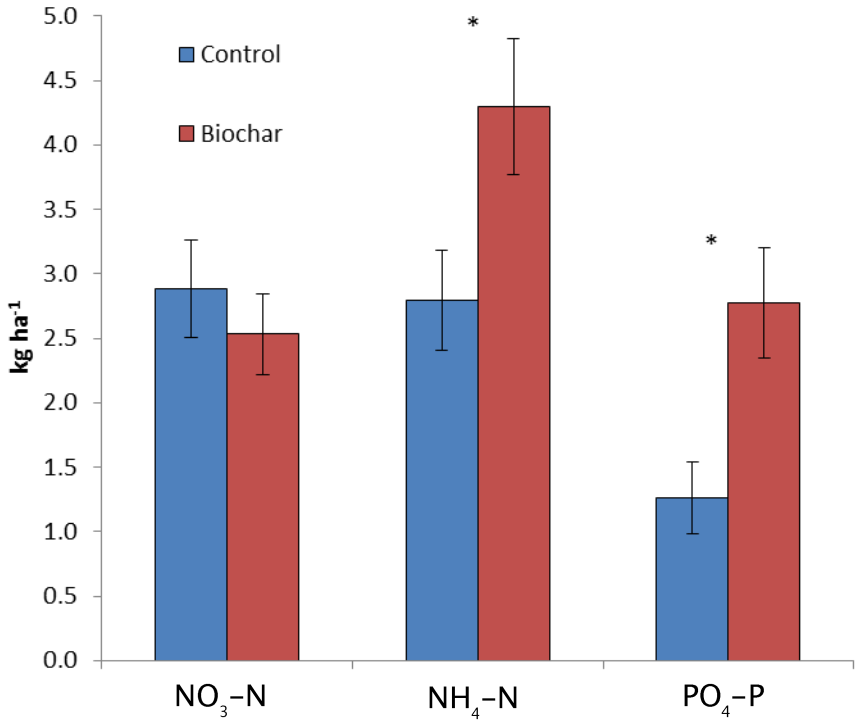


Figure 38 Mean leachate of nitrate (NO_3–N), ammonium (NH_4–N) and phosphate (PO_4–P) in biochar and control plots ± S.E.M. Asterisks represent a significant difference (p = <.05)

4.3.4 The effect of biochar application on enzyme activity

Results of enzyme activity analysis (Figure 39) indicated no change in dsDNA concentration between treatments (p = 0.074). dsDNA is a proxy for microbial biomass, and as such total biomass of microbial communities did not significantly change due to treatment. Acid phosphomonoesterase significantly decreased in biochar treatments (p = 0.017), whilst alkaline phosphomonoesterase activity significantly increased (p = 0.001). Both enzymes are responsible for the removal of phosphate groups by hydrolysis of O-P bonds. Arylsulfatase also indicated a decrease in activity in biochar samples compared with controls (p = 0.008). This enzyme is responsible for the reduction of sulphates from complex molecules. No significant changes were detected in beta-glucosidase (related to polysaccharide degradation), or leucine-aminopeptidase (which hydrolyses amino acids from polypeptides).

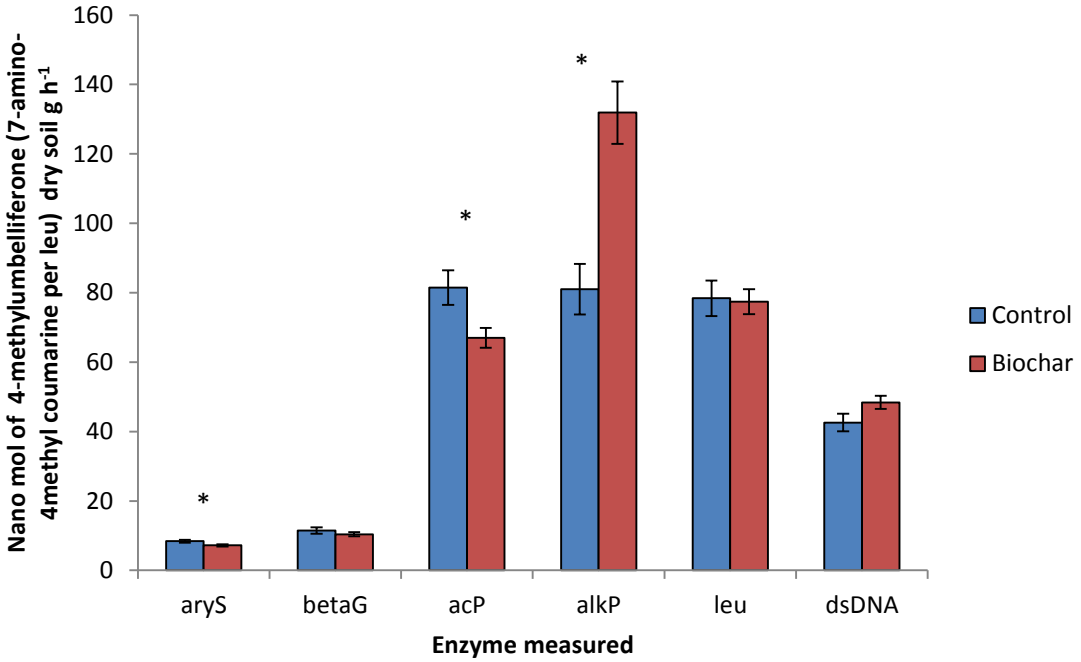


Figure 39 Mean changes in enzyme activity due to biochar treatment in UK1Y samples ± S.E.M. Arylsulphatase = aryS, Beta-glucosidase = betaG, acid phosphomonoesterase = acP, alkaline phosphomonoesterase = alkP, leucine aminopeptidase = leu. Asterisks represent a significant difference (p = <.05)

4.4 Discussion

4.4.1 Does biochar increase the rate of soil respiration?

Application of biochar produced using a range of feedstocks and methods can result in elevated soil respiration (Smith et al. 2010; Purakayastha et al. 2015; Bamminger et al. 2014; Bruun et al. 2014). Increased carbon mineralization can be linked to both biotic and abiotic variables. For example, low pH soils can cause the release of CO_2 from carbonates contained within biochars (Bruun *et al.* 2014). This effect will occur only within the first few days of application in very acid soils, unlike those present at the FR, IT and UK sites (Bruun *et al.* 2014). The breakdown and metabolism of readily bioavailable portions of biochar by soil microbes is responsible for increased soil respiration in the short-term (Lehmann *et al.* 2011; McCormack *et al.* 2013), whilst long-term respiration increases will result from gradual degradation of stable carbon portions into bioavailable C (Major *et al.* 2010).

During the first 165 days after application, increased rates of respiration were detected in biochar treated total respiration plots (Ventura *et al.* 2015). This may represent a short-term increase in respiration as labile portions of biochar are mineralised. This effect was also detected in the additional data collected, suggesting that biochar application has the potential to increase longer-term total respiration, which may occur due to changes in the activity and structure of root associated microbial consortia (Farrell *et al.* 2013; Prayogo *et al.* 2013), through stimulation of extant SOM breakdown by biochar (Zimmerman *et al.* 2011; Cross & Sohi 2011) or through interactions with root exudates degrading labile portions of biochar (Lehmann *et al.* 2011).

Total respiration was significantly higher than heterotrophic regardless of treatment. This in itself is unsurprising, as root biomass will respire and exudates can increase availability of SOC which can be utilised by rhizosphere communities (Swift *et al.* 1998; Michalet *et al.* 2013). Whilst partitioning of total and heterotrophic respiration was carried out by root exclusion, it is not possible to determine the source of respiration within that partition. For example, total respiration may have been increased through a combination of amplified root respiration and rhizosphere respiration from associated fungi and bacteria (Warnock *et al.* 2007; Lehmann *et al.* 2011).

Isotopic analysis of mineralisation taken during the first 165 days showed differences between heterotrophic and total respiration of biochar (Ventura *et al.* 2015). Biochar showed a slow rate of mineralisation, which was increased in the presence of roots. This suggested a priming effect of roots on biochar, possibly due to microbially associated root communities (Warnock *et al.* 2010); the release of exudates from roots (Prendergast-Miller *et al.* 2014); biomechanical processes leading to physical perturbation and breakdown of biochar (Lehmann *et al.* 2011) or a combination thereof. Therefore, whilst no change was detected in the rate of heterotrophic respiration in biochar plots, it is possible that the source of the mineralized C varied. Unfortunately, the lack of isotopic analysis in the extended dataset meant that it was not possible to determine how biochar was decomposed during this time period.

4.4.2 Does the stability of biochar (and subsequent rate of respiration) change with time?

Results of the extended dataset corroborate those reported in Ventura *et al.* 2015, in that total respiration was significantly increased following biochar application. Heterotrophic respiration on the other hand, remained unaffected. Mean total respiration during the extended measurements was lower than that recorded during the initial 165 days, possibly due to decreased available labile biochar mineralization. This indicates no increase in biochar associated respiration with longer exposure time. It has been suggested that weathering effects may further breakdown biochar through oxidation and subsequent mineralization (Cheng *et al.* 2008; Naisse *et al.* 2013). This does not appear to be reflected in these results. This may be due to the comparatively short time span of the study. Furthermore, the large percentage of canopy cover within the SRC site may afford some protection against water and wind erosion (Holland *et al.* 2015). However, due to the lack of isotopic data during the extended period of measurement, it is not possible to determine whether there were changes in the rate of biochar mineralization.

4.4.3 Does biochar application affect soil nutrient status?

Changes in nutrient availability have been associated with biochar application. For example, the nitrogen cycle in biochar treated soils has been disrupted,

with reductions in N_2O emissions (Anderson *et al.* 2011), decreased nitrate leaching (Ventura *et al.* 2013) and increased nitrification (Prommer *et al.* 2014) detected. Elevated ammonium leachate was noted within biochar treated samples, representing an increase in the concentration of ammonium present, or a decrease in the ability of the soil to sorb and hold nitrogen. This may be due to declining rates of nitrification, decreasing the rate of ammonium to nitrite conversion (Cayuela *et al.* 2013; Prommer *et al.* 2014). However, no change in the abundance of taxa associated with nitrification was detected (See Section 3.3).

Changes in phosphate cycling have also been identified, with increased mobilisation of phosphates after biochar application (Fox *et al.* 2014; Hammer *et al.* 2014). This may be linked to shifts in fungal community structure (Section 3.3), as mycorrhizal fungi are essential in the supply of phosphorous to plant root systems (Smith *et al.* 2003; Chen *et al.* 2008; Rooney *et al.* 2009). Activity of alkaline phosphatase was increased in biochar treatments, whilst control plots showed higher rates of acid phosphatase activity. This relative decline in the activity of acid phosphatase, and increased activity of alkaline phophatase may suggest a shift in response to soil pH. It has been suggested that the type of phosphatase prevalent in an environment is linked not only to pH, but also to the taxa which produce them. For example, bacterial phosphatases have alkaline and neutral optimal pHs, whilst extracellular enzymes from plants and fungi require acidic conditions (Caldwell 2005). Therefore a switch in the activity of phosphatases may reflect an impact on their source community, which in this case would suggest an increased role of bacterially derived enzymes in the P cycle in biochar plots. However, this requires further study to be confirmed, as the relationship between alkali and acid phosphatases and their source taxa is not fully understood. Combined with the results of the resin lysimeters, increased P availability within the soil was noted, indicating an impact of biochar on phosphate mobilisation. Previous work studying this phenomenon in biochar implies that phosphate mobilisation results from mycorrhizal hyphae liberating phosphate from within biochar micropores, which are not accessible to root tips (Hammer *et al.* 2014). Combined with biochars ability to adsorb leachate reductions of runoff and downward percolation of phosphates may occur, improving phosphate availability for mycorrhizal symbionts (Beesley *et al.* 2011).

Taken together, it appears that application of biochar increases availability of essential plant nutrients, through reduction of rates of leachate, or through altering the activity of microbial enzymes. The switch from acid to alkaline phosphatase suggests an increase in the role of bacterial phosphate cycling (Caldwell 2005). Furthermore, the lack of significant change in soil respiration compared with control samples suggests biochar is a viable method of carbon sequestration. However, more complex interactions in the source of the carbon mineralisation may be occurring, although this cannot be determined by the current dataset. Increased enzymatic activity coupled with no change in microbial biomass suggests shifts in ecological function may be a result of changes in taxonomic identities or behaviours of soil microorganisms.

4.4.4 Conclusions

Biochar application has implications for P and N cycling, and significantly increased total respiration. This result was still detected in an extended dataset, which included the winter of 2012 until June of 2014, although to a lesser extent. Additionally, isotopic analysis carried out at the same site during the 165 day period indicated a negative priming effect of biochar on native SOM, protecting it from mineralization, and that the increase in total respiration was due to an 8% degradation of biochar (Ventura *et al.* 2015). Whilst no significant increase in respiration was detected due to biochar application alone, in line with the previous study (Ventura *et al.* 2015), the source of the respiration (biochar of extant SOM) cannot be determined due to the lack of isotopic data. Further isotopic analysis at the site would clarify this determining whether the rate of biochar mineralisation changes with time, and whether the protective effect on native SOM continues. This would help further the understanding of biochar stability and degradation effects in SRC scenarios, which can be used to design more carbon efficient SRC management techniques.

Chapter 5: Comparing Grassland to SRC: Differences between set-aside and bioenergy crop soil microbial communities.

5.1 Introduction

Climate change is now accepted as a serious global threat by policy makers and governments (IPCC 2014). This increased interest outside of the scientific community has enhanced awareness of renewable energy, specifically energy from biomass. First generation bioenergy crop systems concentrated on production of biofuels from food crops. These methods relied on conversion of sugars present within crops to bioethanol, which could be used as biofuels (Naik *et al.* 2010). However, first generation biofuels required intensive agricultural methods. Whilst producing relatively little CO_2 themselves, the need for transport, land use change, fertiliser application and land management techniques dramatically increased the carbon footprint of first generation biofuels (Gomez *et al.* 2008). Additionally, first generation fuels compete directly with food crops, with implications for food security (Gomez *et al.* 2008; Sims *et al.* 2010).

Concern over competition between first generation biofuels and food production led to increased interest in second generation non-food biofuels. These are produced using crops which can be grown in marginal land, thereby reducing competition with food crops. Second generation biofuels are reliant on lingocellulosic biomass, which can either be produced as a waste product of other agricultural practises (straw, forest residue and sugar cane bagasse), or by growth of woody bioenergy crops (Poplar, Miscanthus and Willow) (Rowe *et al.* 2009; Sims *et al.* 2010). Dedicated crops such as poplar or willow are grown using short rotation coppice (SRC) methods, maximising yield (Department for Environment Food and Rural Affairs 2004). In the first year after establishment, trees can grow as tall as 4m in height, prior to initial coppicing. Coppicing encourages the growth of multiple stems from a single plant, increasing yield at the next harvest (Department for Environment Food and Rural Affairs 2004). Harvests can be conducted on a three yearly basis for up to thirty years before replanting of stands is required, providing not only a long-term source of biomass, but also increasing soil organic matter (SOM) and carbon sequestration when compared with traditional first generation biofuels (Kahle *et al.* 2013). Biomass has the advantage of being viable for use in co-fired power-stations (after pelletisation), or in the production of ethanol derived biofuels through digestion of lignocellulosic biomass (Rowe *et al.* 2009).

In the context of reducing CO_2 emissions, SRC has several potential advantages. Like first generation biofuels, SRC crops have low emissions rates (Volk *et al.* 2004). Species used are often hardier, requiring less intensive agricultural practises than first generation biomass crops, reducing emissions associated with growth and establishment (Dimitriou *et al.* 2009). As SRC stands are present in the long-term (30+ years), they can continue to sequester carbon to soil through root system growth and annual leaf fall, with the further benefit of enhancing soil quality through augmenting SOM (Baum *et al.* 2009). Similarly, the interaction of SRC roots with fungal networks can lead to further C sequestration via fungal biomass (Rooney *et al.* 2009). There is the potential to combine SRC systems with localised power generation, through which energy and biochar production could be undertaken. Small scale, locally produced biochar could be reapplied to fields, sequestering carbon at source, and potentially increase future biomass yield (see Section 1.3).

Whilst a promising method for energy production, it is not clear what impacts the establishment of SRC stands in marginal land would have on soil microbial communities, due to the wide range of biotic and abiotic shifts that may occur. For example, declines in ecosystems services may occur due to conversion of grassland originally used for livestock grazing, or increased competition for water resources (Holland *et al.* 2015). However, these must be balanced with the range of improvements in pollination, soil and water quality expected due to SRC establishment (Holland *et al.* 2015).

Comparisons of SRC and grassland biodiversity suggest SRC canopy increases the abundance of winged invertebrates. Furthermore, the presence of headlands around the crop increase the variety of habitats available (Rowe *et al.* 2011). Coupled with increased abundances of flora within the SRC stands compared with traditional arable sites, it appears that heterogeneity of habitat positively influences biodiversity and ecological complexity. Soil bacterial and fungal diversity is directly influenced by the complexity of vegetative communities (Garbeva *et al.* 2004; Tedersoo *et al.* 2012; de Vries *et al.* 2012; Mendes *et al.* 2013). Changes in the abundance of plants may influence the community structure of associated bacteria, arbuscular and ecto-mycorrhizal fungi (AMF, EMF respectively) (Baum *et al.* 2009; Crowther *et al.* 2014). Therefore the increased environmental complexity associated with SRC establishment should increase microbial diversity and structural complexity.

Addition of tree roots will likely affect the soil environment, increasing aeration, reducing soil water content, augmenting available SOC and producing root exudates, all of which may further influence microbial community composition (Saleem & Moe 2014).

Macroecological studies have long been aware of successional effects, with disturbance events creating new niches for organisms to exploit, gradually leading to long-term shifts in the structure and function of a community. LUC triggers environmental disturbance, driving successional events, and is known to be associated with shifts in microbial diversity and abundance. Chronosequencing of rainforest land-use-change showed distinct microbial communities for forested, deforested, agricultural and pasture soils (Mendes *et al.* 2015). Land use change from forested areas to grassland can result in decreased total microbial biomass, but increased species richness as new taxa colonise disturbed areas (Crowther *et al.* 2014). Conversely, afforestation of grassland with conifer species can increase P availability, which may influence (and be influenced by) associated microbial consortia (Chen *et al.* 2008). Deforestation decreases the abundance of the bacterial phylum *Acidobacteria,* and the fungal phylum *Basidiomycota*. Furthermore, the abundance of known EM and AM fungi decline after deforestation (Crowther *et al.* 2014). Therefore it is possible that establishment of SRC may lead to increased abundance of the aforementioned taxonomic groups.

There is limited information currently available on the effects of SRC establishment on microbial community structure. The introduction of *Populus* SRC to Canadian sites increased the ratio of fungal biomarkers within the soil and elevated total fungal biomass (Yannikos *et al.* 2014). This may be due to greater availability of woody biomass and litter for saprotrophic decomposition, or through the introduction of *Populus* root associated EMF. Similarly, a 2004 study showed that establishment of short rotation forestry (SRF) willow genotypes (*S. Viminalis L.* and *S. dasyclados Wimm.*) in poor quality mineral soils increased abundance of EMF such as *Laccaria* and *Hebeloma* (Püttsepp *et al.* 2004). Therefore establishment of SRC may introduce associated fungi into the soil microbiome, which may in turn have implications for bacterial and fungal community structure. This could occur through competition, manipulation of nutrient profiles and subsequent variation of edaphic variables (Hofmann-Schielle *et al.* 1999).

Whilst SRC crops are less intensively farmed than first generation biofuel and food crops, their establishment requires ground preparation. This includes weed removal using glyphosate herbicides, sub-soiling and ploughing, and application of manure or fertilisers (Department for Environment Food and Rural Affairs 2004). Each of these may have implications for the microbial communities present. Application of glyphosate herbicides can influence respiration rates (Imfeld & Vuilleumier 2012), and may cause shifts in microbial community structure (Kent & Triplett 2002). Subsoiling and tillage methods overturn the soil, increasing aeration, decreasing compaction and water holding capacity, which can influence community structure and function (Baum *et al.* 2009).

In an effort to determine the differences between bacterial and fungal communities by land use, a comparison of adjacent grassland and SRC soils, 4 years post-conversion was undertaken. Using next generation amplicon sequencing, a high resolution understanding of differences in community richness, diversity and abundance could be detected. Furthermore, the abundances of taxa were studied to elucidate specific taxa that are not shared between the two land uses as a proxy measure to estimate the taxa which may change in abundance as a result of land-use-change. Whilst there is little information available on the effects of SRC establishment on the structure of microbial communities, based on a previous study of the effects of deforestation on microbial communities (Crowther *et al.* 2014), it was hypothesised that SRC would display increased abundances of the fungal phylum *Basidiomycota*, probably as a result of EMF taxa associated with tree roots. Elevated *Acidobacteria* abundance may be detected in SRC, as these are oligotrophic and associated with decomposition of complex carbon sources, such as leaf matter and woody biomass. Given that variation in two phyla were expected, it was assumed that significant differences in α diversity (species richness) and β diversity (community composition and abundance) would be present between the two land uses as niches and trophic interactions would vary between them.

5.2 Method

The SRC site used was located in Pulborough, West Sussex (Figure 7). This was the same site utilised for the UK biochar treatment described in Chapter 3: .

Further information about the site can be found in Section 3.2.2. Control plots located within the SRC were used as established SRC samples. Grassland samples were collected from an adjacent fallow field, designated as Set Aside agricultural land since July 2000, and redesignated as EK3 in the entry level stewardship scheme in 2005 (very low input grassland). Vegetation was comprised of mixed grassland, dominated by *Lolium* spp., *Schedonorus* spp. and *Dactylis* spp (Harris *et al.* 2016).All meteorological and temperature averages are the same as those given in Section 3.2.2.

5.2.1 Soil sampling in SRC and grassland

Sampling was carried out during summer 2012 on the same day as the collection of the UK1M biochar samples (Section 3.2.3). Soil samples were collected simultaneously to those collected for biochar analysis. Each set of 5 samples was collected from three randomly selected plots, surrounding existing soil flux chambers (associated with a separate project) located within fallow grassland, using the methodology described in Section 3.2.3. Briefly, 5 samples were collected from each plot, 1.5m from the plot centre. These were equidistant from one another, in order to capture as much variation within the plot as possible (see Appendix C from a diagram of the sampling pattern). Sterilised soil sampling units were used to collect soil to a depth of 15 cm, prior to sieving to 2mm. The sieved soil was then placed in a 50ml falcon tube, and frozen at -80°C. Samples were transported back to the lab and stored at -80°C until extraction.

5.2.2 DNA Extraction

Extraction of DNA was carried out as described in Section 3.2.5. Briefly, MoBio Powersoil extraction kits were used to the manufacturers specification, with the exception of the initial weight of soil used. This was increased to 0.5g, to improve DNA yield from each sample.

5.2.3 Amplification and sequencing method

Sequencing was carried out using the same method described in Section 3.2.6. Amplification of both the ITS and 16SrRNA regions was undertaken, using the same methodology described in Section 3.2.5.

5.2.4 QIIME analysis

QIIME analysis utilised a modified version of the pipeline described in Section 3.2. Each pair of sequences was combined using PandaSeq prior to subsequent analysis steps. The resulting .fasta files for the grassland were combined with the biochar .fasta file and *de novo* OTU picking undertaken as described in Chapter 3, ensuring OTUs were directly comparable across samples.

The resulting .biom table was filtered to remove biochar treated samples, leaving only control SRC and grassland plots. SRC plots had been subject to a control treatment of water addition at the date of biochar application, but otherwise only differed from the grassland plot in that they had been converted to SRC during 2008. OTU tables for each sample were combined using the "summarize_by_cat.py" process in QIIME, so that each plot was represented by a single sample, containing all reads from each of the 5 phyiscal samples collected, in an effort to minimise any potential effect from pseudo-replication. α and β diversities were rarefied to 90% of the smallest sample as described in the Methods Section of Chapter 3. For 16S samples, the rarefaction value was 58,978 reads, whilst ITS samples were rarefied to 65,111.

Differences in proportional abundance of taxa were calculated using STAMP as described in Section 3.2.7.1, analysing only those taxa which had >5 sequences, with difference between proportions of at least 0.5% and a ratio of proportions of at least 2.

5.3 Results

An initial summary of the 16S dataset after singleton removal reported a total of 574,160 reads in the six pooled samples collected (65,532 to 114,750reads per sample). The ITS dataset produced 848,853 reads (72,346 to 203,208 reads per sample). Total reads per pooled sample can be seen below.

Table 22 Total sequences per pooled sample for SRC and grassland

	SRC Sample 1	SRC Sample 2	SRC Sample 3	Grassland Sample 1	Grassland Sample 2	Grassland Sample 3
16S	99,599	91,804	65,532	88,512	113,693	114,750
ITS	144,944	203,208	116,208	72,346	132,702	179,445

5.3.1 Differences in bacterial community structures in grassland and SRC soils

5.3.1.1 16SrRNA α diversity

No significant difference was detected in Observed Species, PD or Chao1 diversity metrics of α diversity for 16SrRNA data. Average observed species bacterial diversity was higher in SRC samples (*Table 23*). Based on Chao1 diversity, (which estimates the true number of OTUs present based on the number of singleton and doubleton OTUs within the dataset) approximately 15,000 OTUs were present in SRC samples, compared with an estimated 12,000 in grassland. Similarly, PD reflects the relatedness of the OTUs detected, with lower values representing less phylogenetic diversity. Grassland had a slightly lower diversity on average, but this was not significantly different from SRC. Therefore, grassland and SRC have similar bacterial species richness.

Table 23 Results of t-tests with Monte-Carlo permutations (999) for 16SrRNA α diversity in Pulborough, comparing SRC and grassland

16SrRNA	SRC Mean	Grassland Mean	SRC StDev	Grassland StDev	t-stat	p-value
Pulborough	Obs= 9107	7293	187.7	110.7	-11.77	0.072
	Chao1= 14868.4	12211.9	416.7	339.9	6.98	0.114
	PD= 386.6	325.5	4.04	7.11	-10.55	0.05

5.3.1.1 16SrRNA β diversity

β diversity analyses also showed no significant difference in bacterial identities (unweighted UNIFRAC) and their abundances (weighted UNIFRAC) between grassland and SRC PCoA plots of weighted and unweighted UNIFRAC results display a potential clustering effect by treatment, although these are not significant based upon ADONIS testing (Figure 40). Thus, grassland and SRC both contain both similar bacterial taxa in similar proportions.

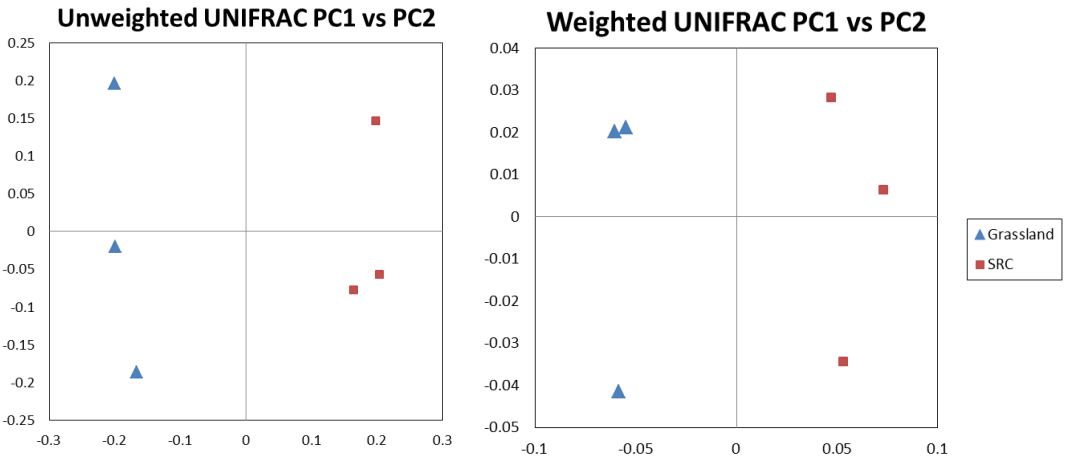


Figure 40 PCoA of weighted and unweighted UNIFRAC distances for bacterial grassland/SRC comparison. Grassland samples (blue) and SRC samples (red)

5.3.2 Differences between bacterial taxonomic abundances in grassland and SRC soils

The dominant phylum present in samples was *Proteobacteria*. Other dominant taxa included *Actinobacteria*, *Planctomycetes* and *Verrucomicrobia*. Five additional phyla were present at levels >1% (*Table 24*). No change in the identities of dominant phyla occurred due to treatment. The phylum contributing the largest proportional shift in mean abundance was *Proteobacteria*, with a 4% decrease in mean SRC abundance when compared with grassland samples (Figure 41).

STAMP analysis of the pooled bacterial community data showed no significant difference in the relative abundances of any taxa between grassland and SRC,

indicating that whilst there appear to be differences in the dominant taxa (Figure 41), even the largest of these are not significant.

Table 24 Mean proportional abundance (± 95% CI) of dominant bacterial phyla in SRC and grassland

Phylum	SRC	Grassland
Proteobacteria	26.19±0.61%	29.82±0.73%
Actinobacteria	17.18±1.10%	16.44±1.30%
Acidobacteria	13.95±0.31%	14.66±0.67%
Planctomycetes	10.03±0.36%	10.21±0.38%
Verrucomicrobia	7.81±0.36%	6.53±0.27%
Bacteroidetes	4.11±0.44%	4.30±0.38%
Chloroflexi	5.61±0.24%	6.42±0.36%
Nitrospirae	3.14±0.30%	2.16±0.24%
OD1	2.07±0.20%	1.04±0.13%
WS3	1.61±0.13%	1.71±0.15%

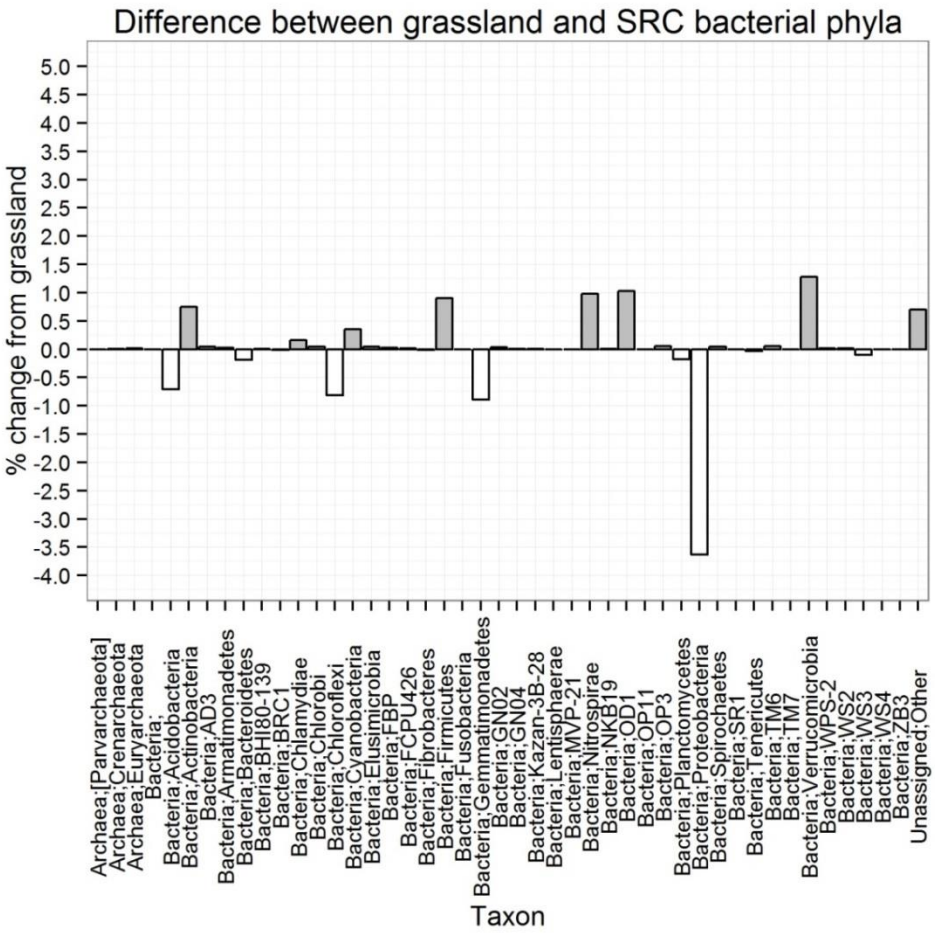


Figure 41 Mean differences in proportional abundance of bacterial phyla between SRC and grassland.

Therefore, it appears that there is no significant difference between the bacterial community in SRC or grassland soils, indicating that the taxa present, and their abundances are homogenous.

5.3.3 Differences between fungal community structure in grassland and SRC soils

5.3.3.1 ITS α diversity

Results of ITS analysis also suggest no significant difference in the species richness between land use type on the soil microbiome. Soils in the SRC willow bioenergy field had slightly higher Observed Species, Shannon and chao1 metrics (*Table 25*). Average observed species within grassland indicates approximately 1220 OTUs present compared with 1500 in SRC.

Table 25 Results of t-tests with Monte-Carlo permutations (999) for ITS α diversity in Pulborough, comparing SRC and grassland

ITS	SRC Mean	Grassland Mean	SRC StDev	Grassland StDev	t-stat	p-value
Pulborough	Obs= 1518.3	1295.2	81.3	160.3	-1.75	0.28
	Chao1= 2247.4	1876.2	74.2	193.4	-2.53	0.066
	Shannon= 6.7	6.3	0.4	0.8	-0.61	0.714

5.3.3.2 ITS β diversity

ITS β diversity was measured using Bray-Curtis distance dissimilarity, due to difficulty aligning ITS sequences. Therefore, no phylogenetic measurement was used, preventing assertions being made about diversity and abundance separately. Due to the way in which Bray-Curtis distance dissimilarity is calculated the method is not capable of determining whether differences are due to variation in the abundance of taxa or a result of changes in the identities of taxa present.

PCoA of Bray-Curtis distances showed dissociation between SRC and grassland samples, although ADONIS testing revealed that this was not

significant. Therefore the identities and abundances of fungal communities do not appear to differ between land use types.

5.3.4 Differences in fungal taxonomic abundances in grassland and SRC soils

A cursory investigation of mean proportional abundances revealed fungal communities were dominated by unidentified fungi, *Ascomycota* and *Basidiomycota*. Additionally, *Glomeromycota* and *Zygomycota* were detected only in grassland plots. A 20% increase in *Basidiomycota* was detected in SRC plots, which appears to be due to a 13% increase in an unknown *Agaricales* genus and a 5% increase in an unknown genus of *Cortinariaceae*. A further 17% decline in unassigned reads in SRC samples was noted (Figure 42). These are reads which whilst amplified by the ITS primers, do not align with fungal sequences.

Table 26 Mean proportion (± 95% CI) of dominant fungal phyla in SRC and Grassland

Phylum	SRC	Grassland
Unidentified Fungi	36.95±3.74%	37.46±7.74%
Ascomycota	27.46±5.80%	30.15±5.85%
Basidiomycota	27.86±8.08%	7.00±3.38%
Glomeromycota	0.02±0.01%%	0.74±0.31%
Zygomycota	0.18±0.10%	0.87±0.39%

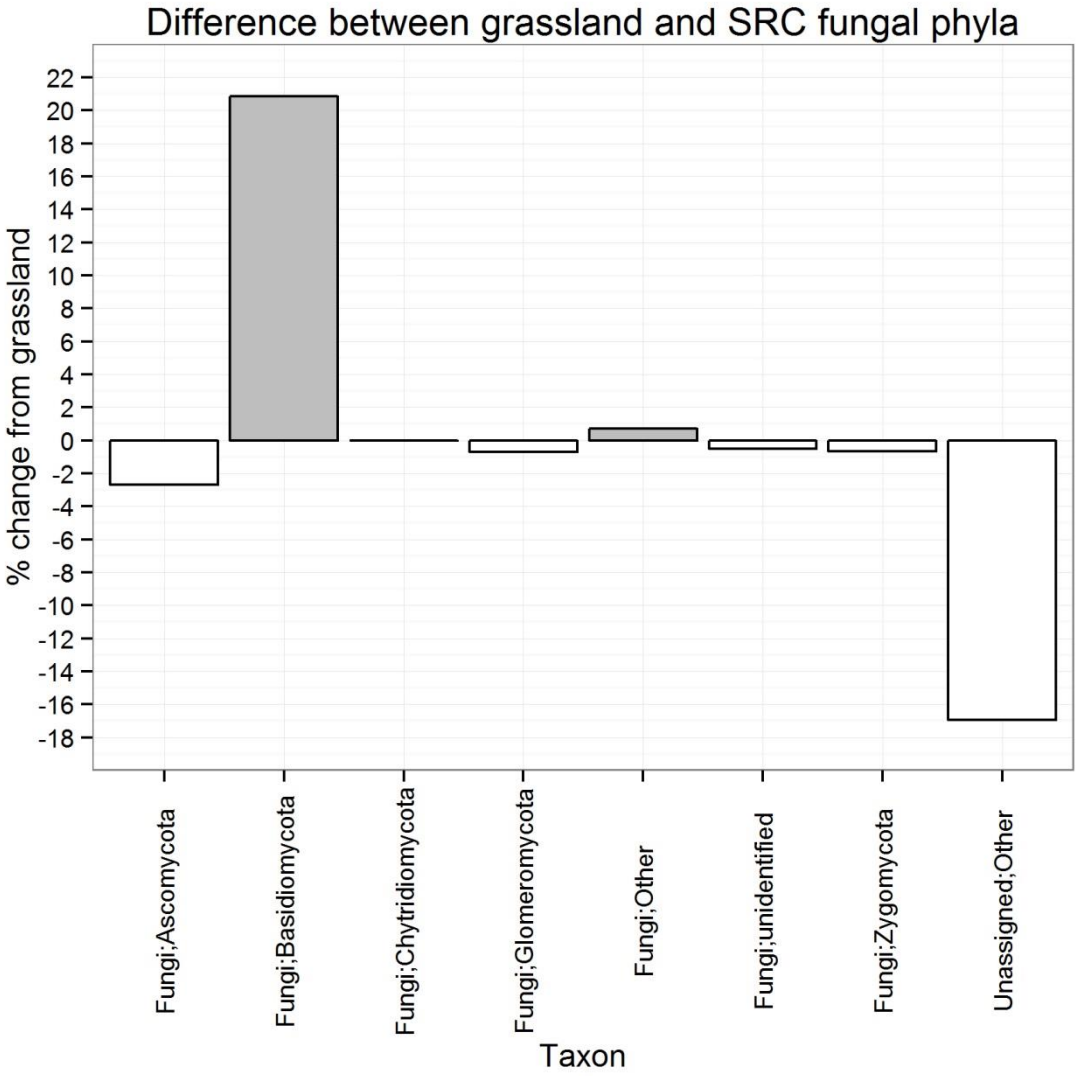
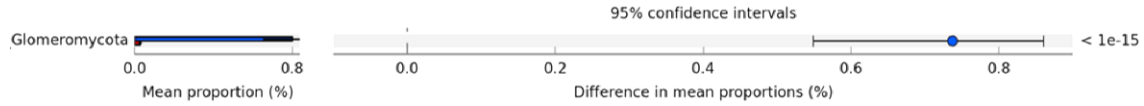


Figure 42 Mean differences in proportional abundance of fungal phyla between SRC and grassland.

STAMP analysis demonstrated that there was a single significant shift in the phylum *Glomeromycota*, which was enriched by 0.8% in grassland samples. This was driven by enrichments of the *Glomerales* (Figure 43). No other significant differences were detected.

A)



B)

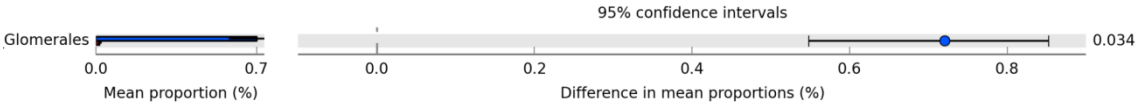


Figure 43 Extended error bar plot showing fungal a) phyla, b) order, with significant q-values (>0.05). red = SRC, blue = grassland, * = unidentified taxa within the taxon denoted.

5.4 Discussion

5.4.1 Are there differences in the microbial communities of grassland and SRC land use types?

Grassland and SRC showed no significant differences in the structure of microbial communities. In both land uses, taxonomic richness, identity and abundance were similar were not significantly different. However, STAMP analysis of differential abundance of specific fungal taxa revealed differences in the order *Glomerales* within the phylum *Glomeromycota*, which had a demonstrably higher proportional abundance in grassland samples. Forest destruction often leads to a decline in the dominance of *Basidiomycete* fungi and a corresponding increase in bacterial and fungal richness as disturbance creates new niches (Crowther *et al.* 2014). It was therefore theorised that SRC soils would display decreased α diversity related to the dominance of a single vegetation type, and increased *Basidiomycete* dominance resulting from tree/fungi symbiosis (Mendes *et al.* 2015). Increased dominance of fungal taxa was also expected to coincide with a difference in the abundances of bacterial taxa, representing the new climax community formed in response to fungal competition for resources. However, no shift from bacterial to fungal dominance was detected. In macro-ecological studies, reductions of α diversity would be detrimental to ecosystem function. However, declines in microbial diversity can continue to maintain functional contributions due to the ability of multiple taxa to undertake similar functional roles (Mendes *et al.* 2015). This functional redundancy may limit differences between ecosystem function under different land uses. β diversity also revealed no significant difference between land uses. This indicates that the identities of taxa present, as well as their abundances were similar. It has been previously suggested that arable land planted with woody biomass crops causes a switch to a fungally dominated community (Stauffer *et al.* 2014). This may reflect an increase in fungal/root symbiosis due to greater reliance on lignocellulosic biomass as a main source of organic carbon. In this experiment, no such chronosequence was undertaken, but it was still expected that the numbers and identities of fungal taxa would differ between sites due to increased lignin availability from litterfall and differences between grassland and SRC edaphic variables. For example, methods for the establishment of SRC rely upon extensive use of

heavy machinery, which compacts soils, reducing aeration and increasing bulk density (Souch *et al.* 2004). Compacted soils contain less oxygen and moisture due to decreased pore space, which could select for taxa that are tolerant to low moisture, anaerobic conditions. Alternatively, SRC stands lead to gradual declines in nutrient availability as trees utilise nutrients for growth and are harvested year on year, particularly N, P and K (Hangs *et al.* 2014). It was therefore expected that there may have been different bacterial taxa involved in the cycling of nutrients in grassland and SRC samples. This indicates that the influence of SRC may not be as large as hypothesised, as the communities were very similar when compared to those in grassland. In addition, this supports the suggestion that bacterial taxa are ubiquitous geographically (Fierer & Jackson 2006; Livermore & Jones 2015), as the two fields were separated by approximately 750m and yet have similar community profiles. How do grassland and SRC fungal communities differ?

Fungal α and β diversity did not significantly differ between land uses, indicating that the hypothesised elevation of the phylum *Basidiomycota* in SRC samples did not occur. Forest soils are often dominated by the *Basidiomycete* class *Agaricomycetes* (Buée *et al.* 2009b), which contains ectomycorrhizal fungi, wood saprotrophs and several plant pathogens (Hibbett *et al.* 2014).Similarly, a study of mycorrhizal interactions in SRC plantations indicated that EMF fungi may be introduced into soil communities during SRC establishment (Baum *et al.* 2009). Results for the present study indicate the impact of SRC growth may not always lead to the levels of fungal dominance previously discussed, as STAMP analysis showed no difference in SRC versus grassland *Basidiomycete* abundance. However, STAMP did reveal elevated proportions of the *Glomeromycete* order *Glomerales*, the taxon containing the arbuscular mycorrhizal fungi (AMF) in grassland soils. AMF taxa form arbuscules, hyphae running intracellularly through plant roots in order to exchange nutrients (usually phosphorous and nitrogen), with the host plant in return for simple sugars (Bever *et al.* 2001; Rooney *et al.* 2009; Hodge & Storer 2014). Conversion of arable land to SRC can lead to increased AMF biomass of AMF in PLFA studies (Yannikos *et al.* 2014). This is the opposite of the result within this chapter, which indicates that grassland contains a greater proportion of AMF compared with SRC. The difference may arise in the state of nutrient availability within the two soils. AMF are less successful in environments where plant roots have high nutrient availability. The SRC field

had been fertilised prior to establishment (during 2008), which may have suppressed the formation of plant-fungi interactions (Verbruggen *et al.* 2013). The last fertilisation of the grassland had been prior to its conversion from arable land in 2005, possibly increasing the requirement for AMF-plant symbiosis. Furthermore, the difference in timescale between the two land uses may act as a confounding variable, as fungal communities can change extensively over time (Caruso *et al.* 2012). By comparing managed grassland with SRC soil 4 years post-conversion it was intended to determine how soil microbiomes differed between the two land uses, a proxy for the impact of land use change. Whilst this has been used in the past to compare the succession effects resulting from land use change (Walker *et al.* 2010; Blaalid *et al.* 2012; Yannikos *et al.* 2014), the effects were not directly measured. No significant differences in total community structure were noted in bacterial or fungal communities. However, an AMF taxon (the *Glomerales*) was detected in great proportions in grassland samples. This may indicate a difference in the nature of fungi/root symbiosis between the two land use types. It is feasible that conversion of grassland to SRC may result in a similar community. However, it should be noted that the effect size was a 0.8% increase in grassland *Glomerales,* and this relatively small difference may have limited biological significance. In order to gain a truly accurate reflection of succession, samples should be collected from replicated sites as SRC was established. This would enable seasonal and successional effects to be separated. Furthermore, this thesis assessed the impacts of a single SRC crop. As variations in rhizosphere can be associated with the host crop (Lambers *et al.* 2009; Hirsch & Mauchline 2012; Hernández *et al.* 2015), it stands to reason that the nature of community variation may differ between crop types. Other methodological limitations include potential primer and database biases, and difficulties resolving the functional implications of taxonomic change as discussed in Section 3.4. Finally, there is the possibility that important differences between land uses may have been missed due to the limited replication of the study. 15 subsamples were collected per treatment, these were pooled by plot to prevent potential pseudoreplication due to geographic proximity. This resulted in a total of 3 replicates per treatment, which may lack the power to detect significant changes in highly heterogeneous bacterial and fungal populations. Future studies should therefore attempt to increase sample

numbers, and ensure that sampling design includes a measure of the impact of spatial distribution on community structures.

5.4.2 Conclusion

Previous research suggests that establishment of SRC leads to the disruption of edaphic variables, whilst varying the vegetative community present. Shifts in these variables drive variation in the structure and function of microbial communities (Fierer & Jackson 2006; Tedersoo *et al.* 2012). It was therefore expected that SRC and grassland samples would have radically different bacterial and fungal community structures. However, no significant change in richness, abundance or taxonomic identity was detected between the two land uses, although a slight increase in AMF relative abundance was detected in grassland samples. SRC may therefore not lead to the substantial divergence of communities expected, although further research using a time series approach should be utilised to confirm this.

Chapter 6: Simulating functional metagenomes using 16S data

6.1 Introduction

Whilst powerful, metagenomics suffers from high costs of sample preparation and sequencing, often limiting its use (Shokralla *et al.* 2012; Langille *et al.* 2013). Combined with the large numbers of replicates required to gain meaningful metagenomic results, this can pose a limitation to the method (Knight *et al.* 2012). Metabarcoding is considerably cheaper than metagenomics, but cannot provide information about changes in the proportion of functional genes present within a sample. However, there have been attempts to remedy this, through use of models relying on 16S bacterial proportional abundance data. PiCrust (Phylogenetic Investigation of Communities by Reconstruction of Unobserved STates) is one such method (Langille *et al.* 2013).

PiCrust makes use of 16SrRNA amplicon (metabarcoding) data, for which OTU's are assigned taxonomic identity through comparison with existing reference databases, such as GreenGenes (See 1.6.4.2), enabling the estimation of functional gene content of a sample (Langille *et al.* 2013). PiCrust reasons that closely related bacterial taxa share similar functional genes profiles. Therefore PiCrust comes supplied with a gene content prediction table, consisting of protein coding genes from previously sequenced bacteria along with the identities of the OTUs in which they were found (from the IMG database http://img.jgi.doe.gov). The basic premise of PiCrust is that given proportional abundances of known bacterial genera within a sample, it should be possible to model their functional content if closely related taxa have been previously sequenced and protein coding genes identified.

For most accurate results, PiCrust requires a closed reference OTU table, that is to say it contains only identified taxa. This is usually carried out through use of existing pipelines, with QIIME being recommended by the authors (See 1.6.4.2 and 3.2.7 for further descriptions of QIIME). PiCrust then undertakes a normalisation step to estimate the actual abundance of bacteria present. This is because 16SrRNA copy numbers can vary between taxa. To control for this, copy number normalisation is carried out, dividing the total of 16S counts per taxon by the associated 16S copy number in the provided precalculated files. Again, these are based on the number of copies of the 16SrRNA gene found in each taxon as documented in the IMG database (Markowitz *et al.* 2014). The

result of the normalisation is an abundance count for each taxon, rather than a proportional abundance of the 16S gene. Whilst appropriate for taxonomic estimations of proportional abundance, uncorrected data is not appropriate for the estimation of gene profiles as functions may otherwise be overinflated. After 16SrRNA copy number normalisation, PiCrust then generates a predicted metagenome. This step multiplies functional gene counts (as found in the pre-generated table) by the 16S abundance for each taxon, producing an estimated abundance of each functional gene within the sample. Differences between treatments can then be assessed using statistical methods such as STAMP (Parks & Beiko 2010) (Section 3.3).

Given the wealth of 16SrRNA data produced by the analysis of biochar application across Europe (Chapter 3:), PiCrust was used to predict the metagenomes at each site. This aimed to determine whether significant changes in the functional attributes of the microbial communities occurred as a result of biochar treatment. Given the significant shifts in the UK1Y results driven by *Acidobacteria* and *Gemmatimonadetes*, the *Gemmatimonadetes* increase in UK1M samples, and the *Proteobacteria* and *Acidobacteria* enrichment detected in ITBC, shifts were expected in associated functions (increases in taxonomic abundance may lead to increases in associated functional genes present). No taxonomic changes were detected in the FR samples, and as such PiCrust was not expected to detect any changes in functional profile for these samples.

6.2 Method

6.2.1 Qiime "closed reference" pipeline

OTU picking was carried out in QIIME (Caporaso *et al.* 2010b), using the "pick_closed_reference_OTUS.py" script (Section 1.6.4.2). The method removes unidentified taxa, maximising the accuracy of PiCrust's gene prediction step. The closed reference OTU picking method utilised the GreenGenes 13.5 database, the most recent version at the time of writing (DeSantis *et al.* 2006). The method was identical to that described in Section 3.2.7, with the replacement of the "pick_de_novo_otus.py" script with the "pick_closed_reference_otus.py" script. The input files were the same as those detailed in Section 3.2.7, resulting from combination of paired end reads using

PandaSeq (Masella *et al.* 2012). OTUs were picked to 97% identity. This was undertaken for each site (UK1M, UK1Y, FR and IT) independently.

6.2.2 Simulating functional profiles using PiCrust

PiCrust attempts to estimate the number of individual cells by using a normalisation method, which divides each OTU by its associated 16S gene copy number. This was carried out using the command "normalize_by_copy_number.py", defining the input table as the output from the QIIME closed reference OTU picking step for each site. The output from this step was subsequently submitted to the "predict_metagenomes.py" command, which estimated the abundance of functional genes (Langille *et al.* 2013). The resulting file contains an estimated abundance profile for KEGG functions, which were analysed through use of STAMP (Parks & Beiko 2010).

6.2.3 Differential abundance of simulated functional profiles

STAMP analysis of differential abundance of modelled functional gene profiles was undertaken using the settings described in Section 3.2.7, in which results were filtered to exclude those where <5 sequences were present, and in which the effect size was <0.25% of the total functional profile, or a <2 fold change. This filtering method was chosen in order to remove those results which, while statistically significant, were unlikely to have any biological relevance (Parks & Beiko 2010). All filtering was applied after statistical tests and corrections had been carried out.

6.3 Results

6.3.1 Closed reference OTU results

Closed reference OTU picking produced almost identical results as those detected in chapter 3 using the *de novo* method at high levels. This is because the method maintains the 97% identity clustering threshold. Therefore, differences between datasets only arise when a read fails to match any other OTU in the reference database. In these cases, the read is discarded. However,

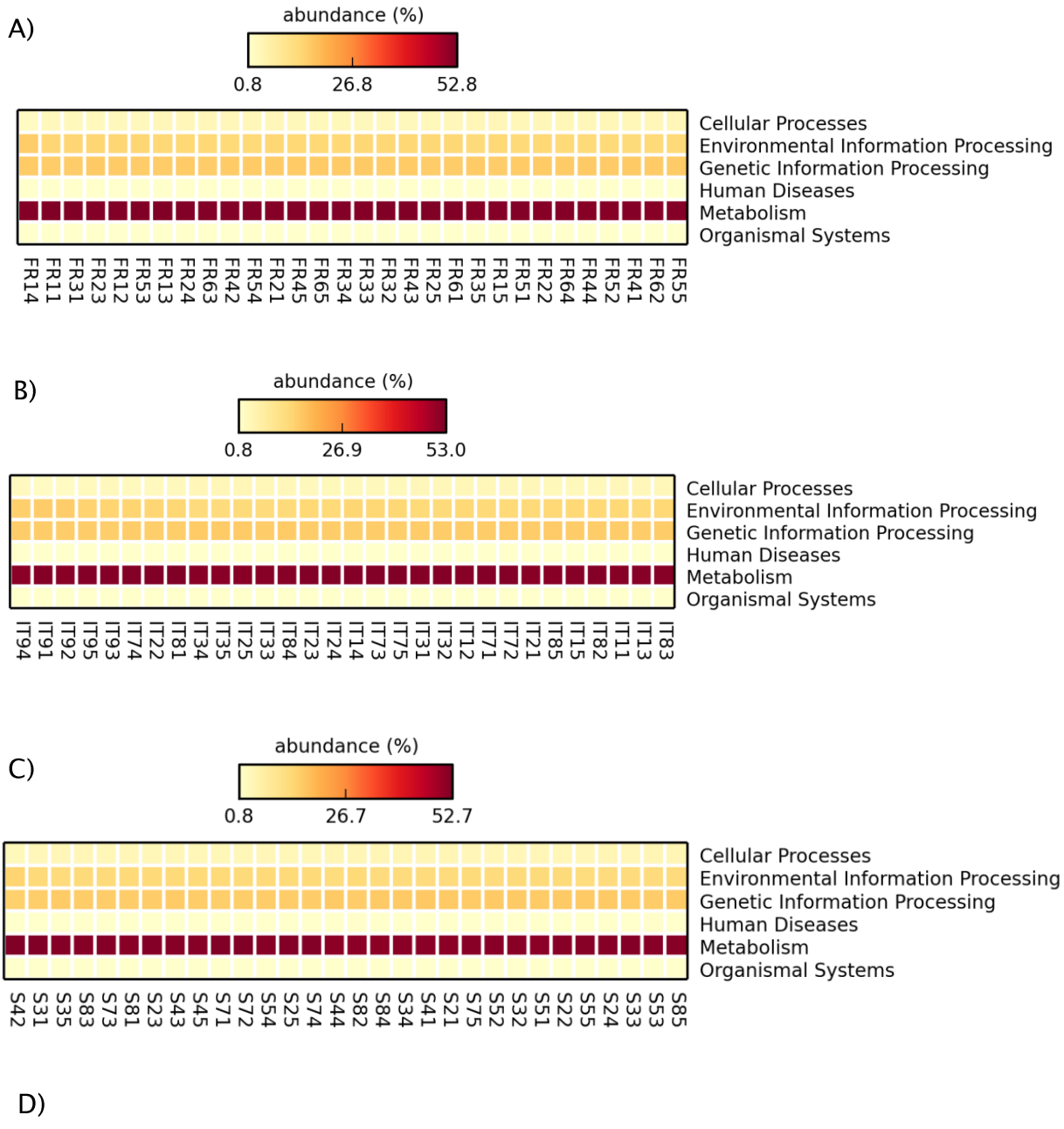
the proportions of OTUs in the remaining identified reads are very similar to those generated by *de novo* methods (Rideout *et al.* 2014). Relative abundance of the phyla at each site can be seen in *Table 27*.

Table 27 Mean abundances of Phyla by treatment and sites produced by closed reference OTU picking

Phylum	UK1MBC	UK1YBC	FRBC	ITBC	UK1MC	UK1YC	FRC	ITC
Acidobacteria	13%	19%	14%	11%	14%	20%	12%	14%
Actinobacteria	17%	8%	32%	35%	17%	9%	36%	32%
Bacteroidetes	4%	7%	3%	2%	4%	4%	0%	2%
Chloroflexi	6%	6%	6%	6%	6%	3%	8%	7%
Nitrospirae	4%	1%	3%	2%	3%	0%	2%	2%
Planctomycetes	8%	8%	9%	8%	10%	10%	6%	11%
Gemmatimonadetes	4%	5%	3%	4%	2%	4%	3%	3%
Proteobacteria	27%	31%	20%	22%	26%	27%	22%	19%
Verrucomicrobia	8%	10%	3%	2%	8%	11%	3%	3%
Cyanobacteria	0%	0%	0%	0%	0%	0%	0%	1%
Other (<1%)	9%	5%	7%	8%	10%	12%	9%	6%

6.3.2 Simulated metagenomes

Of the simulated functional level 1 KEGG groups detected in all sites (UK1M, UK1Y, IT and FR), approximately 50% of both biochar and control samples were associated with Metabolism. Other dominant functions detected included Genetic Information processing, Environmental information processing and cellular processes, contributing approximately 15% each. Functions related to Cellular Processes, Human Diseases and Organismal Systems correspond to <5% of the predicted metagenome (Figure 44). Differential abundance testing in STAMP revealed no significant difference between treatments.



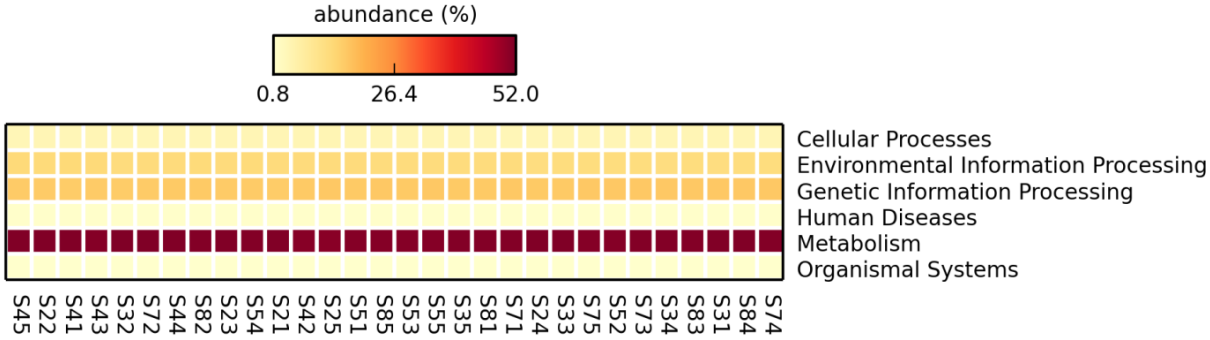
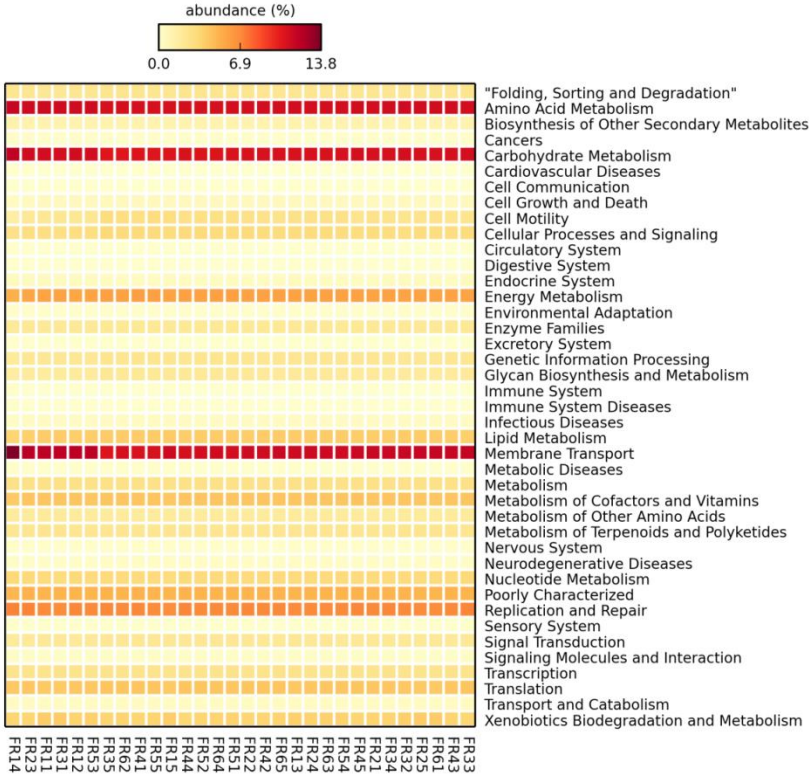


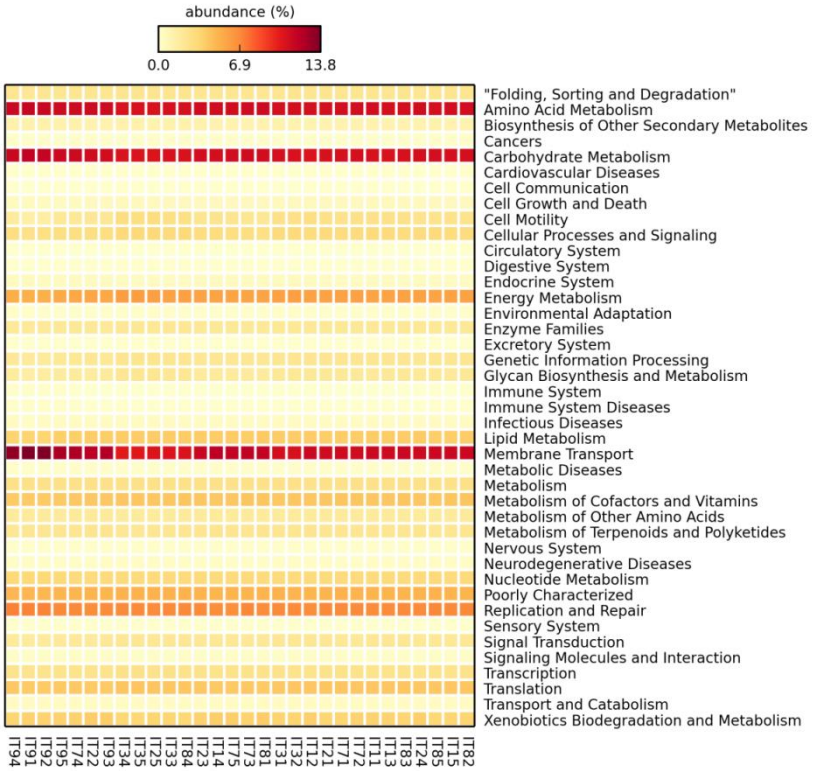
Figure 44 Heatmaps showing proportion of functions within predicted metagenomes for A) FR B) IT C) UK1M and D) UK1Y samples

Level 2 groups were dominated by Membrane transport, Carbohydrate metabolism and Amino Acid metabolism (each accounting for approximately 10%). Other functions detected at levels >1% include Replication and Repair (approximately 7%) Energy metabolism (approximately 4%) and Poorly Characterised functions. Again, no significant differences were detected between treatments .

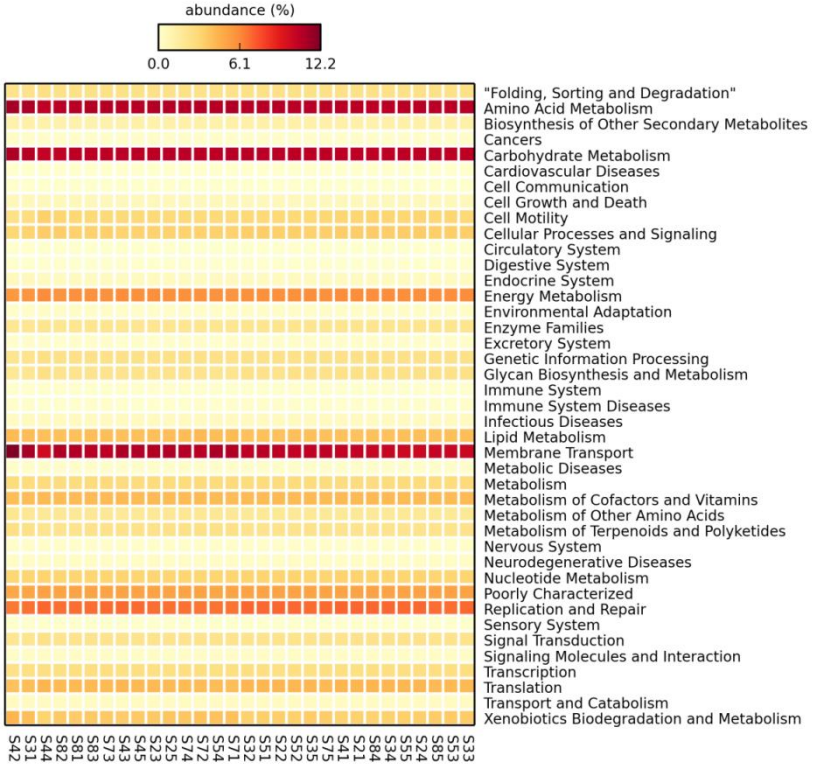
A)



B)


abundance (%)
0.0
6.9
13.8
"Folding, Sorting and Degradation"
Amino Acid Metabolism
Biosynthesis of Other Secondary Metabolites
Cancers
Carbohydrate Metabolism
Cardiovascular Diseases
Cell Communication
Cell Growth and Death
Cell Motility
Cellular Processes and Signaling
Circulatory System
Digestive System
Endocrine System
Energy Metabolism
Environmental Adaptation
Enzyme Families
Excretory System
Genetic Information Processing
Glycan Biosynthesis and Metabolism
Immune System
Immune System Diseases
Infectious Diseases
Lipid Metabolism
Membrane Transport
Metabolic Diseases
Metabolism
Metabolism of Cofactors and Vitamins
Metabolism of Other Amino Acids
Metabolism of Terpenoids and Polyketides
Nervous System
Neurodegenerative Diseases
Nucleotide Metabolism
Poorly Characterized
Replication and Repair
Sensory System
Signal Transduction
Signaling Molecules and Interaction
Transcription
Translation
Transport and Catabolism
Xenobiotics Biodegradation and Metabolism
IT94 IT91 IT92 IT95 IT74 IT22 IT93 IT34 IT35 IT25 IT33 IT84 IT23 IT14 IT75 IT73 IT81 IT31 IT32 IT12 IT21 IT71 IT72 IT11 IT13 IT83 IT24 IT85 IT15 IT82

C)


abundance (%)
0.0
6.1
12.2
"Folding, Sorting and Degradation"
Amino Acid Metabolism
Biosynthesis of Other Secondary Metabolites
Cancers
Carbohydrate Metabolism
Cardiovascular Diseases
Cell Communication
Cell Growth and Death
Cell Motility
Cellular Processes and Signaling
Circulatory System
Digestive System
Endocrine System
Energy Metabolism
Environmental Adaptation
Enzyme Families
Excretory System
Genetic Information Processing
Glycan Biosynthesis and Metabolism
Immune System
Immune System Diseases
Infectious Diseases
Lipid Metabolism
Membrane Transport
Metabolic Diseases
Metabolism
Metabolism of Cofactors and Vitamins
Metabolism of Other Amino Acids
Metabolism of Terpenoids and Polyketides
Nervous System
Neurodegenerative Diseases
Nucleotide Metabolism
Poorly Characterized
Replication and Repair
Sensory System
Signal Transduction
Signaling Molecules and Interaction
Transcription
Translation
Transport and Catabolism
Xenobiotics Biodegradation and Metabolism
S42 S31 S44 S82 S81 S83 S73 S43 S45 S23 S25 S74 S72 S54 S71 S32 S51 S22 S52 S35 S75 S41 S21 S84 S34 S55 S24 S85 S53 S33

D)

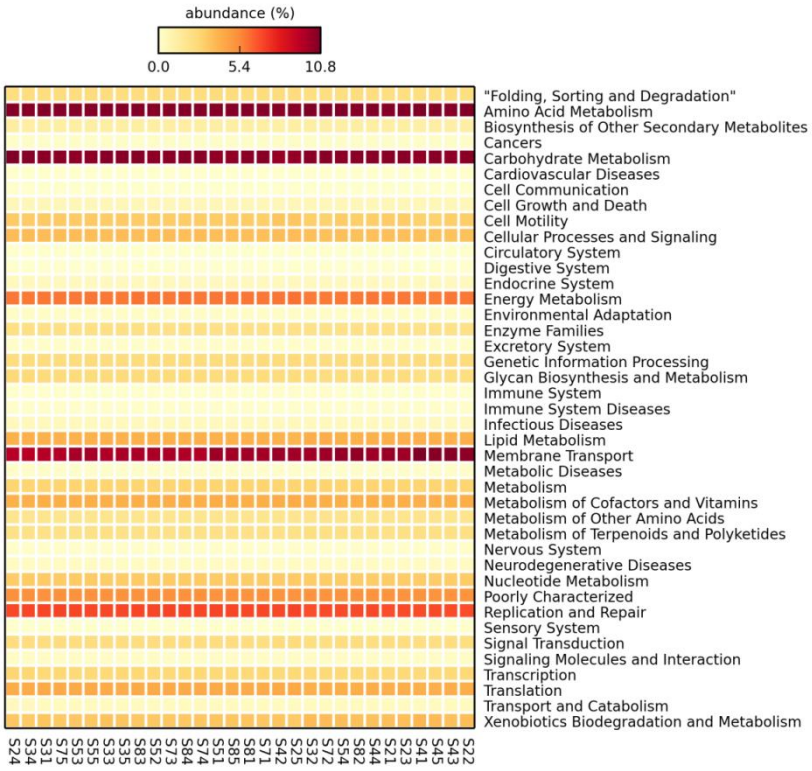


Figure 45 Heatmaps showing the proportion of level 2 functions in each sample in A) FR, B) IT, C) UK1M and D) UK1Y

At level 3, genes associated with transporters were most dominant (a mean of 6% in each treatment/site combination). Unfiltered heatmaps contain several hundred functions, and for this reason are not included here. Other notable functions present in high proportions included genes related to ABC transporters (approximately 5%), DNA repair and recombination proteins (approximately 3-4%), and transcription factors, secretion systems, ribosome biogenesis, purine metabolases, peptidases, other ion-couple transporters and oxidative phosphorylation genes, at around 1% each. STAMP analysis of level 3 functions also revealed no significant difference in simulated metagenomes detected as a result of treatment at any of the sites, or UK time points ($p = 0.05$).

6.4 Discussion

6.4.1 Can PiCrust be used to model functional profiles of complex soil communities?

No significant difference between functions as a result of biochar treatment was detected at any site. This may be interpreted in several ways. It may be that no change occurred in soil function as a result of treatment. Alternately, as PiCrust relies on previously cultured OTUs to assign functional groups to 16S data, the method may not be suitable for highly diverse communities containing many unculturable taxa, such as those found within the soil environment. Previous studies using PiCrust have often focussed on modelling human microbiome metagenomes (Langille *et al.* 2013; Fujimura *et al.* 2014; Seekatz *et al.* 2014), which are comparatively well documented and contain fewer unidentified OTUs compared with soil samples. It has also proved a viable tool in assessing simple acid mine drainage communities, revealing similar predicted metagenomic profiles to those detected in true metagenomic studies of the same communities (Parks & Beiko 2010).

PiCrust requires datasets containing only previously identified OTU's as a way of reducing error caused by unknown data. Using this method enables accurate functional profiles to be generated for those taxa within the dataset. However, this may also result in the loss of large numbers of OTU's which have not yet been identified, thus limiting the power of the method to reconstruct full model metagenomes. Therefore there may be significant functional shifts if unidentified taxa are included. It may be possible to use de novo 16S datasets, but difficulties arise in modelling the expected functional profile for these OTUs due to the lack of closely related taxa within databases. For example, two unidentified taxa within the *Proteobacteria* could contain very different functional genes, due to the wide range of functional groups present within the phylum. PiCrust would estimate both to contain similar genes, as they are both most closely related to the same taxa. This could lead to inflated or unrepresentative counts, further obscuring the true functional profile.

Little change in taxa present was detected as a result of biochar application (Section 3.3). The largest effect sizes present in samples were less than 1% when unfiltered by q-score. Filtering by significance and fold change resulted

in no significant changes. As effect size filters removed all significant samples with an effect of <0.25%, there do not appear to be any biologically meaningful differences between simulated metagenomes due to treatment.

PiCrust is reliant on changes in OTU abundance to estimate functional profiles. Whilst shifts in taxa occurred, (Section 3.3), no biologically meaningful changes in functional abundance occurred. It is unlikely an effect is occurring, as effect sizes are small, and fall within limited confidence intervals. However, PiCrust's inability to model functional profiles from unidentified taxa and its reliance on existing bacterial genomes may mean that it fails to capture much of the detail from soil samples, which contain a wide range of uncultured bacteria. Alternatively, there may be no change in the frequency of genes within the simulated metagenomic profile in response to biochar treatment, although shifts in the rate of transcription may have occurred. These types of shift would not be detected by metagenomic analysis, nor by PiCrust simulations, and would require the use of either metatranscriptomics or extensive qPCR studies in order to assess whether changes in RNA transcripts had occurred. Any such change would be representative of an upregulation of gene activity as a result of treatment, with resulting shifts in protein and enzymatic processes (Carvalhais *et al.* 2012) However, at the present time, extraction techniques and the instability of RNA make these types of study extremely difficult for samples derived from soil environments.

6.4.2 Limitations and conclusion

Without obtaining metagenomic data for the same samples used in the PiCrust analysis and Chapter 3: , it is difficult to determine the validity of the PiCrust method. There appear to be no changes in functional diversity and abundance as a result of biochar treatment, although this may be a result of inherent bias within the PiCrust method, and the limited shifts in diversity detected in 16S data. It should also be noted that PiCrust is only appropriate for bacterial datasets, and can therefore not model full metagenomes, preventing variation in eukaryotic, and viral gene function from being detected. Specifically, this prevented us from modelling fungal functional change, which at some sites showed greater variation in community structure than bacteria (Section 3.3). Therefore significant differences in function driven by fungal community change may have been missed.

Chapter 7: General Discussion

7.1 Introduction

The aim of this thesis was to understand changes in the diversity and abundance of soil microbial communities in response to future climate scenarios, using novel NGS approaches coupled with long-term, realistic field manipulation experiments. After initial assessment of metagenomics technologies, using both drought and biochar samples, it was decided to focus largely on biochar, a treatment previously linked to variation in the structure of microbial communities (Grossman *et al.* 2010; Kolton *et al.* 2011; Xu *et al.* 2014b; Hu *et al.* 2014), with a secondary focus on LUC to SRC bioenergy. Both of these land use changes are highly topical and of global significance and yet represent changes where understanding is limited. To investigate them high resolution sequencing methods were used, in order to understand the specific changes in taxa.

Changes in the identity and abundances of taxa were assessed, and whether these could explain some of the nutritive effects previously associated with biochar application (Lehmann 2007; Mao *et al.* 2012). Studies have used a range of biochars, produced and applied using different methods (Section 1.3 and 2.1.3). By standardising the biochar used at several sites it was confirmed that the different effects previously detected represent real variation in response, probably due to interactions between the biochar, edaphic and environmental variables.

The three biochar sites showed a distinct difference in their dominant phyla, with the UK site differing greatly from the two sites in continental Europe. Whilst the sites in FR and IT had similar community structures at the level of phylum, they displayed very different responses to biochar. By combining nutrient leachate and soil respiration data with NGS samples from the UK time series, the impact of biochar application on microbial activity or nutrient status of the soil was assessed. Through comparison of SRC *Salix* soil samples with adjacent grassland, this thesis aimed to elucidate the effect of land use change on bacteria and fungi communities in these new biomass cropping systems. Finally, identification of potential genes relating to changes in biogeochemical cycling was attempted through use of modelled metagenomes. This was to attempt to estimate the effects of changes in microbial community structure to

ecosystem function, without the use of expensive metagenomic or metatranscriptomic methods.

7.2 Does drought affect microbial community structure and function?

Metagenomic analysis found no effect of drought on either microbial community structure or functional profiles. Subsequent metabarcoding analysis of the same dataset was undertaken, to determine whether using a targeted method would improve analysis. This too failed to confirm an effect. With hindsight it became apparent that three bulk sequenced samples was not a sufficient number of replicates as microbial communities show variation across very small geospatial resolutions, and that the 15 samples per treatment used in the metabarcoding data (Chapter 3:) is more appropriate (Fierer & Jackson 2006; Griffiths *et al.* 2011). This enables clustering based methods (such as PCoA) to resolve more readily, whilst ensuring that variation between samples does not obscure biologically meaningful results. In the literature, drought appears to decrease microbial biomass and activity across all taxa, and communities appear to adapt to the increased water stress by switching their ecological strategy (Sheik *et al.* 2011; Bérard *et al.* 2012; Evans & Wallenstein 2014). This suggests that the functional and taxonomic profiles of these communities may be unchanged, adapting through decreased total biomass and changes in transcription rather than through shifts in community ecology. Therefore, use of PLFA and enzymatic activity assays, possibly coupled with metatranscriptomics may be a more suitable method for detecting changes in community function in these samples. Similarly, in order to confirm that no taxonomic shifts occur, it is suggested that a minimum of 15 samples be collected and sequenced separately per treatment, in order to maintain the required level of replication. Due to time and monetary constraints, biochar was opted as the focus treatment rather than drought, as community response would be greater impacted by this treatment (Kolton *et al.* 2011; Lehmann *et al.* 2011; Anders *et al.* 2013).

7.3 Biochar application causes changes in microbial community structure – but responses vary by site

Initial metagenomic and metabarcoding studies assessing biochar treatment showed no significant difference in communities or functional gene profiles (Chapter 2:). However, secondary metabarcoding study (with samples from three European field sites) determined that biochar always had some effect on the community structure of bacteria, fungi or both (Chapter 3:).

Previous biochar studies have identified shifts in large, coarsely defined groups of taxa determined using PLFA methods (see *Table 16*). Whilst there were suggestions that its application could cause changes in microbial diversity, there was little consensus as to what taxa would be affected (Kim *et al.* 2007; Grossman *et al.* 2010; Hu *et al.* 2014). Furthermore, few studies assessed changes in fungal diversity, richness and community structure (Hu *et al.* 2014). The inclusion of both bacterial and fungal primers to assess diversity attempted to shed some light on the impact of biochar application on these essential organisms.

Studies have suggested that biochar application may have a minimal impact on diversity of microbial communities (Rutigliano *et al.* 2014; Anderson *et al.* 2014). Metagenomic data did not detect any change in taxonomic α diversity in biochar treated plots (Section 2.3.3), nor did the metabarcoding data. Whilst not detected in the metagenomic dataset, the increased number of replicates in the Europe wide metabarcoding data permitted the detection of shifts in β diversity, a measure of the proportional abundances of taxa present (Section 3.3). This is in line with several studies which have suggested that biochar treatment causes significant change in the structure of bacterial communities (Kolton *et al.* 2011; Anderson *et al.* 2011; Anders *et al.* 2013; Chen *et al.* 2013; Xu *et al.* 2014b). Unlike these previous studies, this thesis utilised samples from a field environment, rather than lab based incubation studies. It is therefore likely that the results indicate with greater accuracy the type of responses which could be expected in the field although at the cost of reduced control over confounding variables.

Further analysis of the community shifts showed varying taxa affected differently at each site (Section 3.3.4). Whilst the biochar applied at each site

was identical (the same pH, soil moisture content and production method), the edaphic, ecological and meteorological variables at each site varied. It is this variation in starting conditions which may cause the diverse responses noted. Given that soil moisture, pH and vegetation type are driving variables when it comes to bacterial biogeographical dispersion, this stands to reason (Fierer & Jackson 2006; Tedersoo *et al.* 2012; Fierer *et al.* 2012b; Serna-Chavez *et al.* 2013; Barberán *et al.* 2014).

Whilst bacterial β diversity changed in response to treatment at all sites one year after application (if both weighted and unweighted responses were included), fungal community structure appears more resilient. This is possibly due to differences in the driver of fungal diversity. Whilst soil moisture and pH drive bacterial diversity and abundance, fungal communities are known to be associated with host plants, with temperature and precipitation coming into play in boreal and temperate forest environments (Tedersoo *et al.* 2012; Wardle & Lindahl 2014). Biochar is unlikely to impact upon these driving parameters, although there is potential for changes in soil moisture content and albedo effects (Lehmann *et al.* 2011) to potentially impact upon the abiotic niche of the fungi. It is also suggested that disturbance effects may reduce fungal diversity (Tedersoo *et al.* 2012). Incorporation of biochar may represent one such effect, although no significant difference in diversity was detected one year after treatment. The results may reflect this in the short-term change decline in fungal α diversity 1 month after application, although no significant change in any particular taxa was detected.

7.4 What can be inferred about soil function from changes in taxonomic abundance?

Application of biochar led to changes in the abundance of several fungal and bacterial taxa with known functions within soil communities. Several potential shifts in community function which could occur as a result of these changes were noted. For example, the fungal family *Chaetothyriaceae* in ITBC samples, which was increased in biochar treated plots (Section 3.3) is associated with the formation of leaf surface molds, although information on its ecological niche is limited (Chomnunti *et al.* 2012). This elevated abundance within biochar plots may be linked to previous suggestions of a decrease in plant defences as a result of biochar treatment (Viger *et al.* 2015). IT also showed

elevated abundance of *Proteobacteria*, due to increases in *Rhizobiales.* This may have implications for increased turnover of organic matter to SOM (Spain *et al.* 2009), and plant growth promotion (Bruto *et al.* 2014).

Small adjustments occurred in multiple taxa in UK samples. It appears that shifts were associated with a transition from an oligotrophic to a copiotrophic environment. The associated carbon influx may explain the increased abundance of copiotrophic *Acidobacteria* taxa (Fierer *et al.* 2007). However, attempts to detect changes in functional gene profiles using PiCrust (Chapter 6:) failed to find any significant difference. It may be that no change occurred, or that the reliance of the method on extrapolating profiles from taxonomic summary data missed the range of small adjustments made by the community.

Biochar application in the UK was associated with increases in ammonium and phosphate availability, and increased activity of the enzyme alkaline phosphatase, which may gradually lead to a nutrient rich soil environment (Chapter 4:). Whilst the mechanism by which this took place cannot be confirmed, it is possible that the multiple small shifts in taxonomic abundance and associated shifts in gene activity explain these changes. For example, increased availability of ammonium may be a result of decreased rates of denitrification (Anderson *et al.* 2011), usually associated with declines in the abundance of specific taxa, such as the *Bradyrhizobia*. Whilst no large shift occurred in these taxa, this doesn't rule out a change in unknown denitrifying bacteria, or a shift in the metabolism of known taxa as a result of biochar incorporation.

Previous studies have found that biochar augments nitrous oxide reduction, in turn elevating the rate of N_2 release into the atmosphere (Harter *et al.* 2014). Therefore application of biochar is likely to reduce the formation of nitrous oxide, with the dual effect of reducing the emissions of a potent greenhouse gas and varying soil nitrogen cycling patterns. In this thesis, the total quantity of ammonium in leachate was elevated, implying either an increase in its production, or a decrease in the conversion of ammonium to nitrate. This may be due to the ability of biochar to adsorb ammonium compounds (Gai *et al.* 2014), increasing their presence within the soil. The porosity and presence of surface charges on biochar can result in the retaining and adsorbing charged molecules (Beesley *et al.* 2011; Gai *et al.* 2014). This may reduce the rate at which ammonium ions leach through the soil, increasing their residence time

and thus elevating ammonium concentrations. In order to confirm the suspected shifts in N cycling, qPCR assays of N cycling genes (see Section 7.7) should be undertaken. Alternatively, full metatranscriptomics and metagenomics studies would be able to determine whether the abundance and activity of related genes were affected by biochar treatment.

Phosphate too can be adsorbed by biochar (Zhai *et al.* 2015). Increased colonisation of biochar by bacteria and fungi can result in bioavailable phosphates being released, either through solubilizing inorganic forms of P to orthophosphate, or through physical access of P available within biochar pore spaces which root hairs are unable to penetrate (Anderson *et al.* 2011; Fox *et al.* 2014; Hammer *et al.* 2014; Zhai *et al.* 2015). EMF taxa may interact with biochar, reaching nutrients inaccessible to tree root hairs, providing nutrients to the host plant (Hammer *et al.* 2014). Interactions of this type may favour particular taxa which can utilise biochar more readily and reflected in increases in abundance under biochar treatment. Further supporting these assertions are the results of enzymatic activity analysis, which concluded that the activity of alkaline phophomonoestarases were significantly increased in biochar samples, whilst acid phosphomonoesterases were significantly decreased. Differentiation in the type of phosphomonoesterase present within the soil may be a result of shifts in soil pH due to biochar application. Alternatively, the pH requirements of phosphomonoesterases have also been correlated with shifts from fungally dominated P cycling to bacterially dominated communities (Caldwell 2005; Weintraub 2011). However, these enzymes are also released as plant exudates, and determining the exact source of the increased activity is difficult.

It is interesting to note that a significant increase in total soil respiration occurred, probably due to the influx of C and nutrients, which could still be detected in the long term dataset. Partitioning of isotopic data at the same site indicated that biochar treatment limited the rate of respiration from native SOM, illustrating a negative priming effect (Ventura *et al.* 2015). Subsequently, this may protect extant SOM, preventing its decomposition and increasing its role in C sequestration in the presence of biochar, with the additional benefit of increasing soil quality. However, isotopic analysis was only undertaken during the initial sampling period, and so it is not possible to determine whether biochar protective effect continued in the long term. If the trend

detected during the first year remained, carbon sources from biochar may be preferentially respired, protecting native SOM and allowing for its gradual augmentation. To determine this, further isotopic analysis should be carried out.

7.5 Annual differences have a greater impact than biochar application but interactions between time and treatment may occur

The time series experiment showed that changes in communities were fleeting, and varied with time, regardless of treatment (Sections 3.3.5 and 3.3.9). Whilst a significant effect of biochar occurred (Section 7.3), this was smaller than the annual shifts noted in community structure regardless of treatment. Temporal changes in diversity may reflect a pattern of succession with the seasons, and annual events. It is known that seasonal fluctuations in both fungal and bacterial communities occur, as seasonal ecological niches open up (Gadd 2007; Cruz-Martínez *et al.* 2009; Gilbert *et al.* 2010b; DeBruyn *et al.* 2011). Increased summer temperature, and subsequent variation in WHC and pH could drive variation in the diversity and abundance of taxa present (Fierer & Jackson 2006; Castro *et al.* 2010). Similarly, annual variation of the same edaphic variables may also drive temporal variation. For example, an increase of 10% in unidentified *Sordariomycete* fungi in control samples one month after treatment was detected when compared with controls after one year. These are often plant pathogens, although they can also form symbiotic endophytes providing increased resistance to other pathogens and insect herbivory (Zhang *et al.* 2006). This was not observed in biochar treated plots over the same time period, which may indicate an interaction effect between the treatment and time in the case of this taxa. Similarly, whilst bacterial fluctuations were noted in both treatment and control, there were taxa which were significantly different only in one treatment condition.

To determine the likely effects of these succession events for soil function, further seasonal measurements are required. By carrying out annual measurements of the site, it may be possible to determine whether the annual shifts in diversity are due to recurrent shifts in taxa. Increasing the occurrence of sampling, coupled with more detailed measurements of edaphic variables,

meteorological events and flux in annual vegetation may help to unravel the variables driving the changes in diversity. Similarly, increased temporal resolution would enable an understanding of seasonal succession events which may be occurring.

As discussed in Chapter 3: , there is likely to be an interaction between the physico-chemical environment produced by biochar and the drivers of temporal variation. Biochar is known to increase soil aeration, pore space, and SWC (Major *et al.* 2010; Lehmann *et al.* 2011; Jones *et al.* 2012). Coupled with its liming effect, biochar may provide a buffer against annual fluctuations in these variables, subsequently stabilising microbial communities. However, without greater temporal sampling, it is difficult to determine whether such changes occur. Ideally, several annual datasets should be collected including enzymatic activity, metatranscriptomics and further metabarcoding. Production of monthly datasets for those methods described in Chapter 3: would enable correlation of monthly shifts in taxa with transcriptome variation, enzymatic activity and nutrient availability. This would help to resolve the link between soil community ecology and functional change.

7.6 Do grassland and SRC have differently structured microbial communities?

The majority of the experiments were undertaken in short rotation coppice plantations, as biochar application may be introduced to biomass plantations as part of a cyclical carbon sequestration method. By using the same amplicon sequencing techniques for fungal and bacterial surveys described in Chapter 3: it was hoped to determine whether significant differences in the structure and diversity of communities occurred between land use types.

No significant differences were detected in the richness of bacterial or fungal taxa between the two land uses. This may be linked to the adaptability of microbial taxa, being capable of carrying out a range of functions depending upon the availability of resources within their environment. Functional redundancy in many microbial communities may enable taxa to adapt to shifting edaphic variables, by changing their patterns of resource use (Schimel & Schaeffer 2012; Pan *et al.* 2014; Mendes *et al.* 2015).

Grassland samples displayed a single significant difference in relative abundance, within the fungal order *Glomerales* (Section 5.3). This order contains arbuscular mychorrizal fungi, a group of symbiotic fungi which exchange nutrients with plant roots in return for sugars. However, given the limited nature of the difference (0.8%) it is not clear whether this represents a biologically meaningful difference. Whilst differences in fungal relative abundance were expected, it was surmised that these would occur in SRC samples, which have been shown to contain substantially elevated abundances of *Basidiomycete* fungi in the past (Peay *et al.* 2010; Tedersoo *et al.* 2010; Prayogo *et al.* 2013). *Basidiomycete* taxa such as the *Cortinariaceae* have been previously found to form relationships with the roots of *Salix* species, the same genus used for the establishment of the SRC plantation (Püttsepp *et al.* 2004; Baum *et al.* 2009), and it was expected that the establishment of SRC would have led to a difference in their abundance when compared with adjacent grasslands.

Similarly, differences in the types of biomass available to soil microbial communities were expected to exert selection pressures. For example, SRC plantation soils have higher availabilities of lignified biomass, fine root turnover, and litter fall compared with grassland. This was expected to select for taxa capable of utilising complex sugars, such as *Verrucomicrobia,* (Sangwan *et al.* 2004).

The lack of significant differences may indicate that edaphic variables and other land use change related selection pressures may minimally affect microbial communities. However, it should be noted that this study utilised a relatively low level of replication, leading to decreased power. Given the complexity of the data, additional sampling effort should be used in future to ensure that significant changes in bacterial and fungal communities are not missed. It must also be noted that in order to truly understand the potential implications of conversion of grassland to SRC, a genuine time series experiment should be undertaken. This would be able to determine whether short term shifts in community richness and structure occur in response to land use change related disturbance, and would provide data relating to the formation and transition to new climax communities after SRC establishment.

7.7 Conclusions and Future work

To conclude, biochar application leads to considerable changes in bacterial and fungal community structures. The nature of the change varies between sites, probably due to differing edaphic and meteorological variables and initial community structures. Using an identical biochar still resulted in variation of response, most likely due to the complex interactions between biochar properties (water holding capacity, porosity, surface charge, nutrient content, and pH), edaphic variables (soil pH, soil moisture content, bulk density) and environmental variables (meteorological events, vegetation type, initial microbial community structure). The contradictory responses noted in earlier studies (Section 2.1.3) are therefore likely to reflections of variability in response rather than methodological or technical errors.

Microbial communities also respond to an interaction between biochar and time. This appears to be a combination of gradual shifts in edaphic variables caused by the properties of biochar, gradual metabolisation of biochar C and increased soil surface area. Moreover, temporal effects themselves appear to have a substantial effect on community structure alone, with large variations occurring in control samples over time. The use of a single characterised biochar across sites with similar pH has identified the variety of responses which occur as a result of interactions with the aforementioned variables. However, the exact implications of these complex interactions for microbial community structure remain unknown. Future work should attempt to better understand the interactions between environmental, ecological and edaphic factors by using multivariate analyses to unravel these effects.

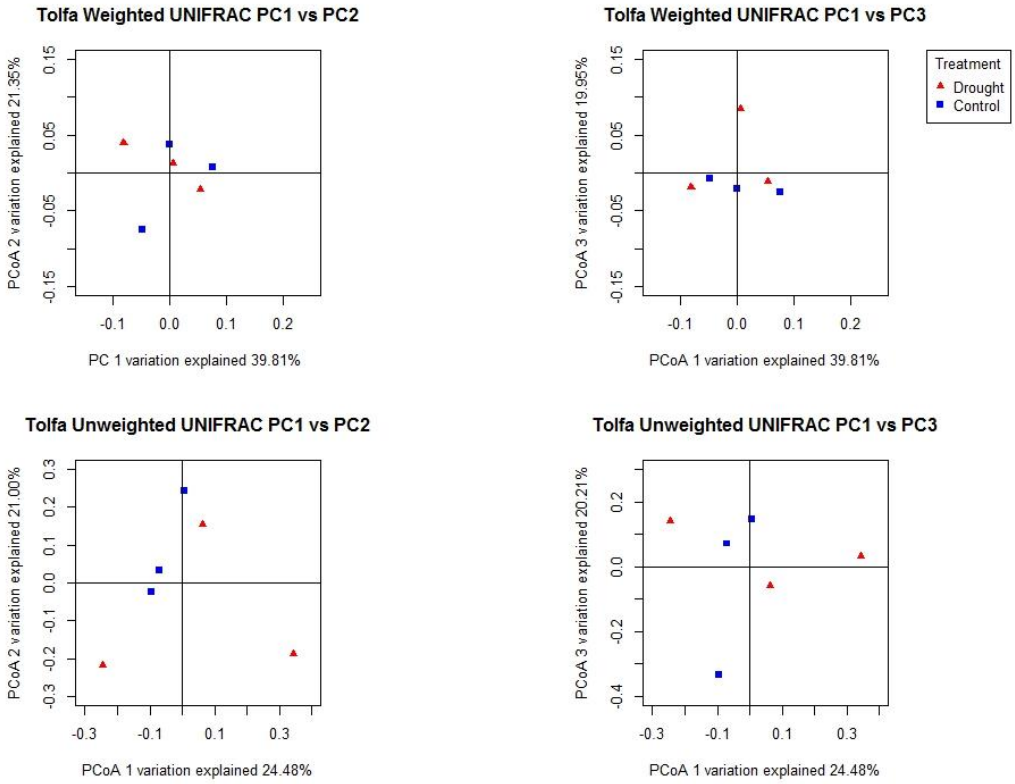
Several taxa found to differ in abundance due to treatment have known ecological functions, including nitrogen cycling, phosphate mobilisation, symbiotic associations or plant growth promotion. This included changes both in the seasonal, biochar and SRC community assemblies. As use of metagenomics did not successfully detect changes in functional profiles, nor did use of simulated metagenomes, future work in this field should concentrate on the use of qPCR and metatranscriptomics techniques. For example, changes in the abundance/rate of transcription in *AmoA* gene fragments would provide insights into the rate of ammonia monooxygenase production, which is responsible for the conversion of ammonia into

hydroxylamine, an intermediate in nitrification. Similarly, *nirS* and *nirK* could be assayed to determine shifts in nitrate reductase production, elucidating shifts in the rate of nitrate to nitrite conversion, whilst *nosZ* assays could determine changes in the rate of nitrous oxide reductase production, an enzyme which reduces nitrous oxide to atmospheric N_2. Similar methods could also illuminate the effects of biochar on P and S cycling, through analysis of *asf* genes (responsible for cleaving S from aromatic sulphonates) or *phn* genes (which lyse C-P bonds found in phosphonates).

Biochar remains an exciting method for geo-engineering, and this thesis contributes to further the understanding of its effects in microbial consortia in long-term, realistic field environments. Better understanding of the implications of biochar application for microbial communities is the first step in designing ways to manipulate such communities to produce more sustainable agricultural practises. However, further work is required to unravel the ways in which these complex communities interact with biochar and the environment.

Appendix A

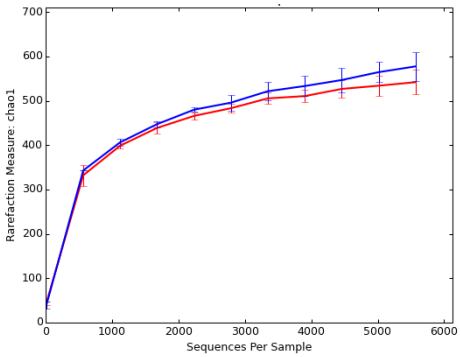
i) PCoA of bacterial β diversity metabarcoding at Tolfa IT



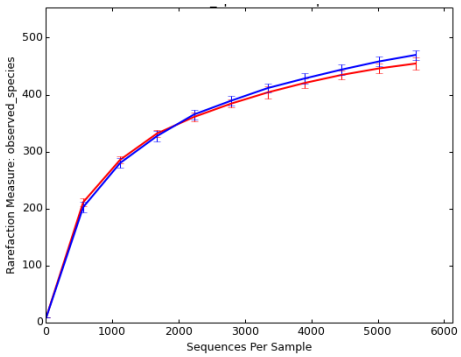
PCoA plots for Tolfa metabarcoding community change

ii) α diversity results for functional metagenomic data from Tolfa IT

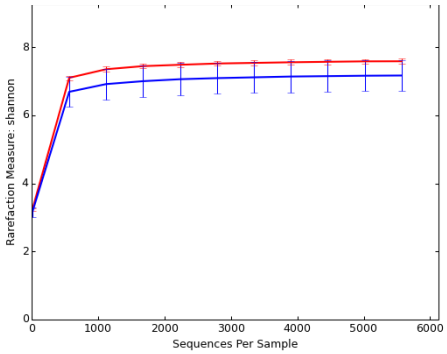
A)



B)

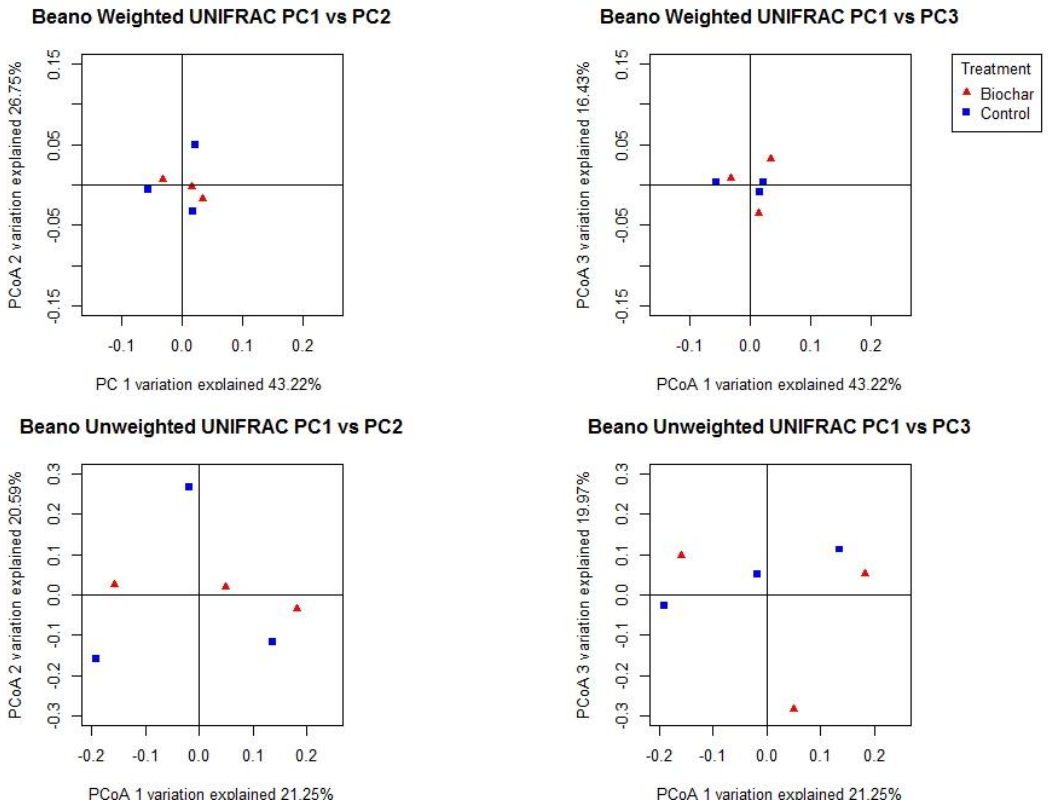


C)



Rarefaction graphs showing α diversity metrics for Tolfa functional data at different sample depths, using a) Chao1, B) Observed species and C) Shannon metrics. Red line represent cumulative drought samples, whilst blue represent cumulative control samples

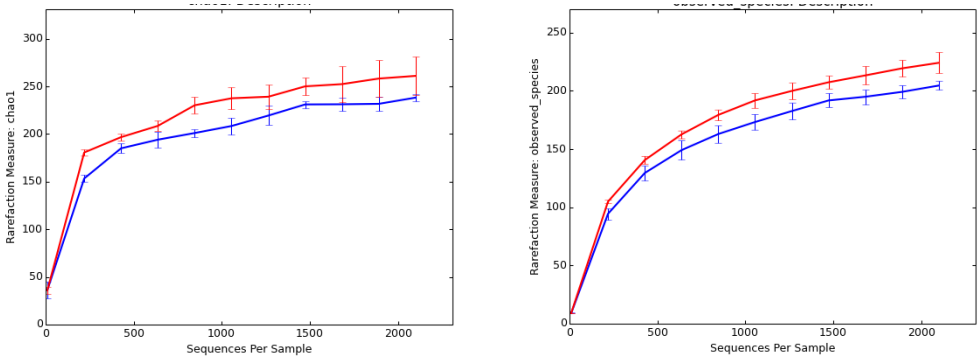
iii) PCoA of bacterial β diversity metabarcoding at Beano IT



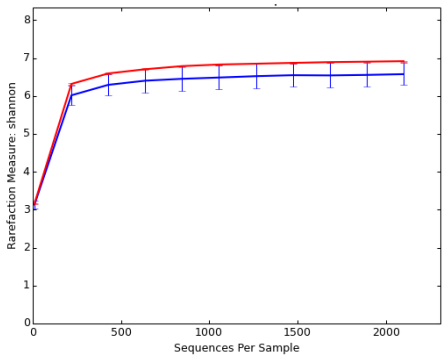
PCoA plots for Beano metabarcoding community change.

iv) α diversity results for functional metagenomic data from Beano IT

A) B)



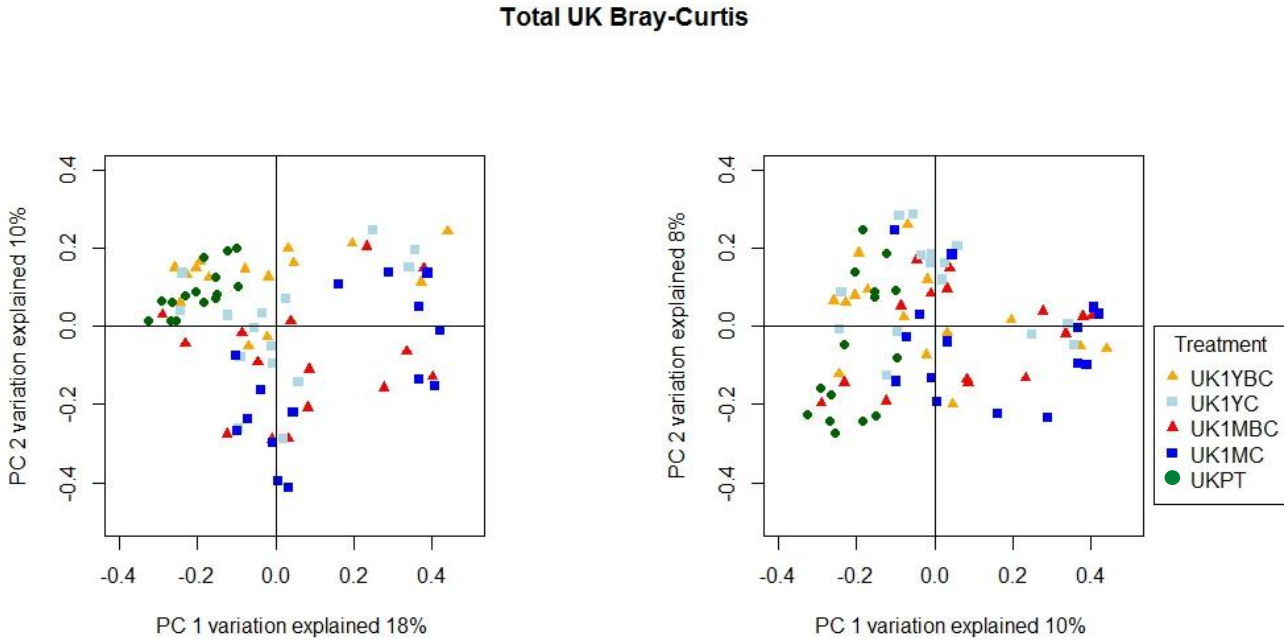
C)



Rarefaction graphs showing α diversity metrics for functional data at different sample depths, using a) Chao1, B) Observed species and C) Shannon metrics. Red lines represent cumulative biochar samples, whilst blue represents cumulative control samples

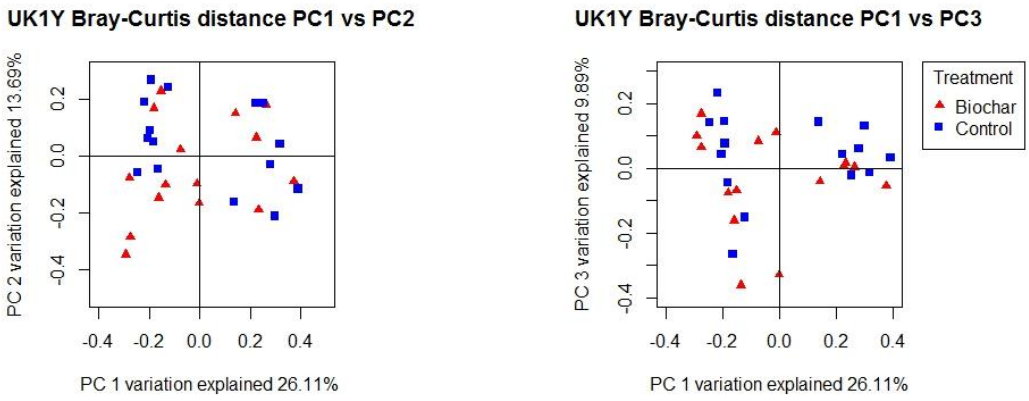
Appendix B

i) PCoA of fungal ß diversity showing differences between time series datasets from UK samples



PCoA of fungal Bray-Curtis dissimilarity for all UK time series samples. Pre-treated, UKPT (green); Biochar 2012, UK1MBC (red); Control 2012, UK1MC (dark blue); Biochar 2013, UK1YBC (orange) and Control 2013, UK1YC (light blue)

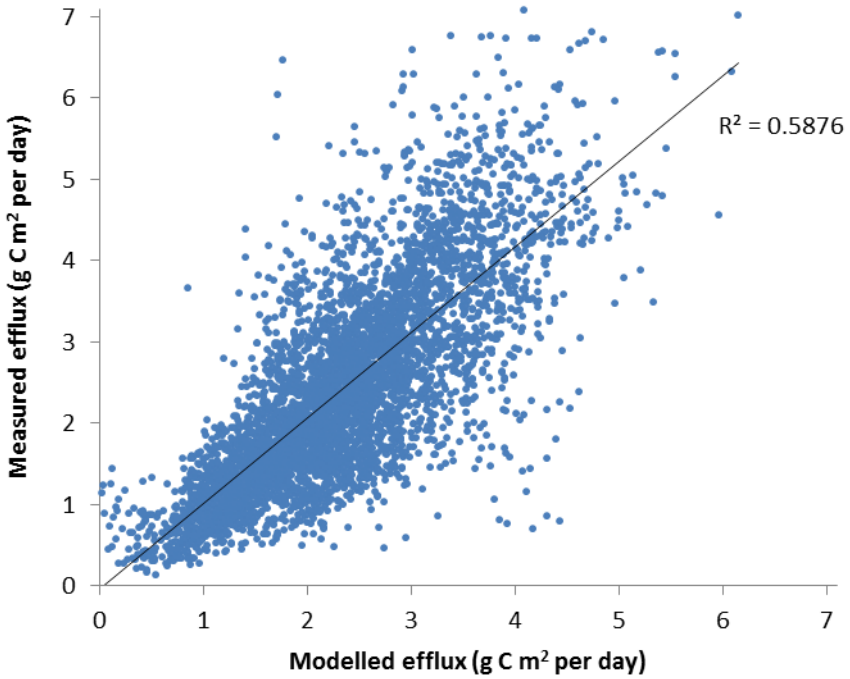
ii) PCoA of fungal β diversity for UK1Y samples



PCoA of ITS fungal Bray-Curtis dissimilarity for UK1Y.

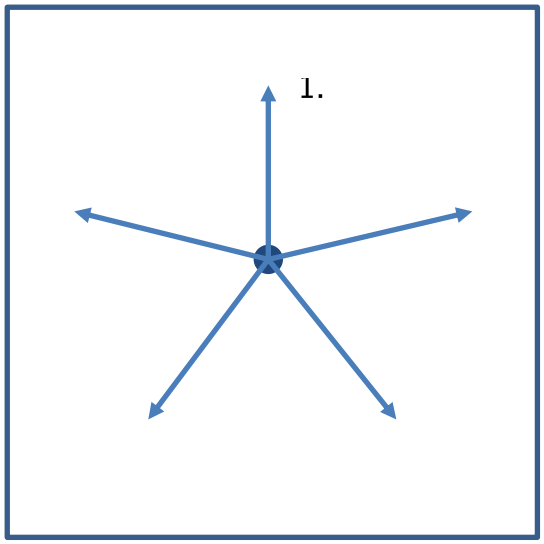
Appendix C

i) Scatter plot displaying the relationship between measured and modelled efflux data



Appendix D

i) Sampling regime for each of the plots, showing the plot centre (blue dot) and the locations of the sampling points 1.5m around it.



Glossary

16SrRNA gene- 16S ribosomal RNA gene, used for amplicon ID of bacteria and archaea

AMF – arbuscular mycorrhizal fungi, symbiotic fungi associated with plant roots, of the phylum *Glomeromycota*. Fungal mycelia penetrate the cells of roots and produce tree like arbuscules to maximise surface area for nutrient exchange.

amoA genes – coding for the ammonia monoxygenase subunit A, found in chemolithotrophic ammonium oxidisers.

amoB genes – coding for the ammonia mono-oxygenase subunit B. Together with *amoA* these combine to produce the ammonia mono-oxygenase enzyme involved in catalysis of ammonia to nitrite

Amplicon – target areas of DNA which can be used for taxonomic identification of sequence data. *e.g.* . 16SrRNA or ITS subregions.

Anthroposols – soils which have been modified by extensive human activity

Binning- to cluster DNA reads into groups by sequence similarity

Biochar – black carbon produced by pyrolysis from biomass or organic waste products, applied to the soil environment

Bp – base pairs, nucleotides in a read

BLAST – basic local alignment search tool, software designed to compare sequence data against databases of existing protein and DNA sequence data

BLAT – BLAST like alignment tool. Similar to BLAST, but utilising a slightly different algorithm which can increase speed of alignment, at the cost of a slight decrease in accuracy

CEC – Cation exchange capacity, the measure of the number of anions and cations present within a soil. May be used as a measure of soil fertility

COI – mitochondrial C oxidase gene, used for amplicon ID of Animalia

DGGE – Denaturing gradient gel electrophoresis

Glossary

EMF – Ectomycorrhizal fungi, fungi which grow in close contact with plant root hairs, with mycelia running between the cells of the plant root forming a Hartig net.

FASTA files – formatted sequence information files

FASTQ files – formatted sequence information files, with the addition of quality information for each associated base within the file

Hartig net – the complex of mycelia produced by EMFs passing between the cells in plant roots, maximising the area in contact between the fungi and the plant.

ITS – internal transcribed spacer region, located between 18S and 28S ribosomal subunit genes. Used for amplicon ID of fungi

LCA – lowest common ancestor

Metabarcoding – extraction and sequencing of taxon specific DNA amplicon regions from environmental samples

Metagenomics – extraction and shotgun sequencing of all DNA from environmental samples

MG-RAST – Metagenomic rapid annotation using subsystems technology. An automated and free pipeline for undertaking shotgun and 16S metagenomic analysis

nifH gene – encodes the *nifH* subunit of nitrogenase, an enzyme responsible for nitrogen fixing

nirK gene – encodes for a Cu nitrite reductase enzyme, capable of reducing nitrite to nitric oxide

nirS gene– encodes for an Fe cytochrome based nitrite reductase enzyme, capable of reducing nitrite to nitric oxide

nosZ– encodes for nitrous oxide reductase, converting nitrous oxide to nitrogen

NGS –Next generation sequencing

OTU- operational taxonomic unit, a binned group of sequences which represent a group of sequences matching to a specific, user defined, percentage similarity

Paired-end reads – reads produced by ligating a primer to each end of a single strand of DNA during sequencing. This method produces a 5'-3' and a 3'-5' read for each single strand of DNA.

PCoA – Principle coordinate analysis

PCR -polymerase chain reaction

PLFA -Phospholipid fatty acid analysis

QIIME- quantitative insights into microbial ecology. a suite of bioinformatics tools and pipelines to analyse metabarcoding data

qPCR -quantitative polymerase chain reaction

read – a single sequence from a metabarcoding or metagenomic dataset

Rhizosphere – soil environment directly associated with plant root systems, in which plant exudates and organic matter can influence microbial communities.

Sequence – the chemical sequence of A, T, C and G present in DNA

SFF files – standard flowgram format files. The standard output from 454 pyrosequencing

SMS – single molecule sequencing

SOC -Soil organic carbon

SOM -Soil organic matter

SRC – short rotation coppice. The practice of coppicing a woody biofuel crop on a semi-annual basis, to increase the quantity of biomass produced.

Terra preta – Amazonian dark soils containing a large proportion of black carbon

List of References

Allen LZ, Allen EE, Badger JH *et al.* (2012) Influence of nutrients and currents on the genomic composition of microbes across an upwelling mosaic. *The ISME journal*, **6**, 1403–14.

Altschul S, Gish W, Miller W (1990) Basic Local Alignment Search Tool. *Journal of Molecular Biology*, **215**, 403–410.

Ameloot N, Sleutel S, Case SDC *et al.* (2014) C mineralization and microbial activity in four biochar field experiments several years after incorporation. *Soil Biology and Biochemistry*, **78**, 195–203.

An D, Caffrey SM, Soh J *et al.* (2013) Metagenomics of Hydrocarbon Resource Environments Indicates Aerobic Taxa and Genes to be Unexpectedly Common. *Environmental Science & Technology*, **47**, 10708–10717.

Anders E, Watzinger A, Rempt F (2013) Biochar affects the structure rather than the total biomass of microbial communities in temperate soils. *Agricultural and Food Science*, **22**, 404–423.

Andersen K, Bird KL, Rasmussen M *et al.* (2012) Meta-barcoding of "dirt" DNA from soil reflects vertebrate biodiversity. *Molecular Ecology*, **21**, 1966–1979.

Anderson MJ (2001) A new method for non-parametric multivariate analysis of variance. *Austral Ecology*, **26**, 32–46.

Anderson IC, Cairney JWG (2004) Diversity and ecology of soil fungal communities: increased understanding through the application of molecular techniques. *Environmental Microbiology*, **6**, 769–779.

Anderson CR, Condron LM, Clough TJ *et al.* (2011) Biochar induced soil microbial community change: Implications for biogeochemical cycling of carbon, nitrogen and phosphorus. *Pedobiologia*, **54**, 309–320.

Anderson CR, Hamonts K, Clough TJ, Condron LM (2014) Biochar does not affect soil N-transformations or microbial community structure under ruminant urine patches but does alter relative proportions of nitrogen cycling bacteria. *Agriculture, Ecosystems & Environment*, **191**, 63–72.

Andreote FD, Jiménez DJ, Chaves D *et al.* (2012) The Microbiome of Brazilian Mangrove Sediments as Revealed by Metagenomics (AM Ibekwe, Ed,). *PLoS ONE*, **7**.

Aronesty E (2011) ea-utls: "Command-line tools for processing biological sequencing data." , http://code.google.com/p/ea-utils.

Aylott MJ, Casella E, Tubby I *et al.* (2008) Yield and spatial supply of bioenergy poplar and willow short-rotation coppice in the UK. *New Phytologist*, **178**, 358–370.

Bai M, Wilske B, Buegger F *et al.* (2013) Degradation kinetics of biochar from pyrolysis and hydrothermal carbonization in temperate soils. *Plant and Soil*, **372**, 375–387.

Baldrian P, Merhautová V, Petránková M, Cajthaml T, Šnajdr J (2010) Distribution of microbial biomass and activity of extracellular enzymes in a hardwood forest soil reflect soil moisture content. *Applied Soil Ecology*,

46, 177–182.

Bamminger C, Marschner B, Jüschke E (2014a) An incubation study on the stability and biological effects of pyrogenic and hydrothermal biochar in two soils. *European Journal of Soil Science*, **65**, 72–82.

Bamminger C, Zaiser N, Zinsser P *et al.* (2014b) Effects of biochar, earthworms, and litter addition on soil microbial activity and abundance in a temperate agricultural soil. *Biology and Fertility of Soils*, **50**, 1189–1200.

Barberán A, Fernández-Guerra A, Bohannan BJM, Casamayor EO (2012) Exploration of community traits as ecological markers in microbial metagenomes. *Molecular Ecology*, **21**, 1909–17.

Barberán A, Ramirez KS, Leff JW *et al.* (2014) Why are some microbes more ubiquitous than others? Predicting the habitat breadth of soil bacteria. *Ecology Letters*, **17**, 794–802.

Barker T (2007) An Assessment of the Intergovernmental Panel on Climate Change. *Climate Change 2007: Synthesis Report*, 12–17.

Barnard RL, Osborne C a, Firestone MK (2013) Responses of soil bacterial and fungal communities to extreme desiccation and rewetting. *The ISME Journal*, **7**, 2229–2241.

Baronti S, Alberti G, Delle Vedove G *et al.* (2010) The Biochar Option to Improve Plant Yields: First Results From Some Field and Pot Experiments in Italy. *Italian Journal of Agronomy*, **5**, 3–11.

Baronti S, Vaccari FP, Miglietta F *et al.* (2014) Impact of biochar application on plant water relations in Vitis vinifera(L.). *European Journal of Agronomy*, **53**, 38–44.

Baum C, Leinweber P, Weih M, Lamersdorf N, Dimitriou I (2009) Effects of short rotation coppice with willows and poplar on soil ecology. *Landbauforschung Volkenrode*, **59**, 183–196.

Beesley L, Marmiroli M (2011) The immobilisation and retention of soluble arsenic, cadmium and zinc by biochar. *Environmental Pollution*, **159**, 474–480.

Beesley L, Moreno-Jiménez E, Gomez-Eyles JL (2010) Effects of biochar and greenwaste compost amendments on mobility, bioavailability and toxicity of inorganic and organic contaminants in a multi-element polluted soil. *Environmental Pollution*, **158**, 2282–2287.

Beesley L, Moreno-Jiménez E, Gomez-Eyles JL *et al.* (2011) A review of biochars' potential role in the remediation, revegetation and restoration of contaminated soils. *Environmental Pollution*, **159**, 3269–3282.

Di Bella JM, Bao Y, Gloor GB, Burton JP, Reid G (2013) High throughput sequencing methods and analysis for microbiome research. *Journal of Microbiological Methods*, **95**, 401–414.

Bellemain E, Carlsen T, Brochmann C *et al.* (2010) ITS as an environmental DNA barcode for fungi: an in silico approach reveals potential PCR biases. *BMC Microbiology*, **10**, 189.

Belyaeva ON, Haynes RJ (2011) Comparison of the effects of conventional organic amendments and biochar on the chemical, physical and microbial properties of coal fly ash as a plant growth medium. *Environmental Earth*

Sciences, **66**, 1987–1997.

Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society*, **57**, 289–300.

Bennett EM, Carpenter SR, Caraco NF (2001) Human Impact on Erodable Phosphorus and Eutrophication: A Global Perspective. *BioScience*, **51**, 227.

Bérard A, Ben Sassi M, Renault P, Gros R (2012) Severe drought-induced community tolerance to heat wave. An experimental study on soil microbial processes. *Journal of Soils and Sediments*, **12**, 513–518.

Berbee ML (2001) The phylogeny of plant and animal pathogens in the Ascomycota. *Physiological and Molecular Plant Pathology*, **59**, 165–187.

Bergmann GT, Bates ST, Eilers KG *et al.* (2011) The under-recognized dominance of Verrucomicrobia in soil bacterial communities. *Soil Biology and Biochemistry*, **43**, 1450–1455.

Bever JD, Schultz P a., Pringle A, Morton JB (2001) Arbuscular Mycorrhizal Fungi: More Diverse than Meets the Eye, and the Ecological Tale of Why. *BioScience*, **51**, 923.

Blaalid R, Carlsen T, Kumar S *et al.* (2012) Changes in the root-associated fungal communities along a primary succession gradient analysed by 454 pyrosequencing. *Molecular Ecology*, **21**, 1897–1908.

Blaalid R, Kumar S, Nilsson RH *et al.* (2013) ITS1 versus ITS2 as DNA metabarcodes for fungi. *Molecular Ecology Resources*, **13**, 218–224.

Braker G, Zhou J, Wu L, Devol AH, Tiedje JM (2000) Nitrite reductase genes (nirK and nirS) as functional markers to investigate diversity of denitrifying bacteria in pacific northwest marine sediment communities. *Applied and Environmental Microbiology*, **66**, 2096–2104.

Bray JR, Curtis JT (1957) An Ordination of the upland forest community of southern Wisconsin. *Ecology Monographs*, **27**, 325–349.

Bruto M, Prigent-Combaret C, Muller D, Moënne-Loccoz Y (2014) Analysis of genes contributing to plant-beneficial functions in plant growth-promoting rhizobacteria and related Proteobacteria. *Scientific Reports*, **4**, 6261.

Bruun S, Clauson-Kaas S, Bobul'ská L, Thomsen IK (2014) Carbon dioxide emissions from biochar in soil: role of clay, microorganisms and carbonates. *European Journal of Soil Science*, **65**, 52–59.

Buckley DH, Schmidt TM (2001) Environmental factors influencing the distribution of rRNA from *Verrucomicrobia* in soil. *FEMS Microbiology Ecology*, **35**, 105–112.

Buée M, Boer W, Martin F, Overbeek L, Jurkevitch E (2009a) The rhizosphere zoo: An overview of plant-associated communities of microorganisms, including phages, bacteria, archaea, and fungi, and of some of their structuring factors. *Plant and Soil*, **321**, 189–212.

Buée M, Reich M, Murat C *et al.* (2009b) 454 Pyrosequencing analyses of forest soils reveal an unexpectedly high fungal diversity. *New Phytologist*, **184**, 449–456.

Busch D, Stark A, Kammann CI, Glaser B (2013) Genotoxic and phytotoxic risk assessment of fresh and treated hydrochar from hydrothermal carbonization compared to biochar from pyrolysis. *Ecotoxicology and Environmental Safety*, **97**, 59–66.

Cabrera a., Cox L, Spokas K *et al.* (2014) Influence of biochar amendments on the sorption–desorption of aminocyclopyrachlor, bentazone and pyraclostrobin pesticides to an agricultural soil. *Science of The Total Environment*, **470–471**, 438–443.

Caldwell B a. (2005) Enzyme activities as a component of soil biodiversity: A review. *Pedobiologia*, **49**, 637–644.

Campbell AG, Schwientek P, Vishnivetskaya T *et al.* (2014) Diversity and genomic insights into the uncultured Chloroflexi from the human microbiota. *Environmental Microbiology*, **16**, 2635–2643.

Caporaso JG, Bittinger K, Bushman FD *et al.* (2010a) PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics*, **26**, 266–7.

Caporaso JG, Kuczynski J, Stombaugh J *et al.* (2010b) QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, **7**, 335–336.

Caporaso JG, Lauber CL, Walters W a *et al.* (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME journal*, **6**, 1621–4.

Carlsson M, Andrén O, Stenström J, Kirchmann H, Kätterer T (2012) Charcoal Application to Arable Soil: Effects on CO 2 Emissions. *Communications in Soil Science and Plant Analysis*, **43**, 2262–2273.

Caruso T, Hempel S, Powell JR, Barto EK, Rillig MC (2012) Compositional divergence and convergence in arbuscular mycorrhizal fungal communities. *Ecology*, **93**, 1115–24.

Carvalhais LC, Dennis PG, Tyson GW, Schenk PM (2012) Application of metatranscriptomics to soil environments. *Journal of Microbiological Methods*, **91**, 246–251.

Castaldi S, Riondino M, Baronti S *et al.* (2011) Impact of biochar application to a Mediterranean wheat crop on soil microbial activity and greenhouse gas fluxes. *Chemosphere*, **85**, 1464–1471.

Castro HF, Classen AT, Austin EE, Norby RJ, Schadt CW (2010) Soil microbial community responses to multiple experimental climate change drivers. *Applied and Environmental Microbiology*, **76**, 999–1007.

Catão ECP, Lopes F a C, Araújo JF *et al.* (2014) Soil Acidobacterial 16S rRNA Gene Sequences Reveal Subgroup Level Differences between Savanna-Like Cerrado and Atlantic Forest Brazilian Biomes. *International Journal of Microbiology*, **2014**, 1–12.

Cavalier-Smith T (1998) A revised six-kingdom system of life. *Biological Reviews of the Cambridge Philosophical Society*, **73**, 203–266.

Cayuela ML, Sánchez-Monedero MA, Roig A *et al.* (2013) Biochar and denitrification in soils: when, how much and why does biochar reduce N2O emissions? *Scientific Reports*, **3**, 1732.

Chapman M, Underwood A (1999) Ecological patterns in multivariate

assemblages: information and interpretation of negative values in ANOSIM tests. *Marine Ecology Progress Series*, **180**, 257–265.

Chen CR, Condron LM, Xu ZH (2008) Impacts of grassland afforestation with coniferous trees on soil phosphorus dynamics and associated microbial processes: A review. *Forest Ecology and Management*, **255**, 396–409.

Chen J, Liu X, Zheng J *et al.* (2013) Biochar soil amendment increased bacterial but decreased fungal gene abundance with shifts in community structure in a slightly acid rice paddy from Southwest China. *Applied Soil Ecology*, **71**, 33–44.

Chen S, Rotaru A-E, Shrestha PM *et al.* (2014) Promoting Interspecies Electron Transfer with Biochar. *Scientific Reports*, **4**, 5019.

Chen MM, Zhu YG, Su YH *et al.* (2007) Effects of soil moisture and plant interactions on the soil microbial community structure. *European Journal of Soil Biology*, **43**, 31–38.

Cheng C-H, Lehmann J, Engelhard MH (2008) Natural oxidation of black carbon in soils: Changes in molecular form and surface charge along a climosequence. *Geochimica et Cosmochimica Acta*, **72**, 1598–1610.

Chmolowska D (2013) A practical introduction to microbial community sequencing. *Central European Journal of Biology*, **8**, 399–409.

Chomnunti P, Ko TWK, Chukeatirote E *et al.* (2012) Phylogeny of Chaetothyriaceae in northern Thailand including three new species. *Mycologia*, **104**, 382–395.

Clark JS, Campbell JH, Grizzle H, Acosta-Martìnez V, Zak JC (2009) Soil Microbial Community Response to Drought and Precipitation Variability in the Chihuahuan Desert. *Microbial Ecology*, **57**, 248–260.

Cotrufo MF, Alberti G, Inglima I *et al.* (2011) Decreased summer drought affects plant productivity and soil carbon dynamics in a Mediterranean woodland. *Biogeosciences*, **8**, 2729–2739.

Cox MP, Peterson D a, Biggs PJ (2010) SolexaQA: At-a-glance quality assessment of Illumina second-generation sequencing data. *BMC Bioinformatics*, **11**, 485.

Cregger M a, Schadt CW, McDowell NG, Pockman WT, Classen AT (2012) Response of the Soil Microbial Community to Changes in Precipitation in a Semiarid Ecosystem. *Applied and Environmental Microbiology*, **78**, 8587–8594.

Cross A, Sohi SP (2011) The priming potential of biochar products in relation to labile carbon contents and soil organic matter status. *Soil Biology and Biochemistry*, **43**, 2127–2134.

Crowther TW, Maynard DS, Leff JW *et al.* (2014) Predicting the responsiveness of soil biodiversity to deforestation: a cross-biome study. *Global Change Biology*, **20**, 2983–2994.

Crowther T, Stanton D, Thomas S (2013) Top-down control of soil fungal community composition by a globally distributed keystone consumer. *Ecology*, **94**, 2518–2528.

Cruz-Martínez K, Suttle KB, Brodie EL *et al.* (2009) Despite strong seasonal responses, soil microbial consortia are more resilient to long-term

changes in rainfall than overlying grassland. *The ISME Journal*, **3**, 738–744.

Czimczik CI, Masiello C a. (2007) Controls on black carbon storage in soils. *Global Biogeochemical Cycles*, **21**, 1–8.

Dai A (2011) Drought under global warming: a review. *Wiley Interdisciplinary Reviews: Climate Change*, **2**, 45–65.

DeBruyn JM, Nixon LT, Fawaz MN, Johnson AM, Radosevich M (2011) Global biogeography and quantitative seasonal dynamics of Gemmatimonadetes in soil. *Applied and Environmental Microbiology*, **77**, 6295–6300.

DeBruyn JM, Radosevich M, Wommack KE *et al.* (2014) Genome Sequence and Methylome of Soil Bacterium Gemmatirosa kalamazoonensis KBS708T, a Member of the Rarely Cultivated Gemmatimonadetes Phylum. *Genome Announcements*, **2**, e00226–14–e00226–14.

Defra (2009) *Safeguarding our Soils A Strategy for England*.

Degnan PH, Ochman H (2011) Illumina-based analysis of microbial community diversity. *The ISME Journal*, **6**, 183–194.

Delle Vedove G, Alberti G, Zuliani M, Peressotti A (2007) Automated Monitoring of Soil Respiration: an Improved Automatic Chamber System. *Italian Journal of Agronomy*, **2**, 377–382.

Delmont TO, Prestat E, Keegan KP *et al.* (2012) Structure, fluctuation and magnitude of a natural grassland soil metagenome. *The ISME Journal*, **6**, 1677–1687.

Dempster DN, Gleeson DB, Solaiman ZM, Jones DL, Murphy D V. (2011) Decreased soil microbial biomass and nitrogen mineralisation with Eucalyptus biochar addition to a coarse textured soil. *Plant and Soil*, **354**, 311–324.

Department for Environment Food and Rural Affairs (2004) *Growing Short Rotation Coppice*.

DeSantis TZ, Hugenholtz P, Larsen N *et al.* (2006) Greengenes, a Chimera-Checked 16S rRNA Gene Database and Workbench Compatible with ARB. *Applied and Environmental Microbiology*, **72**, 5069–5072.

Van Dijk EL, Jaszczyszyn Y, Thermes C (2014) Library preparation methods for next-generation sequencing: Tone down the bias. *Experimental Cell Research*, **322**, 12–20.

Dimitriou I, Baum C, Baum S *et al.* (2009) The impact of Short Rotation Coppice (SRC) cultivation on the environment. *vTI Agriculture and Forestry Research*, **59**, 159–162.

Ding G-C, Pronk GJ, Babin D *et al.* (2013) Mineral composition and charcoal determine the bacterial community structure in artificial soils. *FEMS Microbiology Ecology*, **86**, 15–25.

Dini-Andreote F, Andreote FD, Araújo WL, Trevors JT, van Elsas JD (2012) Bacterial Genomes: Habitat Specificity and Uncharted Organisms. *Microbial Ecology*, **64**, 1–7.

Ducey TF, Ippolito JA, Cantrell KB, Novak JM, Lentz RD (2013) Addition of activated switchgrass biochar to an aridic subsoil increases microbial

nitrogen cycling gene abundances. *Applied Soil Ecology*, **65**, 65–72.

Edgar RC (2010) Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*, **26**, 2460–1.

Eisenhauer N, Cesarz S, Koller R, Worm K, Reich PB (2012) Global change belowground: impacts of elevated CO2, nitrogen, and summer drought on soil food webs and biodiversity. *Global Change Biology*, **18**, 435–447.

Elmer W, Pignatello J (2011) Effect of biochar amendments on mycorrhizal associations and Fusarium crown and root rot of asparagus in replant soils. *Plant Disease*, **95**, 960–966.

Ens J, Farrell R, Bélanger N (2013) Early effects of afforestation with willow (Salix purpurea, "Hotel") on soil carbon and nutrient availability. *Forests*, **4**, 137–154.

Epp LS, Boessenkool S, Bellemain EP *et al.* (2012) New environmental metabarcodes for analysing soil DNA: potential for studying past and present ecosystems. *Molecular Ecology*, **21**, 1821–33.

Esposito A, Kirschberg M (2014) How many 16S -based studies should be included in a metagenomic conference? It may be a matter of etymology. *FEMS Microbiology Letters*, **351**, 145–146.

Evans SE, Wallenstein MD (2011) Soil microbial community response to drying and rewetting stress: does historical precipitation regime matter? *Biogeochemistry*, **109**, 101–116.

Evans SE, Wallenstein MD (2014) Climate change alters ecological strategies of soil bacteria. *Ecology Letters*, **17**, 155–164.

Faith DP, Baker AM (2006) Phylogenetic diversity (PD) and biodiversity conservation: some bioinformatics challenges. *Evolutionary Bioinformatics Online*, **2**, 121–8.

Falkowski PG, Fenchel T, Delong EF (2008) The Microbial Engines That Drive Earth's Biogeochemical Cycles. *Science*, **320**, 1034–1039.

Fang Y, Singh B, Singh BP, Krull E (2014) Biochar carbon stability in four contrasting soils. *European Journal of Soil Science*, **65**, 60–71.

Farrell M, Kuhn TK, Macdonald LM *et al.* (2013) Microbial utilisation of biochar-derived carbon. *Science of The Total Environment*, **465**, 288–297.

Fierer N, BarberÃ¡n A, Laughlin DC (2014) Seeing the forest for the genes: using metagenomics to infer the aggregated traits of microbial communities. *Frontiers in Microbiology*, **5**, 1–6.

Fierer N, Bradford MA, Jackson RB (2007) Toward an ecological classification of soil bacteria. *Ecology*, **88**, 1354–1364.

Fierer N, Jackson RB (2006) The diversity and biogeography of soil bacterial communities. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 626–31.

Fierer N, Lauber CL, Ramirez KS *et al.* (2012a) Comparative metagenomic, phylogenetic and physiological analyses of soil microbial communities across nitrogen gradients. *The ISME Journal*, **6**, 1007–1017.

Fierer N, Leff JW, Adams BJ *et al.* (2012b) Cross-biome metagenomic analyses of soil microbial communities and their functional attributes. *Proceedings*

of the National Academy of Sciences, **109**, 21390–21395.

Fornasier F, Ascher J, Ceccherini MT, Tomat E, Pietramellara G (2014) A simplified rapid, low-cost and versatile DNA-based assessment of soil microbial biomass. *Ecological Indicators*, **45**, 75–82.

Fornasier F, Margon A (2007) Bovine serum albumin and Triton X-100 greatly increase phosphomonoesterases and arylsulphatase extraction yield from soil. *Soil Biology and Biochemistry*, **39**, 2682–2684.

Fox A, Kwapinski W, Griffiths BS, Schmalenberger A (2014) The role of sulfur- and phosphorus-mobilizing bacteria in biochar-induced growth promotion of Lolium perenne. *FEMS Microbiology Ecology*, **90**, 78–91.

Frostegård Å, Tunlid A, Bååth E (2011) Use and misuse of PLFA measurements in soils. *Soil Biology and Biochemistry*, **43**, 1621–1625.

Fuerst J a, Sagulenko E (2011) Beyond the bacterium: planctomycetes challenge our concepts of microbial structure and function. *Nature Reviews Microbiology*, **9**, 403–413.

Fujimura KE, Demoor T, Rauch M *et al.* (2014) House dust exposure mediates gut microbiome Lactobacillus enrichment and airway immune defense against allergens and virus infection. *Proceedings of the National Academy of Sciences of the United States of America*, **111**, 805–10.

Gadd GM (2007) Geomycology: biogeochemical transformations of rocks, minerals, metals and radionuclides by fungi, bioweathering and bioremediation. *Mycological Research*, **111**, 3–49.

Gai X, Wang H, Liu J *et al.* (2014) Effects of Feedstock and Pyrolysis Temperature on Biochar Adsorption of Ammonium and Nitrate (JA Coles, Ed,). *PLoS ONE*, **9**, e113888.

Galvez a., Sinicco T, Cayuela ML *et al.* (2012) Short term effects of bioenergy by-products on soil C and N dynamics, nutrient availability and biochemical properties. *Agriculture, Ecosystems & Environment*, **160**, 3–14.

Garbeva P, van Veen JA, van Elsas JD (2004) MICROBIAL DIVERSITY IN SOIL: Selection of Microbial Populations by Plant and Soil Type and Implications for Disease Suppressiveness. *Annual Review of Phytopathology*, **42**, 243–270.

Garrity G, H H, R C *et al.* (2001) Phylum BVI. Chloroflexi phy. nov. In: *Bergey's Manual of Systematic Bacteriology*, pp. 427–446. Springer New York.

Gilbert JA, Dupont CL (2011) Microbial Metagenomics: Beyond the Genome. *Annual Review of Marine Science*, **3**, 347–371.

Gilbert J a, Meyer F, Antonopoulos D *et al.* (2010a) Meeting Report: The Terabase Metagenomics Workshop and the Vision of an Earth Microbiome Project. *Standards in Genomic Sciences*, **3**, 243–248.

Gilbert JA, Meyer F, Schriml L *et al.* (2010b) Metagenomes and metatranscriptomes from the L4 long-term coastal monitoring station in the Western English Channel. *Standards in Genomic Sciences*, **3**, 183–193.

Gilbert JA, O'Dor R, King N, Vogel TM (2011) The importance of metagenomic surveys to microbial ecology: or why Darwin would have been a metagenomic scientist. *Microbial Informatics and Experimentation*, **1**, 5.

Gill SR, Pop M, DeBoy RT *et al.* (2006) Metagenomic Analysis of the Human Distal Gut Microbiome. *Science*, **312**, 1355–1359.

Glaser B, Birk JJ (2012) State of the scientific knowledge on properties and genesis of Anthropogenic Dark Earths in Central Amazonia (terra preta de Índio). *Geochimica et Cosmochimica Acta*, **82**, 39–51.

Glaser B, Haumaier L, Guggenberger G, Zech W (2001) The "Terra Preta" phenomenon: a model for sustainable agriculture in the humid tropics. *Naturwissenschaften*, **88**, 37–41.

Glenn TC (2011) Field guide to next-generation DNA sequencers. *Molecular Ecology Resources*, **11**, 759–69.

Gomez JD, Denef K, Stewart CE, Zheng J, Cotrufo MF (2014) Biochar addition rate influences soil microbial abundance and activity in temperate soils. *European Journal of Soil Science*, **65**, 28–39.

Gomez LD, Steele-King CG, McQueen-Mason SJ (2008) Sustainable liquid biofuels from biomass: The writing's on the walls. *New Phytologist*, **178**, 473–485.

Goodrich JK, Di Rienzi SC, Poole AC *et al.* (2014) Conducting a Microbiome Study. *Cell*, **158**, 250–262.

Gosling P, Hodge a., Goodlass G, Bending GD (2006) Arbuscular mycorrhizal fungi and organic farming. *Agriculture, Ecosystems and Environment*, **113**, 17–35.

Graber ER, Meller Harel Y, Kolton M *et al.* (2010) Biochar impact on development and productivity of pepper and tomato grown in fertigated soilless media. *Plant and Soil*, **337**, 481–496.

Graber ER, Tsechansky L, Gerstl Z, Lew B (2011) High surface area biochar negatively impacts herbicide efficacy. *Plant and Soil*, **353**, 95–106.

Griffiths RI, Thomson BC, James P *et al.* (2011) The bacterial biogeography of British soils. *Environmental Microbiology*, **13**, 1642–1654.

Grigoriev I, Cullen D, Goodwin S (2011) Fueling the future with fungal genomics. *Mycology*, **2**, 192–209.

Grossman JM, O'Neill BE, Tsai SM *et al.* (2010) Amazonian anthrosols support similar microbial communities that differ distinctly from those extant in adjacent, unmodified soils of the same mineralogy. *Microbial Ecology*, **60**, 192–205.

Guo J, Peng Y, Wang S *et al.* (2013) Pathways and Organisms Involved in Ammonia Oxidation and Nitrous Oxide Emission. *Critical Reviews in Environmental Science and Technology*, **43**, 2213–2296.

Gurwick NP, Moore L a, Kelly C, Elias P (2013) A Systematic Review of Biochar Research, with a Focus on Its Stability in situ and Its Promise as a Climate Mitigation Strategy (Q Sun, Ed,). *PLoS ONE*, **8**.

Hammer EC, Balogh-Brunstad Z, Jakobsen I *et al.* (2014) A mycorrhizal fungus grows on biochar and captures phosphorus from its surfaces. *Soil Biology and Biochemistry*, **77**, 252–260.

Handelsman J (2004) Metagenomics : Application of Genomics to Uncultured Microorganisms. *Microbiology and Molecular Biology Reviews*, **68**, 669–

685.

Hangs RD, Schoenau JJ, Van Rees KCJ *et al.* (2014) First Rotation Biomass Production and Nutrient Cycling within Short-Rotation Coppice Willow Plantations in Saskatchewan, Canada. *BioEnergy Research*, **7**, 1091–1111.

Hanzel J, Myrold D, Sessitsch A *et al.* (2013) Microbial ecology of biogeochemical interfaces – diversity, structure, and function of microhabitats in soil. *FEMS Microbiology Ecology*, **86**, 1–2.

Harris ZM, Alberti G, Jenkins JR *et al.* (2016) Land use change to bioenergy: grassland to short rotation coppice willow has an improved carbon balance. *GCB Bioenergy*, 1–16.

Harter J, Krause H-M, Schuettler S *et al.* (2014) Linking N2O emissions from biochar-amended soil to the structure and function of the N-cycling microbial community. *The ISME Journal*, **8**, 660–674.

Hastings A, Tallis MJ, Casella E *et al.* (2014) The technical potential of Great Britain to produce ligno-cellulosic biomass for bioenergy in current and future climates. *GCB Bioenergy*, **6**, 108–122.

He Z, Xu M, Deng Y *et al.* (2010) Metagenomic analysis reveals a marked divergence in the structure of belowground microbial communities at elevated CO2. *Ecology Letters*, **13**, 564–575.

Hernández M, Dumont MG, Yuan Q, Conrad R (2015) Different Bacterial Populations Associated with the Roots and Rhizosphere of Rice Incorporate Plant-Derived Carbon (CR Lovell, Ed,). *Applied and Environmental Microbiology*, **81**, 2244–2253.

Hibbett DS, Bauer RB, Binder M *et al.* (2014) Agaricomycetes. In: *Systematics and Evolution* (eds D.J M, J.W S), p. 373. Springer-Verlag, Berlin.

Hiiesalu I, Opik M, Metsis M *et al.* (2012) Plant species richness belowground: higher richness and new patterns revealed by next-generation sequencing. *Molecular ecology*, **21**, 2004–16.

Hinchee M, Rottmann W, Mullinax L *et al.* (2009) Short-rotation woody crops for bioenergy and biofuels applications. *In Vitro Cellular and Developmental Biology - Plant*, **45**, 619–629.

Hirsch PR, Mauchline TH (2012) Who's who in the plant root microbiome? *Nature Biotechnology*, **30**, 961–962.

Hirsch PR, Mauchline TH, Clark IM (2010) Culture-independent molecular techniques for soil microbial ecology. *Soil Biology and Biochemistry*, **42**, 878–887.

Hodge A, Storer K (2014) Arbuscular mycorrhiza and nitrogen: implications for individual plants through to ecosystems. *Plant and Soil*, 1–19.

Hofmann-Schielle C, Jug A, Makeschin F, Rehfuess KE (1999) Short-rotation plantations of balsam populars, aspen and willows on former arable land in the FRG I site-growth relationships. *Forest Ecology and Management*, **121**, 85–99.

Holland RA, Eigenbrod F, Muggeridge A *et al.* (2015) A synthesis of the ecosystem services impact of second generation bioenergy crop production. *Renewable and Sustainable Energy Reviews*, **46**, 30–40.

Horton TR, Bruns TD (2001) The molecular revolution in ectomycorrhizal ecology: Peeking into the black-box. *Molecular Ecology*, **10**, 1855–1871.

Hrynkiewicz K, Toljander YK, Baum C *et al.* (2012) Correspondence of ectomycorrhizal diversity and colonisation of willows (Salix spp.) grown in short rotation coppice on arable sites and adjacent natural stands. *Mycorrhiza*, **22**, 603–613.

Hu L, Cao L, Zhang R (2014) Bacterial and fungal taxon changes in soil microbial community composition induced by short-term biochar amendment in red oxidized loam soil. *World Journal of Microbiology and Biotechnology*, **30**, 1085–1092.

Hueso S, García C, Hernández T (2012) Severe drought conditions modify the microbial community structure, size and activity in amended and unamended soils. *Soil Biology and Biochemistry*, **50**, 167–173.

Huffnagle GB, Noverr MC (2013) The emerging world of the fungal microbiome. *Trends in Microbiology*, **21**, 334–341.

Hughes JB, Hellmann JJ, Ricketts TH, Bohannan BJ (2001) Counting the uncountable: statistical approaches to estimating microbial diversity. *Applied and Environmental Microbiology*, **67**, 4399–406.

Huttenhower C (2012) Structure, function and diversity of the healthy human microbiome. *Nature*, **486**, 207–14.

Ihrmark K, Bödeker ITM, Cruz-Martinez K *et al.* (2012) New primers to amplify the fungal ITS2 region - evaluation by 454-sequencing of artificial and natural communities. *FEMS Microbiology Ecology*, **82**, 666–677.

Imfeld G, Vuilleumier S (2012) Measuring the effects of pesticides on bacterial communities in soil: A critical review. *European Journal of Soil Biology*, **49**, 22–30.

IPCC (2014) *Climate change 2014: synthesis report. Summary for policymakers*.

James TY, Letcher PM, Longcore JE *et al.* (2006) A molecular phylogeny of the flagellated fungi (Chytridiomycota) and description of a new phylum (Blastocladiomycota). *Mycologia*, **98**, 860–871.

Janssen PH (2006) Identifying the Dominant Soil Bacterial Taxa in Libraries of 16S rRNA and 16S rRNA Genes. *Applied and Environmental Microbiology*, **72**, 1719–1728.

Jansson J (2011) Towards "Tera-Terra" Terabase Sequencing of Terrestrial Metagenomes. *Microbe*, **6**, 309–315.

Jeffery S, Verheijen FG a, van der Velde M, Bastos a. C (2011) A quantitative review of the effects of biochar application to soils on crop productivity using meta-analysis. *Agriculture, Ecosystems and Environment*, **144**, 175–187.

Jentsch A, Kreyling J, Elmer M *et al.* (2011) Climate extremes initiate ecosystem-regulating functions while maintaining productivity. *Journal of Ecology*, **99**, 689–702.

Jindo K, Sánchez-Monedero M a, Hernández T *et al.* (2012) Biochar influences the microbial community structure during manure composting with agricultural wastes. *Science of The Total Environment*, **416**, 476–481.

Jones DL, Edwards-Jones G, Murphy DV (2011a) Biochar mediated alterations in herbicide breakdown and leaching in soil. *Soil Biology and Biochemistry*, **43**, 804–813.

Jones MDM, Forn I, Gadelha C *et al.* (2011b) Discovery of novel intermediate forms redefines the fungal tree of life. *Nature*, **474**, 200–203.

Jones DL, Murphy DV, Khalid M *et al.* (2011c) Short-term biochar-induced increase in soil CO2 release is both biotically and abiotically mediated. *Soil Biology and Biochemistry*, **43**, 1723–1731.

Jones DL, Rousk J, Edwards-Jones G, DeLuca TH, Murphy DV (2012) Biochar-mediated changes in soil quality and plant growth in a three year field trial. *Soil Biology and Biochemistry*, **45**, 113–124.

Jorgensen T, Haile J, Moller P *et al.* (2012) A comparative study of ancient sedimentary DNA, pollen and macrofossils from permafrost sediments of northern Siberia reveals long-term vegetational stability. *Molecular Ecology*, **21**, 1989–2003.

Kahle P, Möller J, Baum C, Gurgel A (2013) Tillage-induced changes in the distribution of soil organic matter and the soil aggregate stability under a former short rotation coppice. *Soil and Tillage Research*, **133**, 49–53.

Kent WJ (2002) BLAT---The BLAST-Like Alignment Tool. *Genome Research*, **12**, 656–664.

Kent AD, Triplett EW (2002) Microbial Communities and Their Interactions in Soil and Rhizosphere Ecosystems. *Annual Review of Microbiology*, **56**, 211–236.

Kersters K, De Vos P, Gillis M *et al.* (2013) Introduction to the Proteobacteria. In: *The Prokaryotes: a Handbook on the Biology of Bacteria*, pp. 3–37. Springer New York.

Khodadad CLM, Zimmerman AR, Green SJ, Uthandi S, Foster JS (2011) Taxa-specific changes in soil microbial community composition induced by pyrogenic carbon amendments. *Soil Biology and Biochemistry*, **43**, 385–392.

Kim J-S, Sparovek G, Longo RM, De Melo WJ, Crowley D (2007) Bacterial diversity of terra preta and pristine forest soil from the Western Amazon. *Soil Biology and Biochemistry*, **39**, 684–690.

Kinney TJ, Masiello C a., Dugan B *et al.* (2012) Hydrologic properties of biochars produced at different temperatures. *Biomass and Bioenergy*, **41**, 34–43.

Klindworth A, Pruesse E, Schweer T *et al.* (2013) Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research*, **41**.

Knief C (2014) Analysis of plant microbe interactions in the era of next generation sequencing technologies. *Frontiers in Plant Science*, **5**, 1–23.

Knight R, Jansson J, Field D *et al.* (2012) Unlocking the potential of metagenomics through replicated experimental design. *Nature Biotechnology*, **30**, 513–520.

Kolb SE, Fermanich KJ, Dornbush ME (2009) Effect of Charcoal Quantity on Microbial Biomass and Activity in Temperate Soils. *Soil Science Society of*

America Journal, **73**, 1173.

Kõljalg U, Nilsson RH, Abarenkov K *et al.* (2013) Towards a unified paradigm for sequence-based identification of fungi. *Molecular Ecology*, **22**, 5271–5277.

Kolton M, Meller Harel Y, Pasternak Z *et al.* (2011) Impact of Biochar Application to Soil on the Root-Associated Bacterial Community Structure of Fully Developed Greenhouse Pepper Plants. *Applied and Environmental Microbiology*, **77**, 4924–4930.

Kreyling J, Beierkuhnlein C, Elmer M *et al.* (2008) Soil biotic processes remain remarkably stable after 100-year extreme weather events in experimental grassland and heath. *Plant and Soil*, **308**, 175–188.

Kuenen JG (2008) Anammox bacteria: from discovery to application. *Nature reviews. Microbiology*, **6**, 320–326.

Kunin V, Copeland A, Lapidus A, Mavromatis K, Hugenholtz P (2008) A Bioinformatician's Guide to Metagenomics. *Microbiology and Molecular Biology Reviews*, **72**, 557–578.

Kuramae EE, Yergeau E, Wong LC *et al.* (2012) Soil characteristics more strongly influence soil bacterial communities than land-use type. *FEMS Microbiology Ecology*, **79**, 12–24.

Kuzyakov Y, Bogomolova I, Glaser B (2014) Biochar stability in soil: Decomposition during eight years and transformation as assessed by compound-specific 14C analysis. *Soil Biology and Biochemistry*, **70**, 229–236.

Lambers H, Mougel C, Jaillard B, Hinsinger P (2009) Plant-microbe-soil interactions in the rhizosphere: an evolutionary perspective. *Plant and Soil*, **321**, 83–115.

Landesman WJ, Dighton J (2010) Response of soil microbial communities and the production of plant-available nitrogen to a two-year rainfall manipulation in the New Jersey Pinelands. *Soil Biology and Biochemistry*, **42**, 1751–1758.

Langille MGI, Zaneveld J, Caporaso JG *et al.* (2013) Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nature Biotechnology*, **31**, 814–821.

Langmead B, Trapnell C, Pop M, Salzberg SL (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome biology*, **10**, R25.

Lehmann J (2007) A handful of carbon. *Nature*, **447**, 143–144.

Lehmann J, Gaunt J, Rondon M (2006) Bio-char Sequestration in Terrestrial Ecosystems – A Review. *Mitigation and Adaptation Strategies for Global Change*, **11**, 395–419.

Lehmann J, Rillig MC, Thies J *et al.* (2011) Biochar effects on soil biota – A review. *Soil Biology and Biochemistry*, **43**, 1812–1836.

Ley RE, Peterson D a., Gordon JI (2006) Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell*, **124**, 837–848.

Li W, Godzik A (2006) Cd-hit: A fast program for clustering and comparing

large sets of protein or nucleotide sequences. *Bioinformatics*, **22**, 1658–1659.

Lima HN, Schaefer CE., Mello JW., Gilkes RJ, Ker JC (2002) Pedogenesis and pre-Colombian land use of "Terra Preta Anthrosols" ("Indian black earth") of Western Amazonia. *Geoderma*, **110**, 1–17.

Linn DM, Doran JW (1984) Effect of water-filled pore space on carbon dioxide and nitrous oxide production in tillied and nontilled soils. *Soil Science Society of America Journal*, **48**, 1267–1272.

Lipiec J, Doussan C, Nosalewicz A, Kondracka K (2013) Effect of drought and heat stresses on plant growth and yield: a review. *International Agrophysics*, **27**, 463–477.

Livermore JA, Jones SE (2015) Local – global overlap in diversity informs mechanisms of bacterial biogeography. *The ISME Journal*, 1–10.

Lorenz K, Feger K-H, Kandeler E (2001) The response of soil microbial biomass and activity of a Norway spruce forest to liming and drought. *Journal of Plant Nutrition and Soil Science*, **164**, 9–19.

Lozupone C, Knight R (2005) UniFrac: a New Phylogenetic Method for Comparing Microbial Communities. *Applied and Environmental Microbiology*, **71**, 8228–8235.

Luo C, Rodriguez-R LM, Johnston ER *et al.* (2014) Soil Microbial Community Responses to a Decade of Warming as Revealed by Comparative Metagenomics. *Applied and Environmental Microbiology*, **80**, 1777–1786.

Luo C, Tsementzi D, Kyrpides N, Read T, Konstantinidis KT (2012) Direct Comparisons of Illumina vs. Roche 454 Sequencing Technologies on the Same Microbial Community DNA Sample (F Rodriguez-Valera, Ed,). *PLoS ONE*, **7**.

Mackelprang R, Waldrop MP, DeAngelis KM *et al.* (2011) Metagenomic analysis of a permafrost microbial community reveals a rapid response to thaw. *Nature*, **480**, 368–371.

Major J (2010) *Guidelines on Practical Aspects of Biochar Application to Field Soil in Various Soil Management Systems*.

Major J, Lehmann J, Rondon M, Goodale C (2010) Fate of soil-applied black carbon: downward migration, leaching and soil respiration. *Global Change Biology*, **16**, 1366–1379.

Manyà JJ (2012) Pyrolysis for biochar purposes: A review to establish current knowledge gaps and research needs. *Environmental Science and Technology*, **46**, 7939–7954.

Manzoni S, Schimel JP, Porporato A (2012) Responses of soil microbial communities to water stress: Results from a meta-analysis. *Ecology*, **93**, 930–938.

Mao J-D, Johnson RL, Lehmann J *et al.* (2012) Abundant and Stable Char Residues in Soils: Implications for Soil Fertility and Carbon Sequestration. *Environmental Science & Technology*, **46**, 9571–9576.

Maraseni TN (2010) Biochar: maximising the benefits. *International Journal of Environmental Studies*, **67**, 319–327.

Markowitz VM, Chen I-MA, Palaniappan K *et al.* (2014) IMG 4 version of the integrated microbial genomes comparative analysis system. *Nucleic Acids Research*, **42**, 560–567.

Martin F, Kohler A, Duplessis S (2007) Living in harmony in the wood underground: ectomycorrhizal genomics. *Current Opinion in Plant Biology*, **10**, 204–10.

Martin F, Tuskan G., DiFazio S. *et al.* (2004) Symbiotic sequencing for the populous mesocosm. *New Phytologist*, **161**, 321–324.

Masella AP, Bartram AK, Truszkowski JM, Brown DG, Neufeld JD (2012) PANDAseq: paired-end assembler for illumina sequences. *BMC Bioinformatics*, **13**, 31.

Masiello CA, Chen Y, Gao X *et al.* (2013) Biochar and Microbial Signaling: Production Conditions Determine Effects on Microbial Communication. *Environmental Science & Technology*, **47**.

Mason OU, Hazen TC, Borglin S *et al.* (2012) Metagenome, metatranscriptome and single-cell sequencing reveal microbial response to Deepwater Horizon oil spill. *The ISME Journal*, **6**, 1715–1727.

McCormack SA, Ostle N, Bardgett RD, Hopkins DW, Vanbergen AJ (2013) Biochar in bioenergy cropping systems: Impacts on soil faunal communities and linked ecosystem processes. *GCB Bioenergy*, **5**, 81–95.

McGuire KL, Payne SG, Palmer MI *et al.* (2013) Digging the New York City Skyline: Soil Fungal Communities in Green Roofs and City Parks (JA Gilbert, Ed,). *PLoS ONE*, **8**.

Mendes R, Garbeva P, Raaijmakers JM (2013) The rhizosphere microbiome: significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. *FEMS Microbiology Reviews*, **37**, 634–663.

Mendes LW, Tsai SM, Navarrete A a *et al.* (2015) Soil-Borne Microbiome: Linking Diversity to Function. *Microbial Ecology*.

Mendes G de O, Zafra DL, Vassilev NB *et al.* (2014) Biochar Enhances Aspergillus niger Rock Phosphate Solubilization by Increasing Organic Acid Production and Alleviating Fluoride Toxicity. *Applied and Environmental Microbiology*, **80**, 3081–3085.

Meyer A, Focks A, Radl V *et al.* (2013) Different Land Use Intensities in Grassland Ecosystems Drive Ecology of Microbial Communities Involved in Nitrogen Turnover in Soil (H Smidt, Ed,). *PLoS ONE*, **8**.

Michalet S, Rohr J, Warshan D *et al.* (2013) Phytochemical analysis of mature tree root exudates in situ and their role in shaping soil microbial communities in relation to tree N-acquisition strategy. *Plant Physiology and Biochemistry*, **72**, 169–177.

Mikheyev AS, Tin MMY (2014) A first look at the Oxford Nanopore MinION sequencer. *Molecular Ecology Resources*, **14**, 1097–1102.

Morales SE, Holben WE (2011) Linking bacterial identities and ecosystem processes: can “omic” analyses be more than the sum of their parts? *FEMS Microbiology Ecology*, **75**, 2–16.

Naik SN, Goud V V., Rout PK, Dalai AK (2010) Production of first and second generation biofuels: A comprehensive review. *Renewable and Sustainable*

Energy Reviews, **14**, 578–597.

Naisse C, Alexis M, Plante A *et al.* (2013) Can biochar and hydrochar stability be assessed with chemical methods? *Organic Geochemistry*, **60**, 40–44.

Neilson JW, Jordan FL, Maier RM (2013) Analysis of artifacts suggests DGGE should not be used for quantitative diversity analysis. *Journal of Microbiological Methods*, **92**, 256–263.

Oleszczuk P, Jośko I, Futa B *et al.* (2014) Effect of pesticides on microorganisms, enzymatic activity and plant in biochar-amended soil. *Geoderma*, **214–215**, 10–18.

Op den Camp HJM, Islam T, Stott MB *et al.* (2009) Environmental, genomic and taxonomic perspectives on methanotrophic Verrucomicrobia. *Environmental Microbiology Reports*, **1**, 293–306.

Orgiazzi A, Lumini E, Nilsson RH *et al.* (2012) Unravelling Soil Fungal Communities from Different Mediterranean Land-Use Backgrounds (M Moora, Ed,). *PLoS ONE*, **7**.

Osono T (2007) Ecology of ligninolytic fungi associated with leaf litter decomposition. *Ecological Research*, **22**, 955–974.

Owen JG, Reddy BVB, Ternei M a *et al.* (2013) Mapping gene clusters within arrayed metagenomic libraries to expand the structural diversity of biomedically relevant natural products. *Proceedings of the National Academy of Sciences*, **110**, 11797–11802.

Pan Y, Cassman N, de Hollander M *et al.* (2014) Impact of long-term N, P, K, and NPK fertilization on the composition and potential functions of the bacterial community in grassland soil. *FEMS Microbiology Ecology*, **90**, 195–205.

Parks DH, Beiko RG (2010) Identifying biologically relevant differences between metagenomic communities. *Bioinformatics*, **26**, 715–721.

Pascault N, Ranjard L, Kaisermann A *et al.* (2013) Stimulation of Different Functional Groups of Bacteria by Various Plant Residues as a Driver of Soil Priming Effect. *Ecosystems*, **16**, 810–822.

Paz-Ferreiro J, Gascó G, Gutiérrez B, Méndez a. (2011) Soil biochemical activities and the geometric mean of enzyme activities after application of sewage sludge and sewage sludge biochar to soil. *Biology and Fertility of Soils*, 511–517.

Peay KG, Kennedy PG, Davies SJ, Tan S, Bruns TD (2010) Potential link between plant and fungal distributions in a dipterocarp rainforest: Community and phylogenetic structure of tropical ectomycorrhizal fungi across a plant and soil ecotone. *New Phytologist*, **185**, 529–542.

Penton CR, Johnson T a, Quensen JF *et al.* (2013) Functional genes to assess nitrogen cycling and aromatic hydrocarbon degradation: primers and processing matter. *Frontiers in microbiology*, **4**, 279.

Philippot L, Andersson SGE, Battin TJ *et al.* (2010) The ecological coherence of high bacterial taxonomic ranks. *Nature Reviews Microbiology*, **8**, 523–529.

Prayogo C, Jones JE, Baeyens J, Bending GD (2013) Impact of biochar on mineralisation of C and N from soil and willow litter and its relationship with microbial community biomass and structure. *Biology and Fertility of*

Soils, **50**, 695–702.

Prendergast-Miller MT, Duvall M, Sohi SP (2014) Biochar-root interactions are mediated by biochar nutrient content and impacts on soil nutrient availability. *European Journal of Soil Science*, **65**, 173–185.

Price MN, Dehal PS, Arkin AP (2010) FastTree 2 – Approximately Maximum-Likelihood Trees for Large Alignments (AFY Poon, Ed,). *PLoS ONE*, **5**.

Prommer J, Wanek W, Hofhansl F *et al.* (2014) Biochar Decelerates Soil Organic Nitrogen Cycling but Stimulates Soil Nitrification in a Temperate Arable Field Trial (S Jose, Ed,). *PLoS ONE*, **9**.

Purakayastha TJ, Kumari S, Pathak H (2015) Characterisation, stability, and microbial effects of four biochars produced from crop residues. *Geoderma*, **239-240**, 293–303.

Püttsepp Ü, Rosling A, Taylor AF. (2004) Ectomycorrhizal fungal communities associated with Salix viminalis L. and S. dasyclados Wimm. clones in a short-rotation forestry plantation. *Forest Ecology and Management*, **196**, 413–424.

Qi Y, Xu M (2001) Separating the effects of moisture and temperature on soil CO2 efflux in a coniferous forest in the Sierra Nevada mountains. *Plant and Soil*, **237**, 15–23.

Quast C, Pruesse E, Yilmaz P *et al.* (2013) The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research*, **41**, 590–596.

Quilliam RS, Marsden K a., Gertler C *et al.* (2012) Nutrient dynamics, microbial growth and weed emergence in biochar amended soil are influenced by time since application and reapplication rate. *Agriculture, Ecosystems & Environment*, **158**, 192–199.

Rajendhran J, Gunasekaran P (2008) Strategies for accessing soil metagenome for desired applications. *Biotechnology Advances*, **26**, 576–590.

Ramirez KS, Leff JW, Barberan A *et al.* (2014) Biogeographic patterns in below-ground diversity in New York City's Central Park are similar to those observed globally. *Proceedings of the Royal Society B: Biological Sciences*, **281**, 20141988–20141988.

Rho M, Tang H, Ye Y (2010) FragGeneScan: predicting genes in short and error-prone reads. *Nucleic Acids Research*, **38**.

Rideout JR, He Y, Navas-Molina J a *et al.* (2014) Subsampled open-reference clustering creates consistent, comprehensive OTU definitions and scales to billions of sequences. *PeerJ*, **2**, e545.

Rillig MC, Wagner M, Salem M *et al.* (2010) Material derived from hydrothermal carbonization: Effects on plant growth and arbuscular mycorrhiza. *Applied Soil Ecology*, **45**, 238–242.

Rodriguez A, Sanders IR (2014) The role of community and population ecology in applying mycorrhizal fungi for improved food security. *The ISME Journal*, 1–9.

Rooney DC, Killham K, Bending GD *et al.* (2009) Mycorrhizas and biomass crops: opportunities for future sustainable development. *Trends in Plant Science*, **14**, 542–549.

Rowe RL, Hanley ME, Goulson D *et al.* (2011) Potential benefits of commercial willow Short Rotation Coppice (SRC) for farm-scale plant and invertebrate communities in the agri-environment. *Biomass and Bioenergy*, **35**, 325–336.

Rowe RL, Street NR, Taylor G (2009) Identifying potential environmental impacts of large-scale deployment of dedicated bioenergy crops in the UK. *Renewable and Sustainable Energy Reviews*, **13**, 271–290.

Rutigliano FA, Romano M, Marzaioli R *et al.* (2014) Effect of biochar addition on soil microbial community in a wheat crop. *European Journal of Soil Biology*, **60**, 9–15.

Ryberg M, Nilsson RH, Kristiansson E *et al.* (2008) Mining metadata from unidentified ITS sequences in GenBank: A case study in Inocybe (Basidiomycota). *BMC Evolutionary Biology*, **8**, 50.

Saleem M, Moe L a (2014) Multitrophic microbial interactions for eco- and agro-biotechnological processes: theory and practice. *Trends in Biotechnology*, **32**, 529–537.

Sanford R a, Wagner DD, Wu Q *et al.* (2012) Unexpected nondenitrifier nitrous oxide reductase gene diversity and abundance in soils. *Proceedings of the National Academy of Sciences*, **109**, 19709–19714.

Sanger. F NS and CAR (1977) DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences*, **74**, 5463–5467.

Sangwan P, Chen X, Hugenholtz P, Janssen PH (2004) Chthoniobacter flavus gen. nov., sp. nov., the first pure-culture representative of subdivision two, Spartobacteria classis nov., of the phylum Verrucomicrobia. *Applied and Environmental Microbiology*, **70**, 5875–5881.

Schimel J, Balser T (2007) Microbial stress-response physiology and its implications for ecosystem function. *Ecology*, **88**, 1386–1394.

Schimel JP, Schaeffer SM (2012) Microbial control over carbon cycling in soil. *Frontiers in Microbiology*, **3**, 1–11.

Schloss PD, Handelsman J (2005) No Title. *Genome Biology*, **6**, 229.

Schloss PD, Westcott SL, Ryabin T *et al.* (2009) Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. *Applied and Environmental Microbiology*, **75**, 7537–7541.

Schoch CL, Seifert K a, Huhndorf S *et al.* (2012) Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences of the United States of America*, **109**, 6241–6.

Schoch CL, Sung GH, López-Giráldez F *et al.* (2009) The ascomycota tree of life: A phylum-wide phylogeny clarifies the origin and evolution of fundamental reproductive and ecological traits. *Systematic Biology*, **58**, 224–239.

Schulz H, Glaser B (2012) Effects of biochar compared to organic and inorganic fertilizers on soil quality and plant growth in a greenhouse experiment. *Journal of Plant Nutrition and Soil Science*, **175**, 410–422.

Schüβler A, Schwarzott D, Walker C (2001) A new fungal phylum, the Glomeromycota: phylogeny and evolution. *Mycological Research*, **105**, 1413–1421.

Seekatz AM, Aas J, Gessert CE *et al.* (2014) Recovery of the Gut Microbiome following Fecal Microbiota Transplantation. *mBio*, **5**.

Sellstedt A, Richau KH (2013) Aspects of nitrogen-fixing Actinobacteria , in particular free-living and symbiotic Frankia. *FEMS Microbiology Letters*, **342**, 179–186.

Selsted MB, Linden L, Ibrom A *et al.* (2012) Soil respiration is stimulated by elevated CO2 and reduced by summer drought: three years of measurements in a multifactor ecosystem manipulation experiment in a temperate heathland (CLIMAITE). *Global Change Biology*, **18**, 1216–1230.

Serna-Chavez HM, Fierer N, van Bodegom PM (2013) Global drivers and patterns of microbial abundance in soil. *Global Ecology and Biogeography*, **22**, 1162–1172.

Seshadri R, Kravitz S a., Smarr L, Gilna P, Frazier M (2007) CAMERA: A Community Resource for Metagenomics. *PLoS Biology*, **5**.

Setlow P., J. L, Faeder JR (2012) Heterogeneity in Bacterial Spore populations. In: *Bacterial Spores: Current Research and Applications* (ed Abel-Santos E), pp. 199–215.

Shah N, Tang H, Doak TG, Ye Y (2011) Comparing bacterial communities inferred from 16S rRNA gene sequencing and shotgun metagenomics. *Pacific Symposium on Biocomputing*, 165–76.

Sheik CS, Beasley WH, Elshahed MS *et al.* (2011) Effect of warming and drought on grassland microbial communities. *The ISME Journal*, **5**, 1692–1700.

Shokralla S, Spall JL, Gibson JF, Hajibabaei M (2012) Next-generation sequencing technologies for environmental DNA research. *Molecular Ecology*, **21**, 1794–805.

Sims REH, Mabee W, Saddler JN, Taylor M (2010) An overview of second generation biofuel technologies. *Bioresource Technology*, **101**, 1570–1580.

Singh BP, Cowie AL, Smernik RJ (2012) Biochar Carbon Stability in a Clayey Soil As a Function of Feedstock and Pyrolysis Temperature. *Environmental Science & Technology*, **46**, 11770–11778.

Smith JL, Collins HP, Bailey VL (2010) The effect of young biochar on soil respiration. *Soil Biology and Biochemistry*, **42**, 2345–2347.

Smith SE, Smith FA, Jakobsen I (2003) Mycorrhizal fungi can dominate phosphate supply to plants irrespective of growth responses. *Plant Physiology*, **133**, 16–20.

Snyder CS, Bruulsema TW, Jensen TL, Fixen PE (2009) Review of greenhouse gas emissions from crop production systems and fertilizer management effects. *Agriculture, Ecosystems & Environment*, **133**, 247–266.

Sohi S, Lopez-capel E, Krull E, Bol R (2009a) Biochar , climate change and soil : A review to guide future research. *Civil Engineering*, **6618**, 64.

Sohi S, Lopez-Capel E, Krull E, Bol R (2009b) *Biochar, climate change and soil:*

A review to guide future research.

Souch CA, Martin PJ, Stephens W, Spoor G (2004) Effects of soil compaction and mechanical damage at harvest on growth and biomass production of short rotation coppice willow. *Plant and Soil*, **263**, 173–182.

Souza RC, Cantão ME, Vasconcelos ATR, Nogueira MA, Hungria M (2013) Soil metagenomics reveals differences under conventional and no-tillage with crop rotation or succession. *Applied Soil Ecology*, **72**, 49–61.

Spain AM, Krumholz LR, Elshahed MS (2009) Abundance, composition, diversity and novelty of soil Proteobacteria. *The ISME Journal*, **3**, 992–1000.

Stauffer M, Leyval C, Brun JJ, Leportier P, Berthelin J (2014) Effect of willow short rotation coppice on soil properties after three years of growth as compared to forest, grassland and arable land uses. *Plant and Soil*, **377**, 423–438.

Steinbeiss S, Gleixner G, Antonietti M (2009) Effect of biochar amendment on soil carbon balance and soil microbial activity. *Soil Biology and Biochemistry*, **41**, 1301–1310.

Steiner C, Das KC, Garcia M, Förster B, Zech W (2008) Charcoal and smoke extract stimulate the soil microbial community in a highly weathered xanthic Ferralsol. *Pedobiologia*, **51**, 359–366.

Steinweg MJ, Dukes JS, Paul EA, Wallenstein MD (2013) Microbial responses to multi-factor climate change: Effects on soil enzymes. *Frontiers in Microbiology*, **4**, 1–11.

Steven B, Gallegos-Graves LV, Starkenburg SR, Chain PS, Kuske CR (2012) Targeted and shotgun metagenomic approaches provide different descriptions of dryland soil microbial communities in a manipulated field study. *Environmental Microbiology Reports*, **4**, 248–256.

Sun D, Meng J, Chen W (2013) Effects of abiotic components induced by biochar on microbial communities. *Acta Agriculturae Scandinavica, Section B - Soil & Plant Science*, **63**, 633–641.

Swift MJ, Andren O, Brussaard L *et al.* (1998) Global change, soil biodiversity, and nitrogen cycling in terrestrial ecosystems: three case studies. *Global Change Biology*, **4**, 729–743.

Taberlet P, Coissac E, Hajibabaei M, Rieseberg LH (2012a) Environmental DNA. *Molecular Ecology*, **21**, 1789–93.

Taberlet P, Prud'homme SM, Campione E *et al.* (2012b) Soil sampling and isolation of extracellular DNA from large amount of starting material suitable for metabarcoding studies. *Molecular ecology*, 1816–1820.

Taketani RG, Lima AB, da Conceição Jesus E *et al.* (2013) Bacterial community composition of anthropogenic biochar and Amazonian anthrosols assessed by 16S rRNA gene 454 pyrosequencing. *Antonie van Leeuwenhoek*, **104**, 233–42.

Tapia-Torres Y, López-Lozano NE, Souza V, García-Oliva F (2015) Vegetation-soil system controls soil mechanisms for nitrogen transformations in an oligotrophic Mexican desert. *Journal of Arid Environments*, **114**, 62–69.

Tasse L, Bercovici J, Pizzut-Serin S *et al.* (2010) Functional metagenomics to mine the human gut microbiome for dietary fiber catabolic enzymes.

Genome Research, **20**, 1605–12.

Tedersoo L, Arnold a. E, Hansen K (2013) Novel aspects in the life cycle and biotrophic interactions in Pezizomycetes (Ascomycota, Fungi). *Molecular Ecology*, **22**, 1488–1493.

Tedersoo L, Bahram M, Polme S *et al.* (2014) Global diversity and geography of soil fungi. *Science*, **346**, 1078.

Tedersoo L, Bahram M, Toots M *et al.* (2012) Towards global patterns in the diversity and community structure of ectomycorrhizal fungi. *Molecular Ecology*, **21**, 4160–70.

Tedersoo L, May TW, Smith ME (2010) Ectomycorrhizal lifestyle in fungi: Global diversity, distribution, and evolution of phylogenetic lineages. *Mycorrhiza*, **20**, 217–263.

Temperton B, Giovannoni SJ (2012) Metagenomics: Microbial diversity through a scratched lens. *Current Opinion in Microbiology*, **15**, 605–612.

Thomas T, Gilbert J, Meyer F (2012) Metagenomics - a guide from sampling to data analysis. *Microbial Informatics and Experimentation*, **2**, 3.

Thomas F, Hehemann JH, Rebuffet E, Czjzek M, Michel G (2011) Environmental and gut Bacteroidetes: The food connection. *Frontiers in Microbiology*, **2**, 1–16.

Tiwary A, Namdeo A, Fuenetes J *et al.* (2013) Systems scale assessment of the sustainability implications of emerging green initiatives. *Environmental Pollution*, **183**, 213–223.

Tkacz a., Poole P (2015) Role of root microbiota in plant productivity. *Journal of Experimental Botany*, **66**, 2167–2175.

Toberman H, Freeman C, Evans C, Fenner N, Artz RRE (2008) Summer drought decreases soil fungal diversity and associated phenol oxidase activity in upland Calluna heathland soil. *FEMS Microbiology Ecology*, **66**, 426–436.

Tonge DP, Pashley CH, Gant TW (2014) Amplicon -Based Metagenomic Analysis of Mixed Fungal Samples Using Proton Release Amplicon Sequencing (V Chaturvedi, Ed,). *PLoS ONE*, **9**.

Tringe SG (2005) Comparative Metagenomics of Microbial Communities. *Science*, **308**, 554–557.

Trivedi P, Anderson IC, Singh BK (2013) Microbial modulators of soil carbon storage: integrating genomic and metabolic knowledge for global prediction. *Trends in Microbiology*, **21**, 641–651.

Turnbaugh PJ, Bäckhed F, Fulton L, Gordon JI (2008) Diet-Induced Obesity Is Linked to Marked but Reversible Alterations in the Mouse Distal Gut Microbiome. *Cell Host & Microbe*, **3**, 213–223.

Tyson GW, Chapman J, Hugenholtz P *et al.* (2004) Community structure and metabolism through reconstruction of microbial genomes from the environment. *Nature*, **428**, 37–43.

Vaccari FP, Baronti S, Lugato E *et al.* (2011) Biochar as a strategy to sequester carbon and increase yield in durum wheat. *European Journal of Agronomy*, **34**, 231–238.

Venter JC (2004) Environmental Genome Shotgun Sequencing of the Sargasso

Sea. *Science*, **304**, 66–74.

Ventura M, Alberti G, Viger M *et al.* (2015) Biochar mineralization and priming effect on SOM decomposition in two European short rotation coppices. *GCB Bioenergy*, **7**, 1150–1160.

Ventura M, Canchaya C, Tauch A *et al.* (2007) Genomics of Actinobacteria: Tracing the Evolutionary History of an Ancient Phylum. *Microbiology and Molecular Biology Reviews*, **71**, 495–548.

Ventura M, Sorrenti G, Panzacchi P, George E, Tonon G (2013) Biochar Reduces Short-Term Nitrate Leaching from A Horizon in an Apple Orchard. *Journal of Environment Quality*, **42**, 76.

Ventura M, Zhang C, Baldi E *et al.* (2014) Effect of biochar addition on soil respiration partitioning and root dynamics in an apple orchard. *European Journal of Soil Science*, **65**, 186–195.

Verastegui Y, Cheng J, Engel K *et al.* (2014) Multisubstrate Isotope Labeling and Metagenomic Analysis of Active Soil Bacterial Communities. *mBio*, **5**.

Verbruggen E, van der Heijden MG a., Rillig MC, Kiers ET (2013) Mycorrhizal fungal establishment in agricultural soils: factors determining inoculation success. *New Phytologist*, **197**, 1104–1109.

Verheijen F, Jeffery S, Bastos a C, Van Der Velde M, Diafas I (2010) *Biochar Application to Soils*.

Viger M, Hancock RD, Miglietta F, Taylor G (2015) More plant growth but less plant defence? First global gene expression data for plants grown in soil amended with biochar. *GCB Bioenergy*, **7**, 658–672.

Vitousek PM, Aber JD, Howarth RW *et al.* (1997) Human Alteration of the Global Nitrogen Cycle: Sources and Consequences. *Ecological Applications*, **7**, 737–750.

Volk T a., Verwijst T, Tharakan PJ, Abrahamson LP, White EH (2004) Growing fuel: a sustainability assessment of willow biomass crops. *Frontiers in Ecology and the Environment*, **2**, 411–418.

de Vries FT, Manning P, Tallowin JRB *et al.* (2012) Abiotic drivers and plant traits explain landscape-scale patterns in soil microbial communities (N Johnson, Ed,). *Ecology Letters*, **15**, 1230–1239.

Walker LR, Wardle D a., Bardgett RD, Clarkson BD (2010) The use of chronosequences in studies of ecological succession and soil development. *Journal of Ecology*, **98**, 725–736.

Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naive Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. *Applied and Environmental Microbiology*, **73**, 5261–5267.

Wardle D a., Lindahl BD (2014) Disentangling global soil fungal diversity. *Science*, **346**, 1052–1053.

Warnock DD, Lehmann J, Kuyper TW, Rillig MC (2007) Mycorrhizal responses to biochar in soil – concepts and mechanisms. *Plant and Soil*, **300**, 9–20.

Warnock DD, Mummey DL, McBride B *et al.* (2010) Influences of non-herbaceous biochar on arbuscular mycorrhizal fungal abundances in roots and soils: Results from growth-chamber and field experiments. *Applied*

Soil Ecology, **46**, 450–456.

Watzinger A, Feichtmair S, Kitzler B *et al.* (2014) Soil microbial communities responded to biochar application in temperate soils and slowly metabolized 13 C-labelled biochar as revealed by 13 C PLFA analyses: results from a short-term incubation and pot experiment. *European Journal of Soil Science*, **65**, 40–51.

Weintraub MN (2011) *Phosphorus in Action* (E Bünemann, A Oberson, E Frossard, Eds,). Springer Berlin Heidelberg, Berlin, Heidelberg.

West TO, Post WM (2002) Soil Organic Carbon Sequestration Rates by Tillage and Crop Rotation. *Soil Science Society of America Journal*, **66**, 1930.

White JR, Nagarajan N, Pop M (2009) Statistical Methods for Detecting Differentially Abundant Features in Clinical Metagenomic Samples (CA Ouzounis, Ed,). *PLoS Computational Biology*, **5**.

Whitman T, Enders A, Lehmann J (2014) Pyrogenic carbon additions to soil counteract positive priming of soil carbon mineralization by plants. *Soil Biology and Biochemistry*, **73**, 33–41.

Wiedner K, Rumpel C, Steiner C *et al.* (2013) Chemical evaluation of chars produced by thermochemical conversion (gasification, pyrolysis and hydrothermal carbonization) of agro-industrial biomass on a commercial scale. *Biomass and Bioenergy*, **59**, 264–278.

Wilke A, Glass EM, Bischof J *et al.* (2013) *MG-RAST Technical report and manual for version 3.3.6 – rev. 1*.

Williams M, Arnott J (2010) A Comparison of Variable Economic Costs Associated With Two Proposed Biochar Application Methods. *Annals of Environmental Science*, **4**, 23–30.

Williams M a., Rice CW (2007) Seven years of enhanced water availability influences the physiological, structural, and functional attributes of a soil microbial community. *Applied Soil Ecology*, **35**, 535–545.

Woese CR, Fox GE (1977) Phylogenetic structure of the prokaryotic domain: the primary kingdoms. *Proceedings of the National Academy of Sciences of the United States of America*, **74**, 5088–5090.

Wooley JC, Godzik A, Friedberg I (2010) A Primer on Metagenomics (PE Bourne, Ed,). *PLoS Computational Biology*, **6**.

Woolf D, Amonette JE, Street-Perrott FA, Lehmann J, Joseph S (2010) Sustainable biochar to mitigate global climate change. *Nature Communications*, **1**, 1–9.

Xu J (2006) Microbial ecology in the age of genomics and metagenomics: concepts, tools, and recent advances. *Molecular Ecology*, **15**, 1713–31.

Xu H, Wang X, Li H *et al.* (2014a) Biochar Impacts Soil Microbial Community Composition and Nitrogen Cycling in an Acidic Soil Planted with Rape. *Environmental Science & Technology*, **48**, 9391–9399.

Xu H-J, Wang X-H, Li H *et al.* (2014b) Biochar Impacts Soil Microbial Community Composition and Nitrogen Cycling in an Acidic Soil Planted with Rape. *Environmental Science & Technology*, **48**, 9391–9399.

Xue C, Penton CR, Zhang B *et al.* (2015) Soil fungal and bacterial responses to

conversion of open land to short rotation woody biomass crops. *GCB Bioenergy*.

Yannikos N, Leinweber P, Helgason BL *et al.* (2014) Impact of Populus trees on the composition of organic matter and the soil microbial community in Orthic Gray Luvisols in Saskatchewan (Canada). *Soil Biology and Biochemistry*, **70**, 5–11.

Yoccoz NG (2012) The future of environmental DNA in ecology. *Molecular Ecology*, **21**, 2031–8.

Yuste JC, Peñuelas J, Estiarte M *et al.* (2011) Drought resistant fungi control soil organic matter decomposition and its response to temperature. *Global Change Biology*, **17**, 1475–1486.

Zavalloni C, Alberti G, Biasiol S *et al.* (2011) Microbial mineralization of biochar and wheat straw mixture in soil: A short-term study. *Applied Soil Ecology*, **50**, 45–51.

Zhai L, CaiJi Z, Liu J *et al.* (2015) Short-term effects of maize residue biochar on phosphorus availability in two soils with different phosphorus sorption capacities. *Biology and Fertility of Soils*, **51**, 113–122.

Zhang N, Castlebury LA, Miller AN *et al.* (2006) An overview of the systematics of the Sordariomycetes based on a four-gene phylogeny. *Mycologia*, **98**, 1076–1087.

Zhang Q-Z, Dijkstra F a, Liu X-R *et al.* (2014) Effects of Biochar on Soil Microbial Biomass after Four Years of Consecutive Application in the North China Plain (AC Singer, Ed,). *PLoS ONE*, **9**.

Zhang H, Sekiguchi Y, Hanada S *et al.* (2003) Gemmatimonas aurantiaca gen. nov., sp. nov., a Gram-negative, aerobic, polyphosphate-accumulating micro-organism, the first cultured representative of the new bacterial phylum Gemmatimonadetes phyl. nov. *International Journal of Systematic and Evolutionary Microbiology*, **53**, 1155–1163.

Zimmerman AR, Gao B, Ahn M-Y (2011) Positive and negative carbon mineralization priming effects among a variety of biochar-amended soils. *Soil Biology and Biochemistry*, **43**, 1169–1179.