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Using high-throughput sequencing to investigate microbial mechanisms underlying drought-driven carbon release from peatlands

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Using high-throughput sequencing to investigate microbial mechanisms underlying drought-driven carbon release from peatlands

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Summary

Peatlands represent an important terrestrial pool of carbon, but are threatened by anthropogenic climate change, including a potential increase in drought events. Drought leads to the release of carbon dioxide from peat. It is well established that drought affects microbial communities, but so far insights into the microbial mechanisms underpinning the release of carbon from droughted peat are limited.

In this project, temporally-explicit drought manipulations were carried out in two peatland habitats using a replicated and controlled series of mesocosm cores. ARISA fingerprinting was used to initially delimit shifts in microbial community composition, followed by shotgun metagenomic sequencing of a subset of samples and sequencing of SSU rRNA genes (marker gene analysis; MGA). DNA-based methodologies were accompanied by biogeochemical assays, which confirmed that drought conditions were achieved in treated cores relative to controls.

ARISA fingerprinting demonstrated a significant effect of drought on bacterial and fungal community composition, with the most significant effect during the rewetting period. Conversely, sequencing-based methodologies detected a weak or non-existent effect of drought on overall community composition. However, MGA indicated that a subset of OTUs (operational taxonomic units) responded significantly to drought, particularly in the fen at 5cm depth. Where it was possible to assign taxonomy to drought-responsive OTUs, Proteobacteria and Bacteroidetes were overrepresented relative to their abundances in the community as a whole. In many cases, OTUs exhibiting negative responses to drought were closely related to obligate anaerobes. Rhizaria (a group of protists) also appeared to respond to drought. The abundance of *hydA* (a gene for the enzyme which catalyses the hydrogenic step in fermentation) fell during drought.

To conclude, although the effect of drought on overall communities was weak, the analyses showed that a number of OTUs and functional genes responded to drought. The results provide numerous avenues for future research into the mechanisms underlying drought-driven carbon release from peatlands.

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List of Abbreviations

ARISA	Automated ribosomal spacer analysis
DGGE	Denaturing gradient gel electrophoresis
dicq	3-dihydroindole-5,6-quinone-2-carboxylate
DNRA	Dissimilatory reduction of nitrate to ammonia
DOC	Dissolved organic carbon
GPP	Gross primary productivity
HMM	Hidden Markov Model
KEGG	Kyoto Encyclopaedia of Genes and Genomes
L-DOPA	L-3,4-dihydroxyphenylalanine
MAG	Metagenome-assembled genome
MGA	Marker gene analysis
OTU	Operational taxonomic unit
SEED	A database of genes & subsystems
T1, T2, etc.	Time point 1, time point 2, etc.
TCA	Tricarboxylic acid
T-RFLP	Terminal Restriction Fragment Length Polymorphism
TTGGE	Temporal Temperature Gradient Gel Electrophoresis
W1, W2, etc.	Water sampling time point 1, water sampling time point 2, etc.

Chapter 1

Introduction

1.1 Introduction & Definitions

1.1.1 Climate Change

Anthropogenic climate change poses a serious threat to both human lives and natural ecosystems, and is caused by the concentration of certain gases in the atmosphere ('greenhouse gases'). Greenhouse gases prevent the radiation of energy into space and instead absorb it, increasing the amount of energy in the atmosphere and thus causing warming. In particular, human activities have led to an increase in atmospheric concentrations of carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O). Greenhouse gas emissions have already caused a rise in global average temperatures of 0.5°C over the last fifty years, and temperatures are predicted to continue to rise at a rate of 0.2°C per decade (IPCC 2007).

The combustion of fossil fuels is an important contributor to the problem of climate change, releasing carbon which had been stored for millions of years back into the atmosphere in the form of CO_2. The terrestrial biosphere and the ocean represent large carbon pools (Figure 1.1) and have the potential to absorb a significant proportion of anthropogenic carbon emissions, but natural carbon pools are unable to keep pace with current carbon dioxide emissions. This has led to an overall increase in atmospheric carbon of ~4.3 Gt C yr^{-1} (Falkowski *et al.* 2000).

While the quantity of carbon stored in peat is small in comparison to the ocean and the total terrestrial biosphere (Figure 1.1), peat nonetheless represents a significant carbon pool which is highly threatened by human impacts, including climate change. The size of the peat carbon pool shown in Figure 1.1 (Falkowski *et al.* 2000) is probably lower than the true value: other estimates of the peat carbon pool range from 273 Gt C (Turunen *et al.* 2002) to 547 Gt C (Yu 2012), with the most commonly cited Figure 1.being 455 Gt (Gorham 1991). Climate change is likely to have a negative impact on carbon stores in peat, as many peatlands will shift from being carbon sinks to sources (Clark *et al.* 2010). Therefore, the impact of climate change on peatlands may create a positive feedback loop, further increasing carbon emissions.

In particular, this thesis will focus on the effect of drought on peat carbon fluxes, which has been the subject of much research. Climate change will result in changes in the frequency and severity of drought: modelling studies predict an overall fall in peatland water tables (Roulet *et al.* 1992; Gong *et al.* 2012). Although predictions of future changes in precipitation patterns still contain a large degree of uncertainty, rainfall will probably increase at high latitudes and decrease at low latitudes, with mid-latitudes generally showing a summer decrease and a winter

increase (Douville *et al.* 2002; Bates 2008). Alongside changes in the amount of rainfall, increased evapotranspiration and a trend towards extreme rainfall events separated by long dry periods will contribute to an increase in summer drought frequency (Bates 2008). There is some evidence that drought events are already becoming more frequent in comparison to past centuries (Worrall *et al.* 2006; Briffa *et al.* 2009).

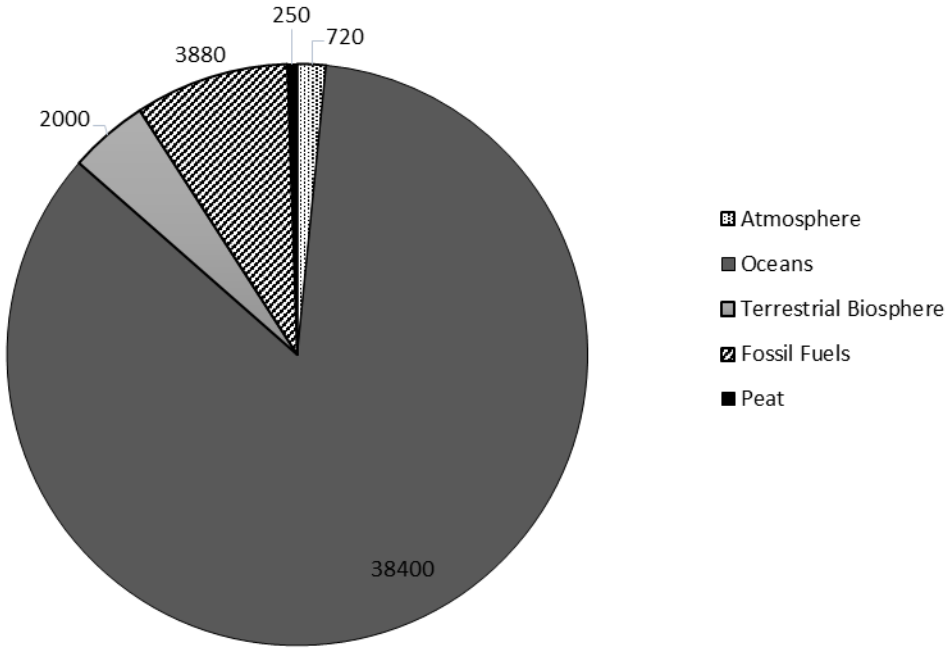

Figure 1.1: Size of major global pools of carbon. The lithosphere (not shown) contains an additional 75 million Gt of carbon. Pool sizes are in gigatons (Gt) and are taken from Falkowski *et al.* (2000).

Conversely, climate change impacts on peatlands may be partially moderated by the effects of restoration and management programmes. The majority of peatlands in the UK are not currently in pristine peat-forming condition: for example, one third of deep peat in England is subject to rotational burning, one fifth is gripped (drainage channels dug) and one seventh affected by gullies (JNCC 2011). Growing awareness of the importance of peatlands has led to a number of efforts to restore damaged peatlands, with the most common interventions being drain blocking ('grip blocking') and removal of invasive vegetation (Holden *et al.* 2008). Grip blocking is of particular relevance to the impact of increasing drought frequency on peat, as it typically leads to a rise in the water table of gripped peatlands (Shepherd *et al.* 2013) and can lead to greater stability of the water table during drought events (Wilson *et al.* 2011). Given

the large proportion of British peatlands which are in a degraded condition, continued peatland restoration and management may be able to minimise the impact of climate change on peatlands.

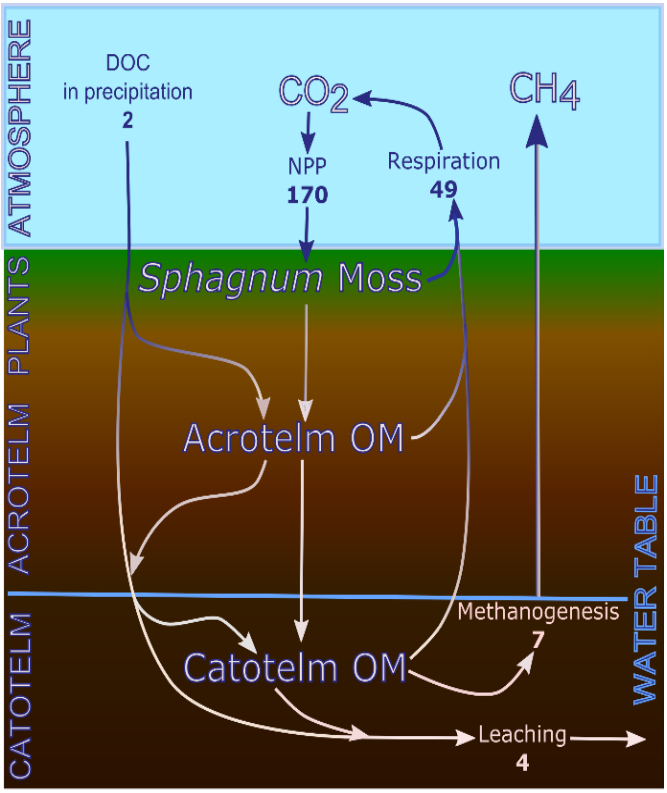

Figure 1.2: Simplified depiction of the carbon fluxes in a typical peatland. Estimated flux sizes are in kg C m^{-2} yr^{-1} and are taken from Worrall *et al.* (2009). OM= organic matter; NPP= Net primary productivity; DOC = dissolved organic carbon.

1.1.2 Definitions and Importance

Peat is defined as soil which has an organic matter content of at least 30% of dry weight, but this is often much higher (Joosten and Clarke 2002).The vast majority of peatlands are situated in the Northern hemisphere, which contains 3.5 million km^2 of peatlands out of an estimated 4 million km^2 worldwide (Gorham 1991; Joosten and Clarke 2002). Particularly important areas for Northern peatlands are Canada, Siberia and Scandinavia, but the UK also contains significant amounts of peat (JNCC 2011), especially in highland areas within Scotland and Wales. In addtion to their role in carbon sequestration, peatlands provide a range of other ecosystem services including species diversity, water purification, nutrient cycling and tourism (JNCC 2011)

Commonly, peatlands are divided into minerotrophic (groundwater-fed) and ombrotrophic (rainwater-fed), reflecting the fact that the water supply has a crucial effect on ecosystem characteristics. In common parlance minerotrophic peatlands are referred to as fens and ombrotrophic as bogs. In general, minerotrophic peatlands are more nutrient-rich than ombrotrophic peatlands, with a higher soil pH due to mineral inputs from groundwater (Joosten and Clarke 2002; Hill *et al.* 2014). However, this trend is not invariably found: Bridgham *et al.* (1998) found that although ombrotrophic peatlands were low in nitrogen and iron, labile phosphorus was relatively high due to higher mineralisation rates and nutrient availability in

acidic fens was similar to that in bogs. However, while the ombrotrophic-minerotrophic definition may not predict nutrient concentration, it corresponds well to differences in pH and the degree of peat decomposition (Bridgham *et al.* 2000).

1.1.3 Carbon Accumulation in Peatlands

Carbon accumulates in peat because the rate of gross primary productivity (GPP) exceeds the rate of respiration, leading to a build-up of carbon. Peatlands exchange carbon in gaseous and aqueous forms (Figure 1.2), with the most significant exchanges being gaseous CO_2 and CH_4 and dissolved organic carbon (DOC). Carbon dioxide exchange is by far the largest flux and results in an overall uptake of carbon, while fluxes of DOC and CH_4 are much smaller and represent net losses (Nilsson *et al.* 2008; Worrall *et al.* 2009; Billet *et al.* 2010; Koehler *et al.* 2010; Christensen *et al.* 2012). Carbon fluxes vary greatly between years (Aurela *et al.* 2004; Koehler *et al.* 2010), seasons (Koehler *et al.* 2010) and microforms (Schneider *et al.* 2012a). Annual variation in fluxes may occasionally result in years where peatlands are net sources of carbon, but overall the majority of peatlands act as carbon sinks (Worrall *et al.* 2009), resulting in peat accumulation (Clymo 1984).

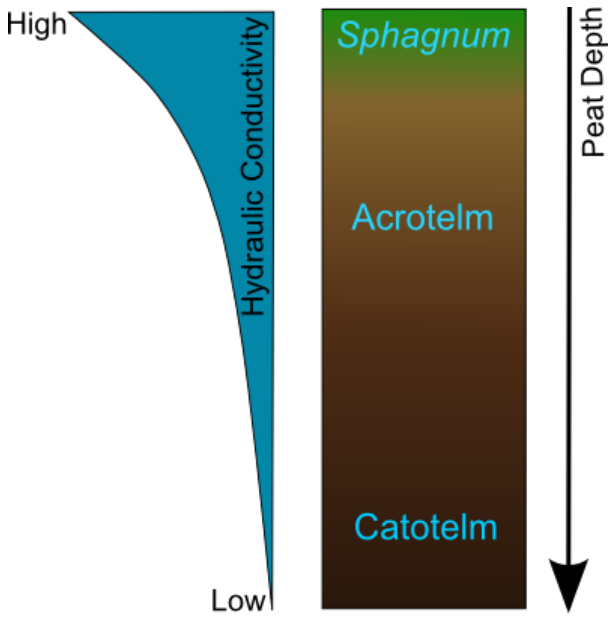

Figure 1.3: Simplified model of peat layers and hydraulic conductivity. Adapted from Lindsay *et al.* (2010).

By definition the water table in peatlands is high, which is a crucial factor in peat formation and maintenance (Clymo 1984). Peat has a low hydraulic conductivity which decreases with depth (Figure 1.3), helping to maintain a high water table (Clymo 1984). The high water table slows decomposition by imposing anaerobic conditions, enabling peat build-up until an equilibrium level is reached. Lowering the water table allows oxygen to penetrate the peat profile and leads to a loss of peat carbon (Sulman *et al.* 2013). However, while drainage and short-term droughts are associated with a loss of stored

carbon, CO_2 fluxes often remain elevated even after rewetting (Laine and Minkkinen 1996; Fenner and Freeman 2011). Elevated fluxes after rewetting suggests that the effect of the water table on decomposition may be mediated by another factor, or factors, at least in part. Phenolic compounds are one candidate: the concentration of phenolic compounds is negatively correlated to the rate of litter decomposition (Aerts and de Caluwe 1997; Bridgham and Richardson 2003) and soil respiration (Wang *et al.* 2015) in peatlands. Conversely, it is possible that elevated levels of electron acceptors (e.g. sulfate) persist following rewetting and allow carbon dioxide fluxes to remain elevated (discussed fully in section 1.3).

1.2 Peat Microbiology

1.2.1 The Structure of Peat Microbial Communities

Peat, and soil in general, is a complex environment which is home to a diverse range of organisms from all three domains of life. Bacteria dominate peat microbial communities in terms of DNA concentration (Lin *et al.* 2014b) and activity (Winsborough and Basiliko 2010), although there is evidence that fungi may be more dominant in bogs than in fens (Golovchenko *et al.* 2007; Amha *et al.* 2015). Fungal abundance decreases rapidly with depth (Lin *et al.* 2012; Lin *et al.* 2014b) and fungi found in deep layers of peat are rarely viable (Golovchenko *et al.* 2013). Bacterial numbers also decrease with depth, although more slowly than fungi, while archaea become more abundant (Lin *et al.* 2012; Lin *et al.* 2014b).

1.2.2 Bacterial Communities in Peat

Most peatland bacterial communities are inhabited by a similar set of bacterial phyla including Acidobacteria, Proteobacteria, Actinobacteria, Verrucomicrobia and Planctomycetes (Figure 1.4). These communities are comparable to the bacterial communities found in other soils (Fierer and Jackson 2006; Barnard *et al.* 2013), although the large degree of within-habitat variation makes generalisations difficult.

The prevalence of Acidobacteria in peat is unsurprising. Many Acidobacteria are slow-growing oligotrophs: they are positively correlated with a high C/N ratio (Jones *et al.* 2009) and are negatively affected by addition of labile carbon (Fierer *et al.* 2007) or litter addition (Nemergut *et al.* 2010). Acidobacteria are more abundant in bogs then fens (Lin *et al.* 2012; Lin *et al.* 2014b), which is unsurprising given the preference of this phylum for acidic habitats (Jones *et al.* 2009).

The characteristics of Proteobacteria are more difficult to define than Acidobacteria, due to the large size and diversity of this phylum. However, in general Proteobacteria appear to be faster growing and to prefer environments with a higher level of nutrients and labile carbon than Acidobacteria (Fierer *et al.* 2007; Nemergut *et al.* 2010; Goldfarb *et al.* 2011; Belova *et al.* 2014). Metagenomic studies show that α-proteobacteria contain the majority of functional genes in bogs and fens, including phenol oxidase genes (Lin *et al.* 2014a). Proteobacteria also possess the majority of genes for phenolic degradation in permafrost (Tveit *et al.* 2013) suggesting a key role in degrading complex aromatics in all peaty soils. Moreover, a diverse range of methanotrophic Proteobacteria are also found in peatlands (Dedysh *et al.* 2006; Bragina *et al.* 2014; Lin *et al.* 2014b).

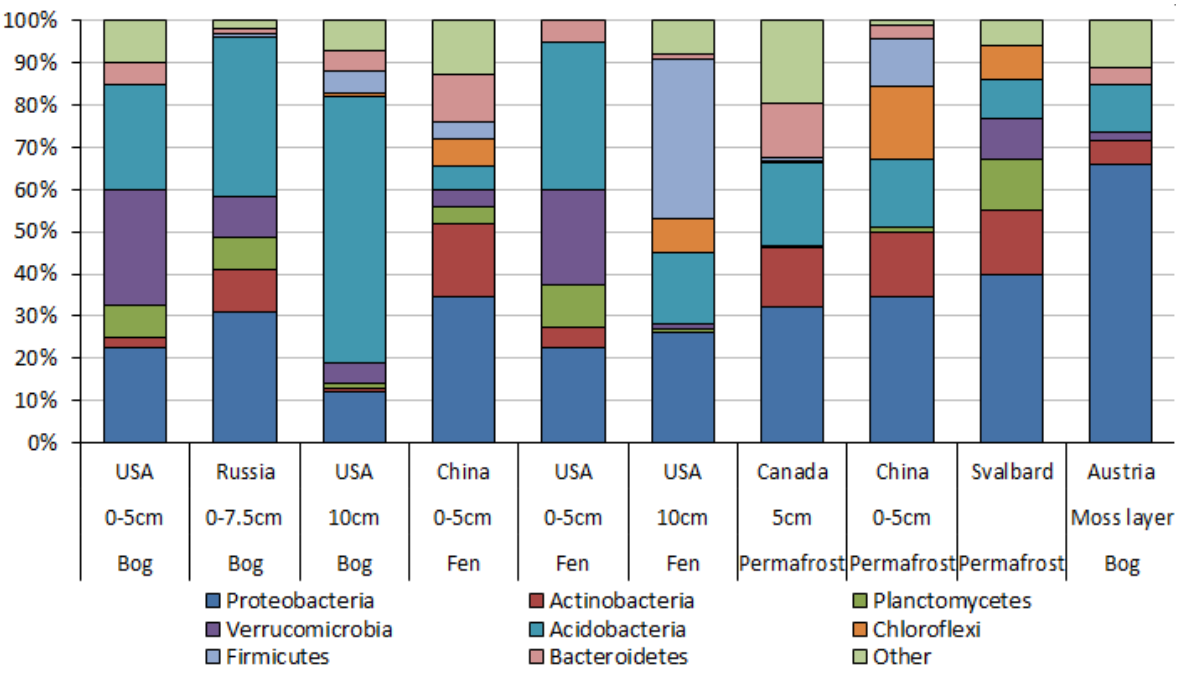

Figure 1.4: Phylum composition of bacterial communities inhabiting peat soils.
Data taken from Lin *et al.* (2012; 2014b), Serkebaeva *et al.* (2013), Deng *et al.* (2014), Frank-Fahle *et al.* (2014), Yun *et al.* (2014) and Tveit *et al.* (2013).

Actinobacteria play an important part in the degradation of complex organic polymers such as cellulose and lignin (Goodfellow and Williams 1983). In bog and fen peats, Actinobacteria usually make up a relatively small proportion of the community (Figure 1.4), but they may nonetheless play a significant role in degradation of phenolic compounds (Lin *et al.* 2014a). Actinobacteria respond strongly to water table and are more abundant under drier conditions (Jaatinen *et al.* 2007; Jaatinen *et al.* 2008; Kotiaho *et al.* 2012; Barnard *et al.* 2013) so it is

likely that the water-saturated conditions in bog and fen peats prevent high Actinobacterial abundances. In permafrost peats, Actinobacteria make up a much larger proportion of the community and are responsible for the majority of cellulose- and hemicellulose-degrading genes (Tveit *et al.* 2013), suggesting that this phylum is relatively tolerant to some peat environments.

Several other bacterial phyla are commonly recovered from peat, but do not dominate the community. Firmicutes may be much more abundant in fens than bogs (Lin *et al.* 2012), although more recent studies have found Firmicutes to show similar abundances in both bogs and fens (Lin *et al.* 2012). Verrucomicrobia tend to be limited to the upper levels of peat (Serkebaeva *et al.* 2013; Tveit *et al.* 2013) suggesting that most peatland Verrucomicrobia are aerobic. Planctomycetes often decrease with depth also, but may exhibit a second 'peak' of abundance in deeper peat (Ivanova and Dedysh 2012; Lin *et al.* 2014a). Both Verrucomicrobia and Planctomycetes are considered to be made up of relatively slow-growing species, which are outcompeted in high-nutrient environments (Ansola *et al.* 2014; Belova *et al.* 2014) and become less abundant in nutrient-enriched environments (Goldfarb *et al.* 2011).

While an attempt has been made above to summarise characteristics of bacterial phyla, it should be noted that a large amount of variation in lifestyle exists among members of each phyla. For example, while the overall abundance of Acidobacteria shows a consistent negative correlation to pH, a number of subdivisions within the Acidobacteria prefer pH values close to neutral (Pankratov *et al.* 2008; Jones *et al.* 2009). A second example is the different subgroups of Planctomycetes inhabiting shallow and deep peat (Ivanova and Dedysh 2012). However, despite these difficulties, many nucleic-acid-based studies continue to analyse bacterial taxa to phylum level due to difficulties with annotation of sequences to a lower taxonomic levels (Garcia-Etxebarria *et al.* 2014) and the lack of species-level information about bacteria.

1.2.3 Archaeal Communities in Peat

While archaea make up a relatively small proportion of the community (Lin *et al.* 2014a; Lin *et al.* 2014b), they increase with depth and are of great interest due to their role in methanogenesis. Previous studies indicate that the most abundant phyla in peatlands are Crenarchaeota and Euryarchaeota, with methanogenic classes of Euryarchaeota typically dominant (Figure 1.5). Methanogenesis can proceed via several pathways of which the most common are acetoclastic and hydrogenotrophic, which use acetate and CO_2 as electron

acceptors, respectively (Le Mer and Roger 2001). Both of these pathways are present in peatlands, and the dominant methanogenesis pathway may vary with depth or habitat (e.g. Lin *et al.* 2014b; Lin *et al.* 2015).

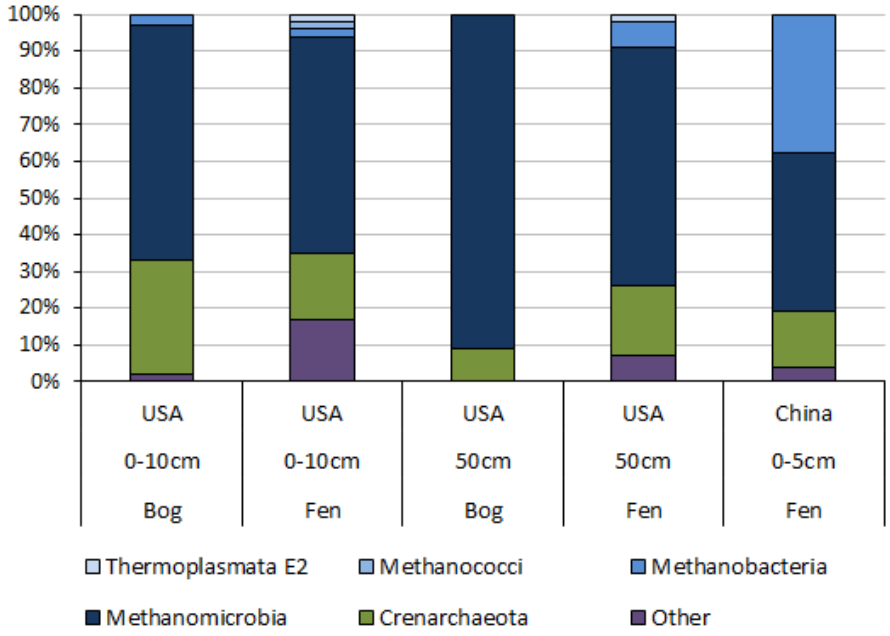

Figure 1.5: Composition of archaeal communities inhabiting peat soils. Data taken from Lin *et al*. (2012) and Deng *et al*. (2014). Thermoplasmata E2, Methanococci, Methanobacteria, and Methanomicrobia are each classes within phylum Euryarchaeota.

1.2.4 Microbial Eukaryotes in Peat

Eukaryotes also make up a relatively small proportion of microbial cells in peat (Lin *et al.* 2014a) but remain important because of their potential importance in the decomposition of complex substrates. In terrestrial habitats fungi are often considered to be the main degraders of complex substrates (Romani *et al.* 2006; Schneider *et al.* 2012a; Schneider *et al.* 2012b) although this paradigm has recently been challenged (Rousk and Frey 2015). In peat however, bacteria play a dominant role in decomposition (Winsborough and Basiliko 2010) and produce a variety of enzymes for degradation of complex substrates including aromatics (Ausec *et al.* 2011) and cellulose (Pankratov *et al.* 2011). Nonetheless, peatland fungi possess the ability to degrade a wide variety of recalcitrant substrates, particularly phenolic compounds (Williams and Crawford 1983; Golovchenko *et al.* 2013), and are likely to respond strongly to climate change (Jaatinen *et al.* 2007; Jaatinen *et al.* 2008; Peltoniemi *et al.* 2015)

Early culture-based studies indicated that the dominant fungal phylum in most peatlands was Ascomycota, with Basidiomycota, Zygomycota and Chytridiomycota also regularly identified (Thormann 2006; Thormann and Rice 2007). More recently, DNA-based studies of soil fungal communities have revealed high levels of diversity in peat fungal communities with Ascomycota and Basidiomycota usually dominant (summarised in Figure 1.6), similar to fungal communities in mineral soils (Kivlin and Treseder 2014; Tedersoo *et al.* 2014), However, the proportion of Basidiomycetes in peatlands is less than the global average

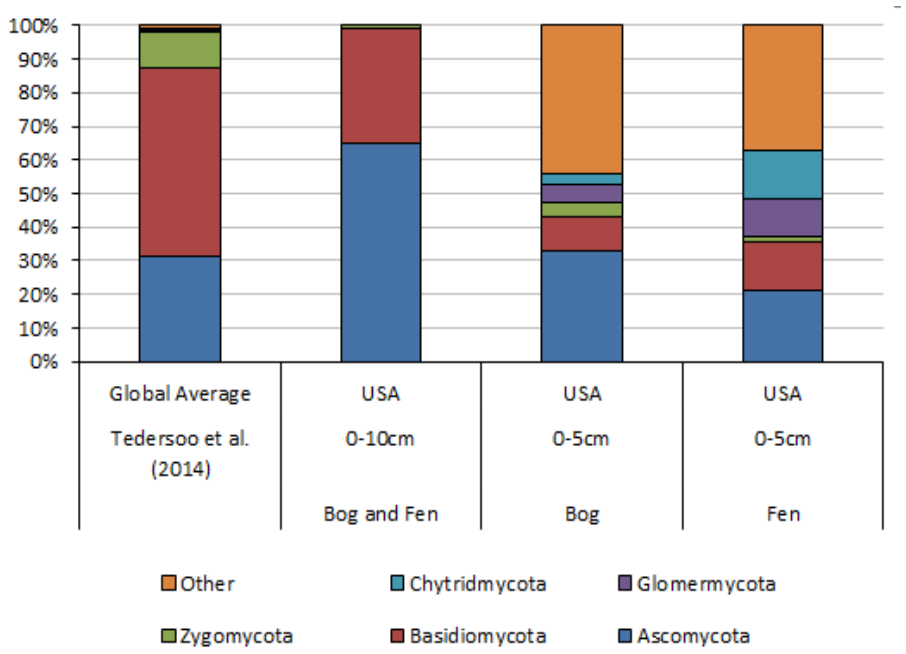

Figure 1.6: Composition of fungal communities inhabiting peat soils. Data taken from Lin *et al*. (2012; 2014b) and Tedersoo *et al.* (2014).

presented by Tedersoo et al. (2014; Figure 1.6). The comparative lack of Basidiomycetes is potentially relevant given the importance role which Basidiomycota have traditionally been thought to play in the degradation of complex substrates such as lignin: Frankland (1998) suggested that a fungal succession occurs from Ascomycetes through to Basidiomycetes during decomposition, since Basidiomycetes are more likely to possess the ability to degrade complex substrates (especially lignin) which remain in the later stages of decomposition. However, the predicted succession is not observed during the decomposition of litter in peatlands (Thormann *et al.* 2003).

While fungi have been the best-studied group of eukaryotes in peat, protozoa may also play an important role in nutrient cycling and as predators of other micro-organisms (Mieczan 2012; Jassey *et al.* 2014). In particular, testate amoeba make up a large proportion of microbial biomass in peat (Gilbert *et al.* 1998; Mieczan 2007; Jassey *et al.* 2013). Ciliates may also contribute significantly to peatland microbial communities (Mieczan 2007; Tveit *et al.* 2013) and a number of other protest taxa are also present in small numbers including heterotrophic flagellates, microalgae, Rhizaria and Strameopiles (Gilbert *et al.* 1998; Jassey *et al.* 2013; Tveit *et al.* 2013).

1.3 The Effects of Short-term Drought

1.3.1 Drought and Carbon Fluxes

There are a number of different definitions of drought, each with different (yet overlapping) impacts (Marsh *et al.* 2007). In particular, 'meteorological' drought can be used to refer to deficiency in rainfall, while 'hydrological' drought refers to accumulated efficiencies in runoff and aquifer recharge. The drought of 1976, sometimes considered as a 'benchmark' drought due to its severity (Marsh *et al.* 2007), was caused by extremely low summer rainfall (81.8mm; summer rainfall data for Wales downloaded from https://data.gov.uk/dataset/regional-climate-values-for-rainfall on 13/10/16) following the driest 16-month period on record in England and Wales. However, the relative severity of droughts depends on the measurement used: for example, the Aridity Index of the 1995 drought is greater than that of the 1976 drought, while the two-year November-April rainfall deficiency of 2006 was greater than that of any other year since the first half of the 20th century (Marsh *et al.* 2007).

In peatlands, where the water table is typically high, 'drought' is typically used to mean a fall in the water table (e.g. Fenner and Freeman 2011; Chen *et al.* 2012; Romanowicz *et al.* 2015). Worrall *et al.* (2006) suggest that when the water table falls below a 'critical' depth of approximately -16 cm, drought has severe effects on biogeochemical processes in the catotelm and fluxes of DOC are disrupted for many years after the drought ends. Between 1958 and 2000, two droughts of this magnitude occurred in Northern England: one in 1976 and one in 1995 (Worrall *et al.* 2006), both corresponding to exceptionally dry years (Jones and Conway 1997; Marsh *et al.* 2007). Similarly, Estop-Aragonés *et al.* (2016) found peatland mesocosms to shift from net carbon sinks to net carbon sources when the water table dropped to approximately –24 cm below the water table, although the 'critical' water table differed

between sites. Duration of drought is also important (Estop-Aragonés *et al.* 2016), although changes in biogeochemistry and carbon fluxes can be observed only days after the water table begins to fall (Fenner and Freeman 2011).

The potential for an increase in drought frequency or intensity with climate change (Bates 2008) gives cause for concern due to the impact of drought on peat carbon stores, as severe droughts can have long-term effects on carbon fluxes in peatlands. Simulated drought conditions invariably increase the rate of CO_2 release from peat, which has been demonstrated in lab mesocosms (Fenner and Freeman 2011; Chen *et al.* 2012), reconstructed peat columns (Moore and Knowles 1989; Moore and Dalva 1993) and field experiments (Bridgham *et al.* 2008; Fenner and Freeman 2011). In many cases CO_2 emissions rise still further after rewetting, and moderate drought can result in elevated CO_2 emissions for 3-4 years (Fenner and Freeman 2011). There is also a significant negative relationship between CO_2 release and water table in natural systems (Chimner and Cooper 2003). Despite the mounting evidence, a limited number of studies have failed to find a significant effect of drought on CO_2 emissions (Aerts and Ludwig 1997; Ellis *et al.* 2009) but this may be due to a high level of variability in CO_2 fluxes under normal conditions.

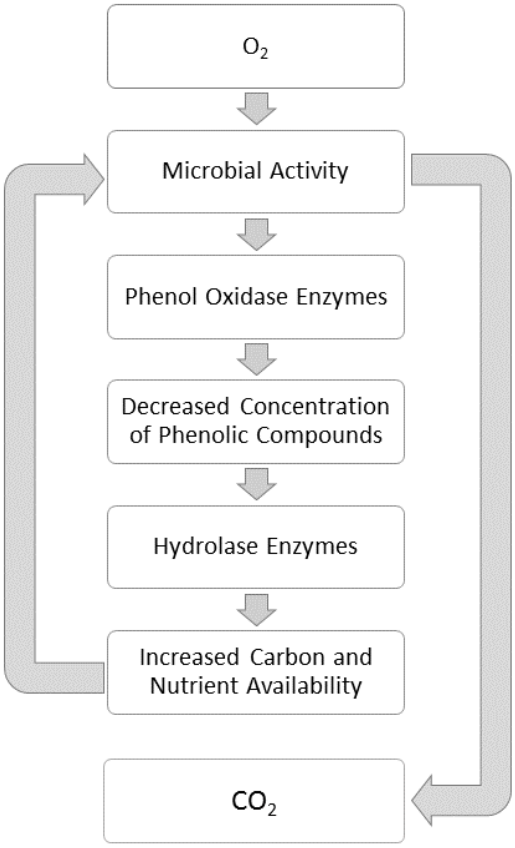

Figure 1.7: The biogeochemical cascade leading to release of CO_2 during drought in peatland. Adapted from Fenner & Freeman (2011).

One influential explanation for increased CO_2 release from peat during drought and rewetting is that the breakdown of inhibitory phenolic compounds enables an increase in the rate of decomposition (Freeman *et al.* 2001). While some anaerobic micro-organisms possess pathways for the degradation of phenolic compounds in the absence of oxygen, anaerobic decomposition of phenolics becomes

energetically unfavourable at low redox potentials (Schink *et al.* 2000). Therefore, the anaerobic conditions found in waterlogged peatlands lead to a build-up of phenolic compounds and these inhibit both enzyme activities and microbial growth. Increased oxygen availability allows the action of phenol oxidase enzymes, which degrade phenolic compounds. In the cascade model presented by Fenner and Freeman (2011; Figure 1.7), increased microbial growth in the presence of oxygen leads to increased production of phenol oxidase enzymes. Phenol oxidases degrade phenolic compounds and thus release other enzymes from inhibition by phenolic compounds, allowing the degradation of soil organic matter and leading to the release of carbon and nutrients which bacteria can then use for growth and respiration.

Freeman *et al*. (2001) provide convincing evidence that phenol oxidases play a key role in drought-driven increases in enzyme activities and CO_2 release: the authors report that the activity of hydrolase enzymes (responsible for breaking down polymeric organic matter) does not increase in direct response to oxygen, but does increase in response to the removal of phenolic compounds or the addition of phenol oxidase (Freeman *et al.* 2001; Freeman *et al.* 2004b). Additionally, in field systems there is a strong negative correlation between peat carbon fluxes and phenolic compounds (Freeman *et al.* 2001; Wang *et al.* 2015). However, a number of other studies have failed to find the expected link between phenol oxidases, water table and decomposition rates. Williams *et al*. (2000) failed to find any correlation between water table depth and phenol oxidase activity, while Xiang *et al.* (2013) found phenol oxidase activity to actually *fall* during drought. Another experiment found than aerobic conditions increased CO_2 release from incubated peat samples, but without affecting the concentration of phenolic compounds (Brouns *et al.* 2014). Most recently, Romanowicz *et al.* (2015) found hydrolase activities to show no correlation to concentration of phenolic compounds. Therefore, it seems likely that phenolic compounds are one of many potentially interacting factors that limit decomposition in peatlands. Brouns *et al.* (2014) found that oxygenation directly led to an increase in CO_2 release from peat, and this effect continued after anoxic conditions had resumed, linked to the increased availability of electron acceptors such as sulfate. This direct effect of oxygenation suggests that in some cases the mechanism underlying drought-driven carbon release may simply be the availability of electron acceptors. Alternatively, increased hydrolase activity during drought could in some cases liberate nutrients such as nitrogen from organic matter (Mettrop *et al.* 2014), thus releasing micro-organisms from nutrient limitation and allowing further growth and respiration.

As well as carbon dioxide, fluxes of methane (CH_4) and nitrous oxide (N_2O), two extremely potent greenhouse gases, are affected by drought. Methane production decreases during drought (Moore and Dalva 1993; Aerts and Ludwig 1997; Blodau *et al.* 2004; Ellis *et al.* 2009; Knorr *et al.* 2009), and often remains depressed following rewetting (Freeman *et al.* 1993; Aerts and Ludwig 1997). Methanogenesis is suppressed by the availability of alternative electron acceptors such as nitrate and sulfate, which are oxidised during drought (Kang and Freeman 2002). Nitrous oxide is produced as a by-product of denitrification, which in peatlands is usually limited by low concentrations of nitrate (Keller and Bridgham 2007). N_2O emissions often increase during drought but rapidly fall following rewetting, as nitrate ions are rapidly depleted (Freeman *et al.* 1993; Aerts and Ludwig 1997; Dowrick *et al.* 1999).

Finally, dissolved organic carbon (DOC) concentrations in pore water fall during drought (Freeman *et al.* 1994; 2004a; Ellis *et al.* 2009; Fenner and Freeman 2011; Clark *et al.* 2012), probably due to the fact that aerobic conditions allow carbon to be fully oxidised and released as carbon dioxide (Acharya 1935). In addition, the decrease in porewater pH which is observed during drought can lead to a decrease in DOC solubility (Clark *et al.* 2012).

1.3.2 Drought, Microorganisms and Carbon Release

Alongside the enzyme dynamics of peatland ecosystems, it is important to understand the microbial mechanisms underlying drought-driven carbon release: microorganisms play a key role in determining gas fluxes (Schimel and Gulledge 1998) and in determining rates of decomposition and nutrient cycling (Madsen 2005). Microbial communities are also strongly affected by environmental conditions, and have been linked to greenhouse gas fluxes (Philippot *et al.* 2011; Nazaries *et al.* 2013; McCalley *et al.* 2014) and shown to mediate the effect of environmental change on decomposition (Allison *et al.* 2013; Wang *et al.* 2015).

While microbial biomass is commonly incorporated into models of decomposition rates, the role of community composition is more complex and thus is less commonly considered (McGuire and Treseder 2010). However, Moorhead and Sinsabaugh (2006) suggest a model in which litter is successively decomposed by three guilds of micro-organisms: ‘opportunists’, ‘decomposers’ and ‘miners’. These three guilds are each associated with different categories of substrate: first opportunists use the most labile substrates (sugars and amino acids), then decomposers degrade moderately recalcitrant substrates (particularly holocellulose), and finally miners degrade the most recalcitrant substrates such as lignin. Under this model, it is

likely that community composition becomes more important as decomposition proceeds: many micro-organisms are able to use sugars for energy, but the ability to degrade lignin is typically limited to white-rot fungi, which are primarily found within class Agaricomycetes (Riley *et al.* 2014). Given that the dominant fractions in peat organic matter are lignin and holocellulose (Yavitt *et al.* 2000), it is therefore likely that microbial community composition is important for the decomposition of organic matter in peatlands. There is evidence that abiotic conditions may influence peatland microbial community composition more strongly than does organic matter type (Preston and Basiliko 2016), and so it is possible that the conditions in peatlands constrain the ability of the community to degrade recalcitrant organic matter. Aeration of peat following drought could potentially alleviate these constraints, and lead to shifts in community composition which allow the decomposition of recalcitrant organic matter. The increases in extracellular enzyme activity which occur during drought lead to an increase in labile carbon and nutrients (Fenner and Freeman 2011). This in turn could potentially lead to a rise in the abundance of fast-growing 'cheaters', using the labile substrates produced by extracellular enzymes in order to fuel rapid growth (Allison 2005). Therefore, drought potentially causes large changes to the selective pressures on microbial communities, and therefore it can be hypothesised that drought will lead to large changes in community composition.

Several studies have found drought to lead to changes in microbial community composition in mineral soils (e.g. Barnard *et al.* 2013; 2015), but the conclusions of these studies are unlikely to apply to peat due to differing hydrological conditions between habitats. In dryland soils drought represents a transition from aerobic to desiccated conditions, while in wetlands the transition is from anaerobic to aerobic conditions. However, few studies have looked at the impact of drought on microbial communities in peatlands. Fenner *et al.* (2005) carried out TTGGE fingerprinting of *xylE* (a phenol oxygenase gene) and found that the diversity and abundance of this gene increased during drought, accompanied by an increase in phenol oxidase activity. Kim *et al.* (2008) looked at three groups of micro-organisms using appropriate markers: total bacteria (16S rRNA), denitrifiers (*nirS*) and methanogens (*mcrA*). Drought reduced the abundance (according to real-time PCR) of all three genes in fens, as well as the abundance of 16S rRNA and *nirS* in bogs. However, T-RFLP fingerprinting failed to find changes in the composition or diversity of the three genes during drought. A more recent study looked at the effect of several years of climate manipulation on Scandinavian fens, and found long-term drying to have a small but significant impact on the composition of community

PLFA and fungal ITS (Peltoniemi *et al.* 2015). However, the effect of short term drought on microbial communities is likely to be different to that of long-term drying, as part of the response during long term drying may be indirect e.g. due to changing vegetation (Wang *et al.* 2015). Additionally, Nunes *et al*. (2015) report reduced bacterial community diversity and changes in bacterial community composition following fluctuations in water content, although this study was carried out on peat at 50cm depth and so does not reveal microbial changes in the more active peat layers closer to the surface.

Therefore, current literature leaves a number of unanswered questions. While T-RFLP may be used to determine species identity in some cases (Horz *et al.* 2000; Dickie and FitzJohn 2007), this has not been utilised for research of peatland microbial communities. T-RFLP is only able to visualise the dominant bands, meaning that much of the diversity within peat is likely ignored by this method. In addition, while Fenner *et al.* (2005) found differences in the diversity and abundance of the *xylE* gene, several other families of phenol oxidase genes exist which have not yet been studied during drought in peatlands (Appel 1993). Other classes of enzyme which are thought to be involved in drought-driven CO_2 release (e.g. hydrolases) have been ignored completely by genetic studies. As with phenol oxidase enzymes, hydrolases represent a large and diverse category, including members from a large number of families and individual genes. Therefore, application of genetic fingerprinting or qPCR for study of functional genes would be improved by prior characterisation of the functional genes in peatland environments. Additionally, the response of microbial eukaryotes to short-term droughts has been little studied. Therefore, there is a great deal of potential for the use of high-throughput sequencing to shed light on the microbial mechanisms underlying CO_2 release following drought in peatlands.

1.4 High-throughput sequencing and microbial ecology

1.4.1 Next-generation Sequencing in Community Ecology

In the last decade a number of high-throughput 'second-generation' sequencing platforms have been developed, vastly reducing sequencing cost per base pair. The first of these to be introduced was the Roche 454 Genome Sequencer in 2005 (Shokralla *et al.* 2012). The introduction of Illumina sequencing gave much higher throughput with a lower error rate, but at the cost of short read lengths (initially only ~35bp). However, Illumina sequencing chemistry has steadily improved, and under ideal conditions the Illumina Miseq is now able to generate

paired-end 300bp reads while the latest Illumina HiSeq can generate up to 1.5Tb of data in a single run (http://www.illumina.com/systems/sequencing-platform-comparison.html; accessed 27/09/15). More recently, 'third-generation' sequencing platforms such as the PacBio RS or the MinION (Oxford Nanopore Technologies) give much longer reads (Branton *et al.* 2008; Ferrarini *et al.* 2013; Quick *et al.* 2014), although to date these are typically used for genome assembly rather than community analysis.

A number of molecular approaches have harnessed the power of second-generation sequencing to study communities from all cellular domains of life (e.g. Fierer and Jackson 2006; Lin *et al.* 2014a; Lallias *et al.* 2015), as well as viruses (e.g. Reavy *et al.* 2015). Currently, the most commonly used methods are DNA-based, including shotgun sequencing (metagenomics) and marker gene analysis (MGA) which will be the focus of this review. RNA-based methods may be better able to distinguish rapid changes in the active community (Barnard *et al.* 2013; Moran *et al.* 2013), as many environmental micro-organisms may be dormant or inactive (Jones and Lennon 2010) and extracellular DNA may be abundant in soils (Carini *et al.* 2016). However, RNA-based methodologies also come with a number of challenges, including the unstable nature of RNA (Prosser 2015) and the fact that the vast majority (95-99%) of environmental RNA is rRNA, meaning mRNA must be enriched prior to sequencing if functional transcripts are of interest (McGrath *et al.* 2008).

1.4.2 MGA Workflows

MGA approaches involve PCR amplification and sequencing of marker genes which are then assigned to OTUs (operational taxonomic units) for downstream analysis (see Figure 1.8 for a simplified workflow). Most commonly, the choice of marker gene and OTU assignment methodology is designed in such a way that OTUs approximate species-level units of diversity. Commonly used marker genes for microbial life include 16S rRNA for bacteria and archaea (e.g. Sogin *et al.* 2006), 18S rRNA for protozoa (e.g. Pawlowski *et al.* 2012) and ITS for fungi (e.g. Epp *et al.* 2012). A number of programs exist which will carry out all steps of MGA analyses, from quality control to hypothesis testing, including QIIME (Caporaso *et al.* 2010) and mothur (Schloss *et al.* 2009).

Approaches to OTU-picking fall into two main groups: *de novo* OTU picking clusters sequences based on percentage similarity, while closed reference OTU picking clusters OTUs based on a reference database. Neither method is ideal: *de novo* OTU-picking may be computationally expensive for large numbers of sequences, and often leads to OTU groupings which do not agree with known species (White *et al.* 2010) or which aggregate organisms which do not share ecological characteristics (Preheim *et al.* 2013; Yamamoto and Bibby 2014). Conversely, closed reference OTU picking is limited by the fact that the majority of biodiversity is still undiscovered (Caporaso *et al.* 2011). Additional problems with MGA come from primer bias (Klindworth *et al.* 2013) and copy-number heterogeneity (Větrovský and Baldrian 2013). Despite these drawbacks, MGA approaches are an efficient way to study community composition in diverse environments such as soil (Lin *et al.* 2014b) or the human gut (Turnbaugh *et al.* 2009). Alternative MGA approaches focus on a single function of interest using relevant functional genes as markers such as *narG*, *nirK/nirS* and *nosZ* for denitrification (Palmer *et al.* 2012) or *cbhI* for cellulose degradation (Mueller *et al.* 2014).

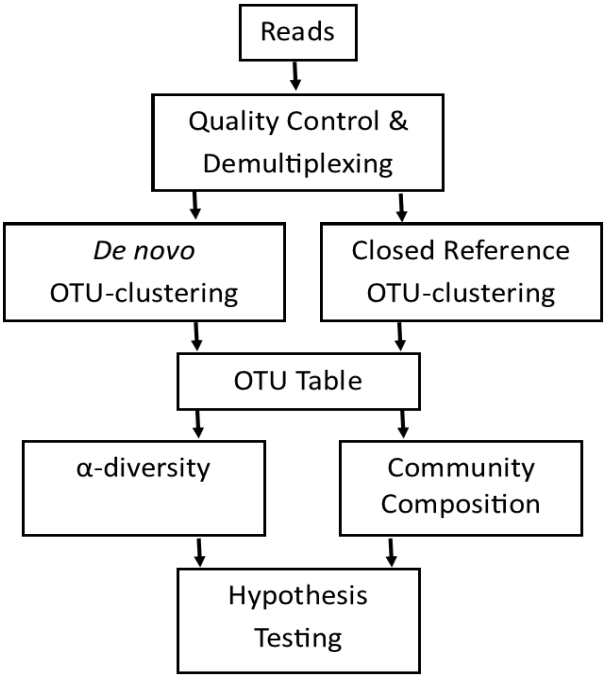

Figure 1.8: Schematic showing a 'typical' workflow for MGA.

1.4.3 Metagenomic Workflows

Rather than using PCR amplification of a gene of interest, metagenomic sequencing involves sequencing a random subset of DNA from the environment. Metagenomic sequencing workflows involve randomly fragmenting all DNA in a sample, before attaching sequencing adaptors, carrying out normalisation steps and sequencing the libraries which result. Metagenomics has a number of advantages over MGA: it enables functional and taxonomic

communities to be examined simultaneously, as well as avoiding the primer bias which is common to all MGA studies (Klindworth *et al.* 2013). Directly analysing functional genes enables insights into the functional potential of the microbial community that taxonomy alone would not. For example, Fierer *et al*. (2012) found that nitrogen concentration affected both taxonomic and functional composition of the bacterial community. Nitrogen addition led to an increase in Proteobacteria, Bacteriodetes and Actinobacteria and a decline in Acidobacteria. At the same time, there was an increase in genes for DNA/RNA replication, electron transport and protein metabolism alongside a decrease in genes involved with urea decomposition. Both analyses allow us to infer that the microbial community shifts from one dominated by oligotrophs to one dominated by copiotrophs as nitrogen is added. However, the functional approach allows us to do so directly, based on the presence of genes involved in growth and respiration. Nitrogen addition also led to a reduction in genes involved in urea decomposition, suggesting a reduction in organic nitrogen usage that would not have been detected by taxonomic analysis. Conversely, metagenomic sequencing is not without disadvantages. A large volume of sequence data is needed for adequate coverage of complex communities (Ni *et al.* 2013), which increases the cost of both sequencing and data analysis. In addition, the majority of metagenomic analyses rely on annotation using existing datasets: for example, Lin *et al*. (2014a) were only able to assign 33% of reads to known proteins, and accuracy of assignment progressively decreases with increasing taxonomic resolution and decreasing read length (Garcia-Etxebarria *et al.* 2014).

An example metagenomic workflow is shown in Figure 1.9. As with MGA, workflows exist to carry out all steps of analysis in a single platform, with the MG-RAST webserver being by far the most commonly used (Glass *et al.* 2010). The first steps in metagenomics workflows are quality control and demultiplexing. Basic quality control can be carried out using relatively simple tools (Zhou and Rokas 2014) to demultiplex samples based on 'barcode' sequences, trim adapters and primers from the ends of reads, and to remove unreliable reads or bases based on quality scores generated by the sequencing instrument during the run. Once these steps have been carried out, software such as FastQC (Zhou and Rokas 2014) may be used to visualise the data in order to detect common problems such as untrimmed barcode sequences or quality score deterioration at the 3' ends of reads.

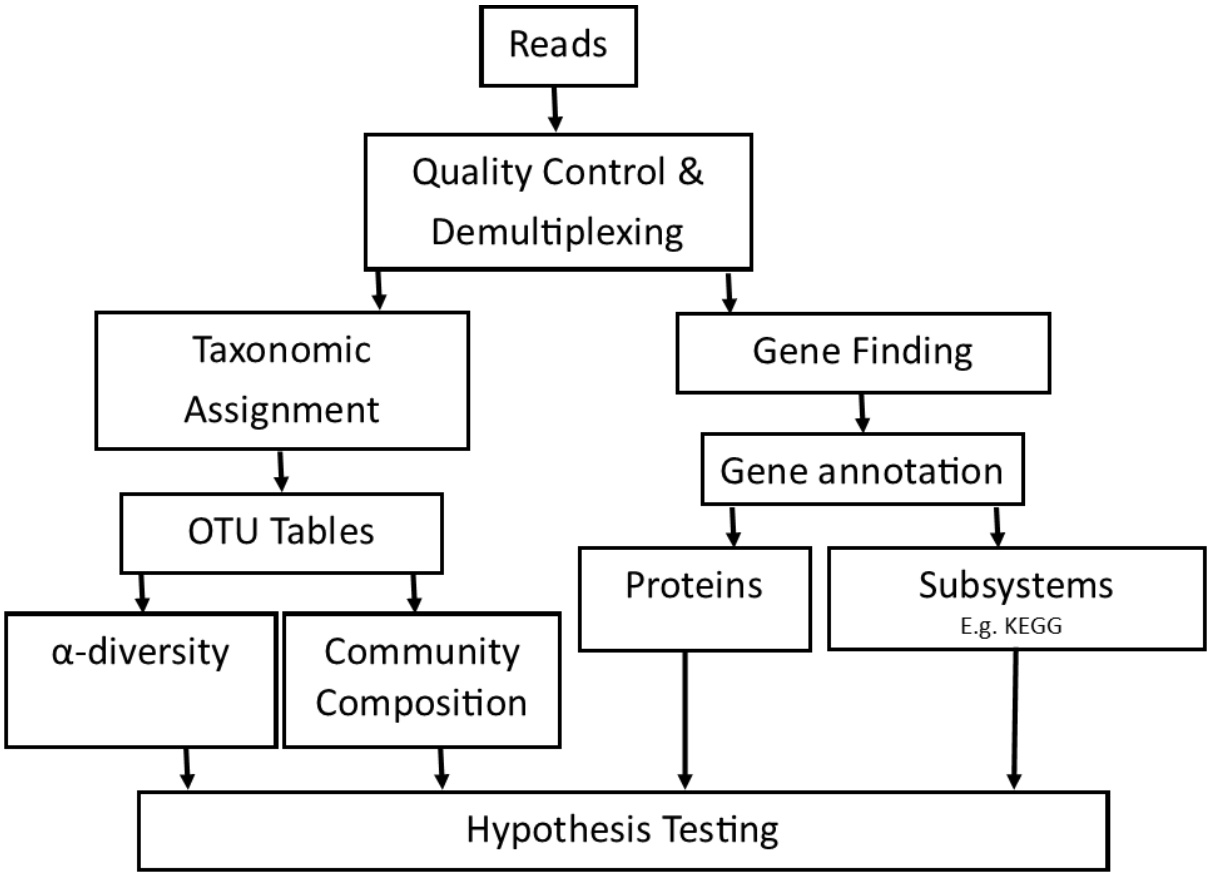

Figure 1.9: Schematic to illustrate steps in a 'typical' metagenomic workflow.

Once suitable quality control has been carried out, analysis proceeds with the goal of inferring both the taxonomic composition and the functional potential of the community in question. Taxonomic assignments can be carried out in several ways. Firstly, marker genes (such as SSU

rRNA) may be extracted from the dataset and annotated, for example using PhylOTU (Sharpton *et al.* 2011). The main advantage of extracting SSU rRNA for analysis lies in the large number of annotated sequences available in supported repositories such as the Ribosomal Database Project (RDP; Maidak *et al.* 1997) or SILVA (Pruesse *et al.* 2007), but analysing marker genes alone ignores a large amount of available data and is subject to problems with SSU sequence analysis, such as copy-number heterogeneity (Větrovský and Baldrian 2013). An alternative approach is to attempt to annotate all sequences, but this approach is computationally expensive, limited by incomplete databases, and may create bias towards well-studied organisms with sequenced genomes Thirdly, composition-based approaches such as PhyloPythia (McHardy *et al.* 2007) or Phymm (Brady and Salzberg 2009) assign taxonomy based on oligonucleotide composition, allowing classification of novel organisms. Recently, a fourth approach has been introduced: inference of the community composition based on a set of 'elite' marker genes, as is carried out in Phylosift (Darling *et al.* 2014). Fairly similar taxonomic profiles are usually obtained from metagenomic data whether 16S rRNA genes or all sequences are used for taxonomic assignment (Delmont *et al.* 2012; Fierer *et al.* 2012), while composition-based methods may provide improved classification of novel organisms but are strongly affected by read length (Brady and Salzberg 2009).

Functional analysis usually begins with a gene finding step, to identify the sequences in the dataset which are most likely to code for proteins and therefore reduce the computational power required for later steps. A number of gene-finding algorithms have been developed specifically for metagenomic analysis, including FragGeneScan (Rho *et al.* 2010), MCG (El Allali and Rose 2013) and Orphelia (Hoff *et al.* 2009). Each of these methods uses several sequence characteristics- e.g. codon bias, stop/start codons, GC content- to calculate a probability that a given read belongs to a protein coding region. Gene finding yields a reduced subset of reads, which are then annotated. In MG-RAST this is carried out using the BLAT algorithm (Wilke *et al.* 2013) but other approaches may be used, such as HMMER (Eddy 2011) which models conserved amino acids in order to find domains belonging to protein families of interest.

The final step is hypothesis testing and presentation of the data. While early metagenomics studies were mostly exploratory and focused on differences between highly distinct environments such as whale carcasses and the open ocean (Tringe *et al.* 2005) or very different biomes (Fierer and Jackson 2006), more recent studies are beginning to use metagenomics to study more subtle differences such as the effects of warming (Luo *et al.* 2014) or fire (Tas *et*

al. 2014). With the continuing increase in sequencing throughput, metagenomic studies are increasingly required to adopt replicated designs, enabling accurate conclusions to be drawn (Knight *et al.* 2012). The demand for proper statistical tests in metagenomics has resulted in the development of a number of statistical programs designed to implement them. For example, STAMP (Parks et al 2014) is a stand-alone program which carries out statistical tests on differences between two or more groups of samples. A number of R packages have also been developed with the specific aim of enabling analysis of shotgun metagenomic data: examples include phyloseq (McMurdie and Holmes 2013), ShotgunFunctionalizeR (Kristiansson *et al.* 2009) or MetaPath (Liu and Pop 2010). Less-specialised R packages may also be used: for example, VEGAN (Dixon and Palmer 2003) for community analysis or DESeq2 (Love *et al.* 2014) to test for differential abundances of proteins or taxa.

In addition to the workflow described in Figure 1.9, metagenomic reads may be assembled into contigs or even draft genomes. A number of assembly algorithms have been developed to overcome challenges specific to metagenomic assembly, such as uneven coverages (caused by the differential abundance of different organisms) and distinguishing sequence errors from interspecies differences. Examples of metagenome assemblers include Ray Meta (Boisvert *et al.* 2012), MetaVelvet (Namiki *et al.* 2012) and Meta-IDBA (Peng *et al.* 2011). Although annotation of contigs rather than raw reads improves annotation accuracy (Magasin and Gerloff 2014), the majority of taxonomic and functional metagenomic analyses use raw reads even where contigs are assembled as a separate step (Delmont *et al.* 2012) because annotation of contigs makes quantitative data analysis inappropriate. A number of studies have assembled draft genomes from metagenomic analyses, even from high-diversity habitats such as sediment or gut samples (Sharon and Banfield 2013; Sangwan *et al.* 2016). Genome assembly may be assisted by digital normalisation to reduce data volume to a manageable level (Howe *et al.* 2012) or by binning contigs based on composition and/or coverage (Albertsen *et al.* 2013; Alneberg *et al.* 2014).

1.5 Summary

Northern peatlands contain a large store of organic carbon which is threatened by climate change. In particular, droughts may become more frequent and intense: periods of drought result in a loss of carbon in the form of CO_2, with the effect continuing for some time after the drought finishes. This is believed to be caused by the release of hydrolase enzymes from

inhibition by phenolic compounds, mediated by phenol oxidases (which are activated by aeration of the peat when the water table falls).

Although microbial communities are critically important in decomposition and CO_2 release, little is known of their response to drought in peatlands. The abundances of methyl coenzyme-M reductase, nitrite reductase and certain phenol oxidases have been studied during drought, but the response of other phenol oxidase genes remains unknown and we know next to nothing about other ways in which the functional and taxonomic composition of the microbial community in Northern peatlands changes during drought. In addition, without a better understanding of which genes are involved in driving decomposition in peat it is difficult to apply qPCR or genetic fingerprinting approaches to determine the effect of drought on the most important genes in degradation of organic matter in peat.

Metagenomics and MGA have the potential to answer these questions. The field of metagenomics has advanced hugely with the invention of next-generation sequencing and the development of user-friendly computational resources for metagenomic analysis. Therefore, a metagenomic study combining functional and taxonomic analyses would advance our understanding of the mechanisms of carbon release from peatlands subjected to drought.

1.6 Thesis Scope and Objectives

The overarching aim of the current PhD thesis is to use second-generation sequencing methodologies to identify whether changes in peatland microbial communities underlie changes to greenhouse gases fluxes which occur in peatlands during drought. This aim was built around the following hypotheses:

i) That the taxonomic composition of microbial communities, both prokaryotic and eukaryotic, changes during drought as a response to changing hydrological and chemical conditions

ii) That changes in the taxonomic composition of microbial communities are associated with changes in community functional potential

iii) That the changes described in (i) and (ii) are linked to the changes in carbon fluxes which have been previously observed during drought in peatlands

Additional aims were to advance current understanding of the composition of peat microbial communities, and to develop and apply an ecologically relevant approach to delimitation of OTUs in marker gene datasets.

To achieve the stated aims, the following techniques were applied:

i) A simulated drought was carried out using peat 'mesocosm cores' from two peatland habitats, half of which were subjected to drought and the remainder maintained at constant water table to serve as controls

ii) Biogeochemical assays were coupled with ARISA fingerprinting in order to identify the timing of changes to microbial communities (Chapter 2)

iii) Marker gene assessment of 18S and 16S SSU markers was used to identify changes in the taxonomic composition of microbial communities during drought (Chapter 3)

iv) Shotgun metagenomic sequencing of a subset of samples, taken from a single habitat and depth, to identify changes in both taxonomic and functional composition of microbial communities during drought and to characterise important functional genes present in this environment (Chapter 4)

v) *De novo* assembly of dominant members of the bacterial community from metagenomic shotgun sequencing (Chapter 5)

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Chapter 2

The effects of simulated drought and rewetting on the biogeochemistry and bacterial and fungal communities within bog and fen mesocosm cores

2.1 Introduction

Peatlands represent a major store of sequestered terrestrial carbon, meaning that the potential impact of climate change on peat cannot be taken lightly (Limpens *et al.* 2008). Anthropogenic climate change will likely lead to an increase in the severity and frequency of drought (Bates 2008), which causes a large release of carbon dioxide from peatlands (Fenner and Freeman 2011). Drought also affects the fluxes of nitrous oxide and methane, two greenhouse gases which are even more potent than carbon dioxide. Nitrous oxide flux commonly increases during drought (Freeman *et al.* 1993; Aerts and de Caluwe 1997; Dowrick *et al.* 1999), while methane flux decreases (Moore and Dalva 1993; Ellis *et al.* 2009) under drought conditions.

Changes in gas fluxes are a result of numerous changes to biogeochemical processes in peat soils during drought and rewetting. Redox potential rises following water table drawdown (Vepraskas and Faulkner 2001) as oxygen penetrates more deeply into the peat, and the concentrations of ferric iron and of nitrate and sulfate ions rises as reduced forms of iron, nitrogen and sulfur are oxidised (Clark *et al.* 2005; Knorr *et al.* 2009; Brouns *et al.* 2014). Changes in nitrate and sulfate availability have a direct effect on gas fluxes: methanogenesis is supressed by the availability of alternative electron acceptors such as sulfate (Kang and Freeman 2002) while increased nitrate availability allows denitrification to proceed, releasing N_2O as a by-product (Keller and Bridgham 2007). In addition, increased concentrations of sulfate and dissolved CO_2 cause a fall in the pH of porewater (Ponnamperuma 1972). Increased sulfate concentrations may also be a factor in the decrease in DOC concentrations which is observed under drought conditions, as decreases in pH decrease DOC solubility (Clark *et al.* 2012; Evans *et al.* 2012). Each of these chemical variables may exert a strong influence on microbial communities. In particular, redox potential has a strong impact on the ratio of fungi to bacteria (Seo and DeLaune 2010), while pH has a strong impact on bacterial growth (Fernández-Calviño and Bååth 2010) and community composition (Rousk *et al.* 2009; 2010).

In many cases, drought-driven carbon dioxide release is likely caused by a decrease in the concentration of phenolic compounds caused by increased phenol oxidase activity under oxygenated conditions (Freeman *et al.* 2001). Phenolic compounds inhibit the activities of extracellular enzymes (Freeman *et al.* 2001) and are toxic to microbial life (Freeman *et al.* 1990; Mellegård *et al.* 2009). The degradation of phenolic compounds allows a rise in the activity of many enzymes, including extracellular hydrolases which contribute to the

degradation of polymeric organic matter (Fenner and Freeman 2011). In particular, hydrolysis of polymeric carbohydrates such as cellulose may contribute to carbon cycling in plant litter and soils. Cellulose is one of the most abundant components of plant matter, making up 15-60% of total mass (Paul and Clark 1989), and consists of long chains of glucose molecules linked by β(1→4) linkages. A simplified model of the degradation of cellulose involves three types of enzyme: endoglucanases, which break cellulose down into shorter chains; exoglucanases, which break these short fragments into cellobiose (a disaccharide) or small oligosaccharides; and finally, β-glucosidases, which break cellobiose into glucose (Singhania *et al.* 2013). As the terminal step in cellulose degradation, therefore, β-glucosidase plays an important role in degradation of organic carbon in plant matter. β-glucosidase activity increases during drought in response to a reduction in phenolic compounds (Freeman *et al.* 2001; Fenner and Freeman 2011), meaning that this enzyme may well play a role in drought-driven carbon loss from peatlands.

A number of studies have found that microbial communities differ between depths and habitats (Lin *et al.* 2012; Serkebaeva *et al.* 2013; Lin *et al.* 2014b). Community differences are related to differences in soil and porewater chemistry, in particular pH, concentration of carbon and nitrogen, and carbon quality (Lin *et al.* 2012). However, there is currently little to no understanding of the microbial mechanisms underlying changing carbon fluxes in peatlands during drought. Fungi and bacteria are responsible for the majority of microbial decomposition in soils and plant litters. Of the two, fungi are considered the most important producers of enzymes under aerobic conditions (Romani *et al.* 2006; Schneider *et al.* 2012) and are especially important producers of phenol oxidase enzymes (Romani *et al.* 2006). However, bacteria dominate carbon mineralisation in peatlands (Winsborough and Basiliko 2010) and play important roles in the degradation of phenolic compounds under anaerobic conditions (Philipp and Schink 2012).

Prior to the large-scale application of second-generation sequencing methodologies to the study of microbial communities, a number of DNA-based 'community fingerprinting' approaches were developed, including T-RFLP, DGGE and ARISA fingerprinting. Each of these approaches can be used to pinpoint shifts in the richness and composition of the microbial community, and in some cases may even be used to infer which species are present (Shyu *et al.* 2007; Slemmons *et al.* 2013). ARISA fingerprinting is able to distinguish the microbial

communities of different habitats (Ranjard *et al.* 2001; Danovaro *et al.* 2006), and is often more sensitive than other microbial fingerprinting techniques (Danovaro *et al.* 2006). Richness estimates obtained using ARISA fingerprinting are strongly correlated to the 'true' richness of a community (Kovacs *et al.* 2010). Therefore, despite the advent of next-generation sequencing, ARISA fingerprinting remains a quick and affordable method to identify where and when changes occur in microbial communities, allowing targeted application of next-generation sequencing.

Aims and Objectives of Chapter

The overarching aim of this chapter was to test the hypothesis that microbial communities in peat change during periods of drought and rewetting. The objectives of this chapter were as follows:

1. To identify changes in gas fluxes, enzyme activities, water chemistry and the concentration of phenolic compounds during a period of drought
2. To identify whether differences exist between the microbial (bacterial and fungal) communities of bogs and fens at two depths
3. To identify whether peatland microbial communities change during simulated drought, and if so, to identify the temporal and spatial location of these changes
4. To compare the responses of peatland bacterial and fungal communities to drought

2.2 Methods

2.2.1 Sample Site

Ten 'mesocosm cores' were collected from two sites representing typical Welsh bog and fen habitats. The chosen fen habitat was Cors Erddreiniog, a low-lying fen in mid-Anglesey (SH461826). This site is a part of the Anglesey Fens complex, which is designated a Special Area of Conservation and represents a nationally important area of alkaline and calcareous fen (http://jncc.defra.gov.uk/protectedsites/sacselection/sac.asp?EUCode=UK0012884, accessed 25/08/15).

Marchlyn Mawr is a small bog on the edge of Snowdonia National park (grid reference SH610625). This site is less well-characterised than Cors Erddreiniog, but the vegetation

belongs to NVC classification M6 (*Carex echinata – Sphagnum recurvum/auriculatum* mire) (Williamson *et al.* 2010).

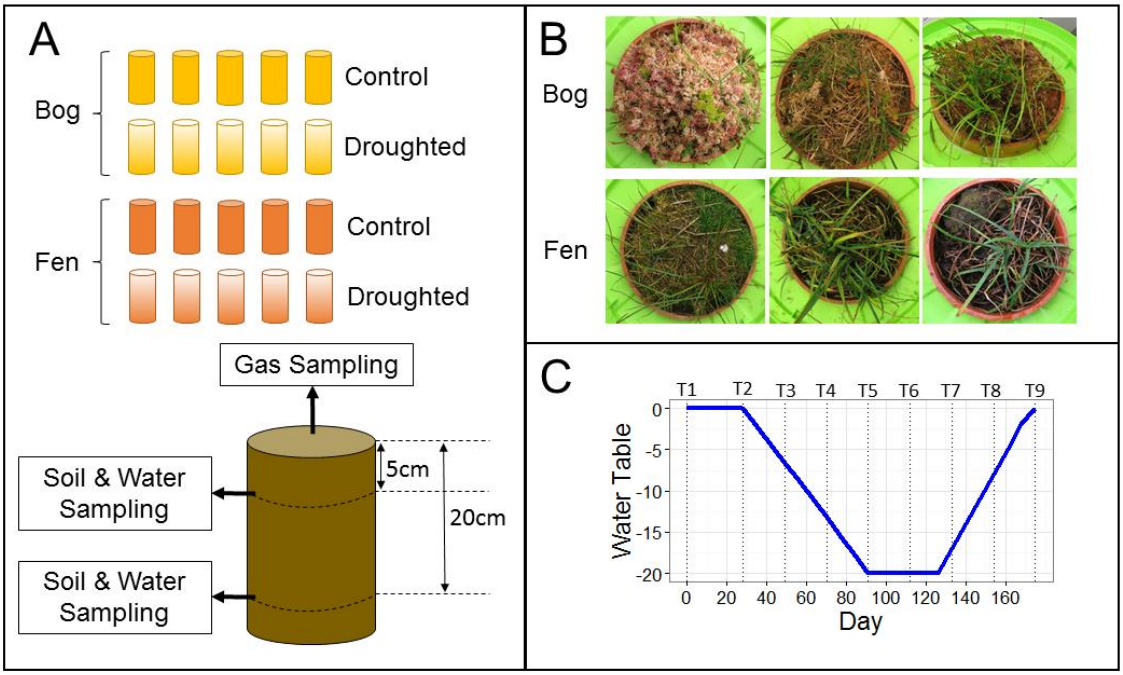

Figure 2.1: Schematic showing aspects of the experimental design. A: Overview of the experiment showing the number of mesocosm cores collected for each treatment and habitat, as well as depths at which sample collection was carried out. B: View of mesocosm cores from above, giving an indication of typical vegetation in bog and fen. C: Position of the water table throughout the experiment, with sampling time points indicated by dotted lines.

2.2.2 'Mesocosm Core' Design and Collection

Peat 'mesocosm cores' were collected in PVC pipes (each 20 cm in diameter and 35 cm in length), following a protocol adapted from Freeman et al. (1993). Peat cores collected in this way have intact peat profiles and plant communities. After collection, mesocosm cores were kept in a controlled temperature room at 8-10°C for the duration of the experiment, and were lit by fluorescent daylight tubes for 16 hours a day (mean PAR: 305.4 µmol m^{-2} sec^{-1}). Cores were placed in bins filled with artificial rainwater for bog cores and artificial groundwater for fen cores, with holes drilled near the base of each core to allow water exchange with the surrounding water. The composition of the rainwater followed a standard recipe described by Fenner (2002) and the groundwater recipe followed the composition of groundwater at Cors

Erddreiniog as closely as was possible (Nina Menichino, pers. comm.). Ion concentrations in both waters are described in Table 2.1. The concentration of calcium (Ca^{2+}) is extremely high in groundwater from Cors Erddreiniog and leads to an apparent charge imbalance, but this is likely due to the fact that bicarbonate was not measured: bicarbonate and calcium are the two dominant ions at Cors Erddreiniog (Farr *et al.* 2014). While the calcium concentrations shown in Table 2.1 are high, they are not exceptional for this habitat (Menichino 2015).

Table 2.1: Final concentration of important ions in artificial rainwater and groundwater, as well as measured concentrations from Cors Erddreiniog (N. Menichino, pers. comm.). Unfortunately, it was not possible to exactly match concentrations from the groundwater using available equipment and salts. Concentrations are given in mg l^{-1}.

Ion	Artificial Groundwater	Artificial Rainwater	Cors E. Groundwater
Na^{2+}	2.4	2.5	-
K^{+}	0.1	0.1	-
Ca^{2+}	0.3	5.2	124.5
Mg^{+}	0.7	2.9	5.5
Cl^{-}	0.7	10.2	15.1
SO_4^{-}	3.1	3.3	3.0
NH_4^{+}	0.1	0.0	0.1
NO_3^{-}	0.0	0.1	0.1

2.2.3 Water Table Manipulation

Within each habitat, five mesocosm cores were assigned to the drought-rewet treatment while the remaining five acted as controls (Figure 2.1A). The water table in the drought cores at each sampling time point is described in Table 2.2 and visualised in Figure 2.1C. The duration and severity of water table drawdown was based on a natural drought which occurred in 2006 in the Cerrig-yr-Wyn catchment in mid-Wales (Nathalie Fenner, pers. comm.). The water level in the control cores was kept constant by topping up with distilled water when necessary. Distilled water, rather than artificial rain or groundwater, was used to maintain the water table in order to avoid increasing ion concentrations as the experiment progressed: water was lost through evaporation, but ions could not leave the system (whereas in a natural system they could be lost by leaching to lower levels). The water level of the drought cores was adjusted twice a week (on Tuesdays and Fridays): during the drought period this involved the removal of water from the bins containing drought cores, and during the rewetting period artificial

rainwater or groundwater was added to bring the water table up to the required level (Table 2.2).

Table 2.2: Depth beneath the surface of the water table in drought cores throughout the experiment. Water table depth was adjusted at twice-weekly intervals by removing water from each bin containing a drought core. 'Soil sample' refers to the label applied to each time point for soil samples, while 'water sample' refers to the label applied to water sample time points.

Day	Date	Water Table (cm)	Soil Sample	Water Sample
0	28/08/2013	0	T1	
7	02/09/2013	0		
14	09/09/2013	0		
21	16/09/2013	0		
28	23/09/2013	0	T2	
35	30/09/2013	-2.2		
42	07/10/2013	-4.4		
49	14/10/2013	-6.6	T3	
56	21/10/2013	-8.8		
63	28/10/2013	-11		
70	04/11/2013	-13.2	T4	
77	11/11/2013	-15.4		W1
84	18/11/2013	-17.6		
91	25/11/2013	-20	T5	
98	02/12/2013	-20		W2
105	09/12/2013	-20		
112	16/12/2013	-20	T6	
119	23/12/2013	-20		
126	30/12/2013	-20		
133	06/01/2014	-17	T7	W3
140	13/01/2014	-14		
147	20/01/2014	-11		
154	27/01/2014	-8	T8	W4
161	03/02/2014	-5		
169	10/02/2014	-2		W5
176	17/02/2014	0	T9	W6

2.2.4 Sample Collection

Mesocosm cores were allowed to acclimatise for one month before sample collection began, after which samples were collected at three week intervals on the dates shown in Table 2.2.

Gas samples were taken by placing a sealed headspace over each core with a rubber septum to allow gas collection. At 0, 15, 30, 60 and 120 minutes, a 20 cm^3 gas sample was removed and injected into an evacuated 12 ml glass vial (Labco Medical Supplies). Gas sampling was carried out between 10 am and 12 noon.

Immediately following gas sampling, samples of peat were collected at two depths in each peat core. Holes were drilled in the pipe at two depths: 5 cm and 20 cm, measured from the peat surface (no mesocosms subsided following sample collection) and a 6 g sample of peat was collected for laboratory analysis using sterile tools. Following removal of the soil sample, a redox probe was inserted into the peat and a measure of redox potential taken using a redox probe with an Ag/AgCl reference electrode in 3 M KCl. To adjust the value obtained to the 'true' value (i.e. that which would have been obtained using a standard hydrogen electrode), a correction factor of +207 was added prior to further analysis (Vepraskas and Faulkner 2001).

Porewater samples were also collected during the second half of the experiment (Table 2.2). Water samples were collected using Rhizon samplers (Rhizosphere Research Products, The Netherlands), which were inserted into the holes from which soil samples had been taken at the first time point. Rhizons were left in place throughout the experiment. Water samples were collected by connecting an evacuated 20 ml syringe and leaving it in place until 20 ml of water was collected. Samples were immediately filtered using a 0.45 µm syringe filter, and stored at 4°C until analysis. Water sample collection was not possible in all cases, especially during drought, and sample size for each combination of treatment, time, depth and habitat is shown in Table 2.3.

2.2.5 Laboratory Analysis of Samples

DNA Extraction

Soil collected from the mesocosm cores was mixed thoroughly using flame-sterilised tools: undecomposed plant matter was cut into small pieces with fine tip dissection scissors, and the peat was stirred using a spatula. DNA was extracted from a 0.25 g subsample with a MoBio PowerSoil kit, following manufacturer's instructions. Following preliminary tests, the MoBio PowerSoil kit was found to give more consistent results than alternative methods.

Table 2.3: Sample size for water chemistry measurements. Five replicate cores were collected within each combination of habitat and treatment, but due to difficulties with extraction of water from cores a balanced set of replicates was not taken at every time point.

		Time		Sample size			
Habitat	**Depth**	**Point**	**Treatment**	**pH**	**Phenolics**	**Nitrate**	**Sulfate**
Bog	5cm	W1	Control	5	5	2	0
Bog	5cm	W1	Drought	3	3	0	0
Bog	5cm	W2	Control	5	5	5	5
Bog	5cm	W2	Drought	0	0	0	0
Bog	5cm	W3	Control	5	5	5	5
Bog	5cm	W3	Drought	0	0	3	3
Bog	5cm	W4	Control	5	5	4	4
Bog	5cm	W4	Drought	1	3	4	4
Bog	5cm	W5	Control	4	4	4	4
Bog	5cm	W5	Drought	2	2	2	2
Bog	5cm	W6	Control	4	4	4	4
Bog	5cm	W6	Drought	3	4	4	4
Bog	20cm	W1	Control	5	5	3	0
Bog	20cm	W1	Drought	5	5	2	0
Bog	20cm	W2	Control	5	5	5	5
Bog	20cm	W2	Drought	5	5	5	5
Bog	20cm	W3	Control	5	5	5	5
Bog	20cm	W3	Drought	4	4	5	5
Bog	20cm	W4	Control	5	5	5	5
Bog	20cm	W4	Drought	5	5	3	3
Bog	20cm	W5	Control	5	5	5	5
Bog	20cm	W5	Drought	5	5	5	5
Bog	20cm	W6	Control	5	5	5	5
Bog	20cm	W6	Drought	5	5	4	5
Fen	5cm	W1	Control	5	5	2	4
Fen	5cm	W1	Drought	5	4	5	3
Fen	5cm	W2	Control	5	5	5	5
Fen	5cm	W2	Drought	4	4	4	4
Fen	5cm	W3	Control	5	5	5	5
Fen	5cm	W3	Drought	3	3	5	5
Fen	5cm	W4	Control	5	5	5	5
Fen	5cm	W4	Drought	5	5	5	5
Fen	5cm	W5	Control	4	4	4	4
Fen	5cm	W5	Drought	5	5	5	5
Fen	5cm	W6	Control	5	5	5	5
Fen	5cm	W6	Drought	5	5	5	5
Fen	20cm	W1	Control	5	5	3	3
Fen	20cm	W1	Drought	5	5	5	2
Fen	20cm	W2	Control	5	5	5	5
Fen	20cm	W2	Drought	5	5	5	5
Fen	20cm	W3	Control	5	5	5	5
Fen	20cm	W3	Drought	5	5	5	5
Fen	20cm	W4	Control	5	5	5	5
Fen	20cm	W4	Drought	5	5	5	5
Fen	20cm	W5	Control	5	5	5	5
Fen	20cm	W5	Drought	5	5	5	5
Fen	20cm	W6	Control	5	5	5	5
Fen	20cm	W6	Drought	4	4	4	4

DNA was eluted with 100 µl sterile Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 7.6) and stored at -80°C prior to further analysis. Samples were further purified using a MoBio PowerClean kit following manufacturer's instructions, as this was found to give more consistent PCR amplification during the ARISA fingerprinting step.

Water Content

Dry weight and water content were obtained by weighing approximately 1 g of peat into a crucible, heating to 108°C for 48 hours, and weighing the mass which remained. No extra mass loss was observed by increasing the duration of heating.

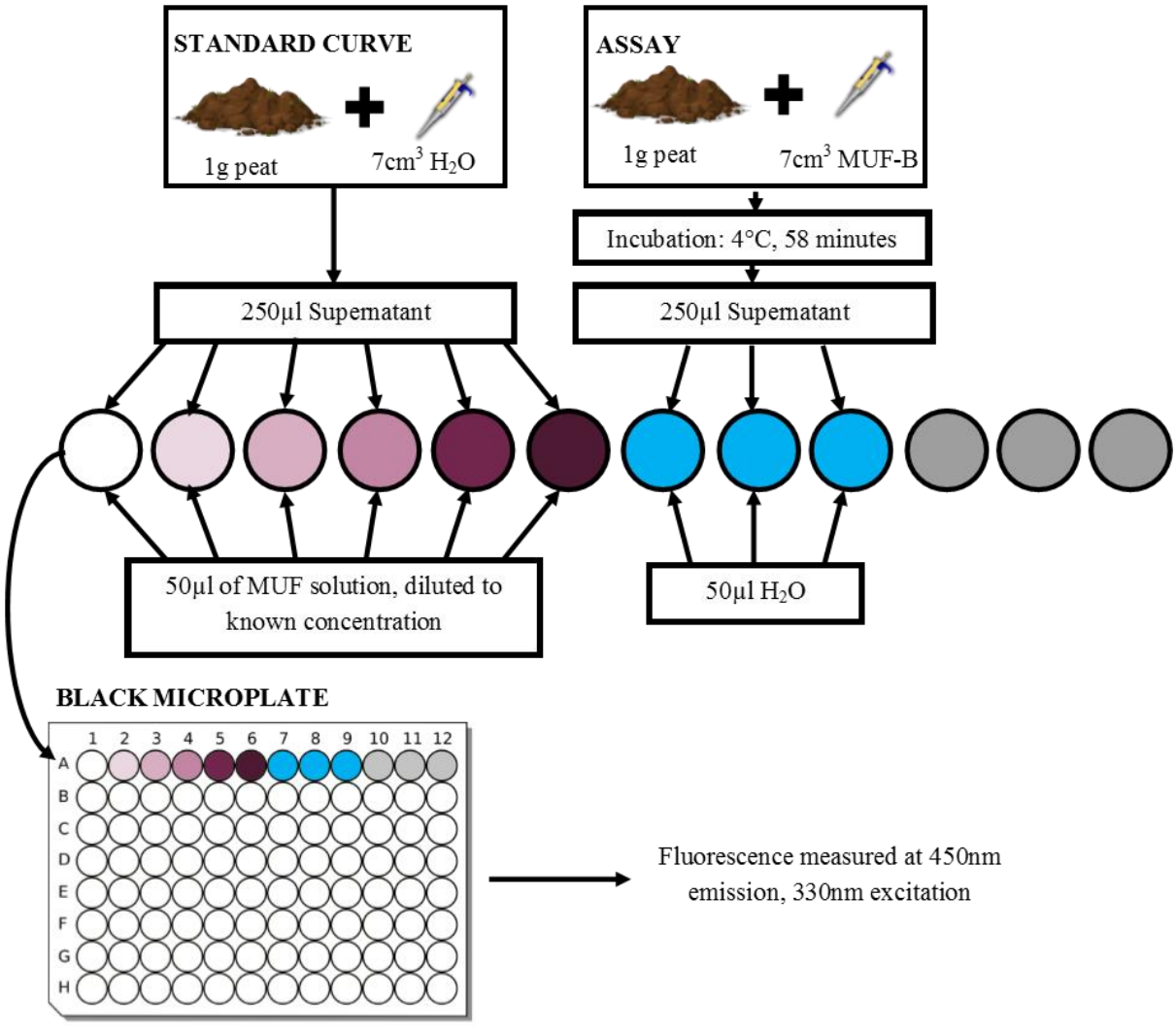

Figure 2.2: Workflow for β-glucosidase assay, using 4-methylumbelliferone-β-D-glucopyranoside (MUF-B) as the substrate. In the presence of β-glucosidase, MUF-B is converted to MUF-free acid, which fluoresces at 450nm excitation, 330nm excitation.

Gas Fluxes

Gas samples were analysed on a Varian 450-GC, fitted with a flame ionisation detector (FID) and methaniser to measure CO_2 and CH_4, and an electron capture device (ECD) for nitrous oxide. At each time point, the machine was calibrated using three gas mixtures of known concentration obtained from Scientific and Technical Gases Ltd (Newcastle under Lyme, Staffordshire, UK). For each gas a regression line was calculated between time and gas concentration, and the slope of the regression was taken as the average flux value. Global warming potential (GWP) was calculated by multiplying each of the three gases by its 100-year global warming potential: 1 for CO_2, 23 for CH_4 and 296 for N_2O (IPCC 2001).

Determination of Enzyme Activities and Concentration of Phenolic Compounds

Phenol oxidase activities were measured using L-3,4-dihydroxyphenylalanine (L-DOPA). In the presence of phenol oxidase, L-DOPA is oxidised to 3-dihydroindole-5,6-quinone-2-carboxylate (dicq), which has a pinkish colour (Pind *et al.* 1994). Briefly, 1 cm^3 of peat was thoroughly mixed with 9 cm^3 of water in a stomacher (Seward). Six 750 µl subsamples of the resulting slurry were removed into Eppendorf tubes. 750 µl of 10 mM L-DOPA was added to half of these tubes to measure phenol oxidase activity, while 750 µl of ultrapure water was added to the remaining three tubes to measure baseline absorbance. All tubes were incubated at 4°C for 9 minutes, then centrifuged at 20,000g for 5 minutes to terminate the reaction. 300 µl of the supernatant from each tube was placed into a clear 96-well microplate and absorbance was measured at 460 nm. Absorbance was used to calculate phenol oxidase activity using Beer's Law and a molar absorption coefficient of 3700 for dicq (Pind *et al.* 1994; modified by Dunn *et al.* 2014).

β-glucosidase activity was measured following Dunn et al. (2014), as summarised in Figure 2.2. This method is modified from Freeman *et al.* (1995) and uses 4-methylumbelliferone-β-D-glucopyranoside (MUF-B) as a substrate, which upon hydrolysis releases 4-methylumbelliferone (4-MUF).

1. Prior to beginning the assay, fresh solutions of MUF-B and 4-MUF were made. MUF-B was brought to the incubation temperature, while dilutions of 4-MUF were

made for the calibration curve (to give final concentrations of 0, 5, 10, 15, 20 and 30 µM).

2. For the assay, 7 cm^3 of 400 µm MUF-B was added to the first peat sample, homogenised, and incubated at 4 °C for 58 minutes. At the same time peat slurry for the calibration curve was prepared by adding 7 cm^3 of water to the second peat subsample, then homogenising and incubating the resulting slurry alongside the assay sample.
3. While the samples were incubated, a black microplate was prepared: 50 µl of the relevant 4-MUF dilution was added to wells in the first six columns, while 50 µl of distilled water was added to three wells to give an equivalent dilution to the wells used to calculate the calibration curves.
4. At the end of incubation, the supernatant was extracted by transferring the slurry to a 2 ml centrifuge tube and centrifuging at 20,000g for 5 minutes. 250 µl of supernatant was added to the relevant microplate wells: adding the peat-MUF-B mix to the assay wells, and the water-peat mix to the calibration curve wells. Absorbance was measured for all wells at 450 nm emission and 330 nm excitation, and the concentration of MUF in the assay wells was calculated using the calibration curve.

The concentration of phenolic substances was measured using Folin-Ciocalteu reagent (Box 1983). Briefly, a 1 cm^3 subsample of peat was taken using a cut-off syringe and weighed, before water-soluble phenolics were extracted by homogenising the peat subsample with 9 ml of water before centrifuging the resulting slurry. 250 µl of supernatant was added to three wells of a clear microplate and baseline absorbance measured prior to addition of 12.5 µl Folin-Ciocalteu reagent and 37.5 µl filtered sodium carbonate solution (200 mg l^{-1}). Samples were mixed, incubated at room temperature for 90 minutes, and absorbance measured at 750 nm. A calibration curve was produced using dilutions of phenol solution in the range of 0-10 mg l^{-1}.

To measure the pH of peat itself, a pH probe was placed in peat slurry made by mixing 1 cm^3 of peat with 9 ml of MilliQ water (Toberman *et al.* 2010). Prior to each use, the pH probe was calibrated using commercial calibration solutions with pH values of 4 and 7 (Sigma Aldrich).

Water Chemistry

The pH of each porewater sample was taken by inserting a pH probe into the sample. Nitrate and sulfate were measured using a Metrohm 850 Ion Chromatograph, with a Metrosep A Supp 5 150 mm anion column and an eluent of 3.2 mM/1.0 mM sodium carbonate/bicarbonate. Concentrations of dissolved phenolic compounds were measured using an identical procedure to that used for soil samples, but substituting porewater for peat slurry.

ARISA Fingerprinting

Automated ribosomal intergenic spacer analysis (ARISA) involves amplifying the intergenic spacer region of microbial ribosomal DNA and analysing the length of the obtained amplicons. The length of the intergenic spacer region is very variable, and amplicons of different sizes are therefore assumed to be separate species (although this is not always the case (Kovacs *et al.* 2010)). A subset of three mesocosm cores for each combination of treatment and time was selected for ARISA fingerprinting. For each chosen core ARISA was carried out for both depths and all nine time points, giving a total of 216 samples for this part of the analysis.

Primers chosen for ARISA of bacterial communities were ITSF (5'-GTCGTAACAAGGTAGCCGTA-3') and ITSReub (5'-GCCAAGGCATCCACC-3'), which have been shown to outperform other commonly used ARISA primers (Cardinale *et al.* 2004). As there was no existing comparison of primer pairs for ARISA fingerprinting of fungal communities, selected primers were tested using Primer Prospector software. Forward primers used in the comparison were 2234C, ITS1, ITS1WH and ITS5, and reverse primers were ITS4 and 3126T (White *et al.* 1990; Ranjard *et al.* 2001). First, primers were compared in all possible combinations, and screened based on the number of matches to sequences in the UNITE database (Abarenkov *et al.* 2010). Reverse primer 3126T was ruled out based on a low number of matches to the database (data not shown). Secondly, the taxonomic distribution of matches and the length distribution of fragments were examined. Matches were distributed across all the major phyla of fungi for all three primer pairs (data not shown). However, the spread of fragment sizes was larger and more even for ITS1WH-ITS4 meaning that this primer pair would distinguish species more clearly (Figure 2.3).

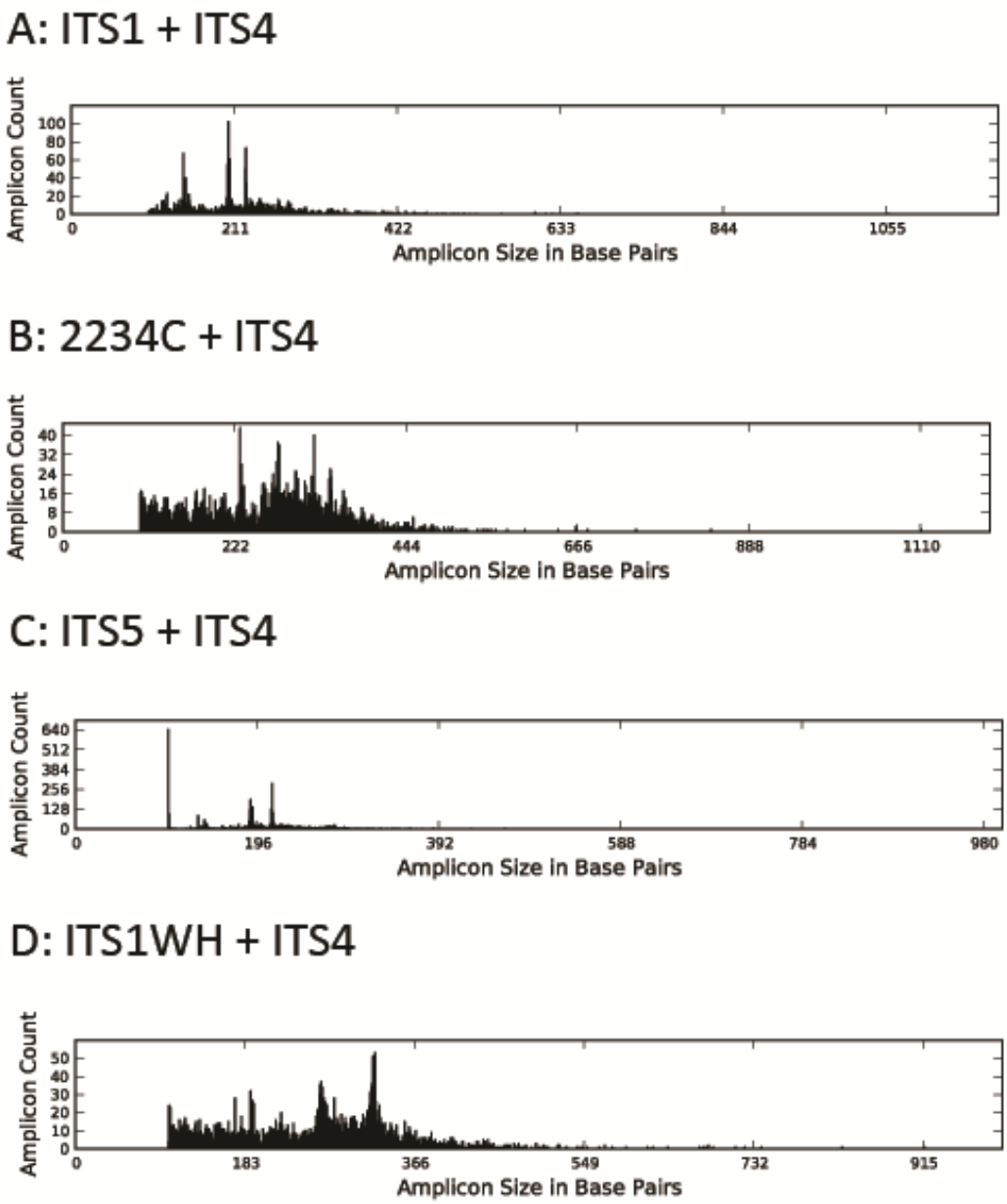

Figure 2.3: Distribution of amplicon sizes generated by four primer pairs tested in PrimerProspector.

Each PCR reaction for ARISA contained 9.45 µl of nuclease -free water, 12.5 µl of PCR Master Mix (Promega), 1 µl of each primer (10 µM), 0.05 µl of molecular grade bovine serum albumin (1mg/ml, Thermo Scientific) and 1 µl of template DNA (diluted to 10 ng/µl) to give a final volume of 25 µl. For ARISA of bacterial communities, the mixture was held at 95°C for 2 minutes for initial denaturation, followed by thirty cycles of 95 °C for one minute

(denaturation), 52°C for 45 seconds (annealing), 72 °C for 1.5 minutes (extension), and a final extension period of five minutes. An annealing temperature of 54.2°C was used for the fungal ARISA primers, with all other steps in the PCR program identical to that for ARISA of bacterial communities.

PCR amplicon lengths were measured on a Qiaxcel Advanced (Qiagen), using a Qiaxcel High Resolution kit and method OM1200 (recommended by the manufacturer for amplicon lengths between 0.5 and 1.5 kbp). Thresholds for peak calling were adjusted to a baseline window of 240 s and a peak identification threshold of 6 % for ARISA of bacterial communities. For ARISA of fungal communities, a baseline window of 40s (the default) was used with a threshold of 6%.

2.2.6 Data Analysis

All biogeochemical variables (enzyme activities, phenol concentration, gas fluxes, pH and redox) were analysed using linear mixed effects models. Linear mixed-effects models are gaining popularity in ecological analyses (e.g. Langenheder *et al.* 2010; Dossena *et al.* 2012; Evans *et al.* 2012), and were required in this case to allow for the effect of core (since multiple samples were taken from each mesocosm core, analyses would otherwise have been compounded by temporal pseudoreplication). Model selection was based on the recommendations of Zuur *et al.* (2009), and was carried out using the 'nlme' package in R (Pinheiro *et al.* 2013).

Firstly, a maximal model was constructed using depth, habitat, treatment, date and all interactions. The random effects were chosen by using restricted maximum likelihood (REML) to fit the maximal model both with no random component and with core as a random component, and comparing between the two using likelihood ratios. The random intercept model was chosen if the p-value obtained by a likelihood ratio test was <0.05 (i.e. indicating that the random effect of 'core' had a significant effect on the model). The residuals from this step were plotted against each factor (including random variables), and where patterns existed a new variance structure was selected. This was done by fitting a new model with a fixed variance structure (i.e. allowing for different variances for each level of the factor causing the pattern). Again, the best model was chosen using a likelihood ratio test. For some variables (dissolved phenolics, nitrate, DOC) a fixed variance structure was insufficient to correct

variance heterogeneity and a log transformation was applied. Finally, the optimal fixed effects structure was chosen using backwards selection. Briefly, the maximal model with the optimal random effects structure was fitted again using maximal likelihood (ML). Interactions were dropped one at a time, and significance of each term was tested by comparison with the previous model using maximum likelihood (terms were removed if the p-value obtained was >0.05). Once all non-significant terms had been removed, the model was refitted using REML and validated by inspection of residual plots. Post-hoc tests were carried out using the package 'lsmeans' (Lenth 2016), in order to determine at which time points a significant treatment-control difference was found.

Data from the ARISA analysis was analysed using the 'vegan' package in R (Oksanen *et al.* 2015). First, fragment sizes were sorted into 5 bp bins and converted to presence-absence data. Richness of bacterial and fungal communities were analysed using generalized linear models fitted using the 'glm' function in R. Models were fitted using time point, treatment and the interaction between time point and treatment as factors. A separate model was fitted within each combination of habitat and depth. Models were initially fitted using Poisson errors, but in cases where evidence of overdispersion (the presence of greater variability than would be expected within the Poisson distribution) was found, standard errors were corrected using a quasi-GLM method in which the variance was the mean multiplied by a dispersion parameter (in the Poisson distribution the variance is assumed to be equal to the mean). The significance of each interaction term was then analysed by analysis of deviance (function 'drop1.glm') using a Chi-squared test.

Jaccard distances were calculated between samples and used to conduct PERMANOVA tests (function 'adonis') as follows:

1. PERMANOVA tests were carried out on all samples to test for the effects of all factors (time, treatment, habitat and depth).
2. As the effect of habitat and depth was very large, PERMANOVA was carried out separately for each combination of habitat and depth to test for effects of time point, treatment and a time: treatment interaction.
3. Where significant effects of treatment were found, time points were broken into four groups of roughly even size to identify when changes occurred (there were not enough replicates at each time point to analyse these separately)- hereafter, these groups are

referred to as pre-drought (time points T1 and T2), drying (T3, T4 and T5), minimum water table (T6 and T7) and rewetting (T8 and T9). Although the water table was at a minimum at time point T5, the water table had only just reached this level and so time point five was grouped with 'drying'.

PCoA plots were generated based on Jaccard distances using function 'cmdscale' in package 'VEGAN'. Samples in which no fragments were detected were excluded from PERMANOVA analysis and NMDS plots (two samples from the bacterial dataset and one from the fungal dataset).

2.3 Results

2.3.1 Water Content and Redox Potential

Water content was significantly different between the two habitats and depths, and was lowest at 20 cm depth and in the fen habitat. There were significant interactions between time point and treatment and between treatment and depth (Table 2.4), representing a lower water content at 5 cm during drought (Figure 2.4). Water content was significantly different between droughted and control mesocosm cores at 5 cm depth during time points 3 (t_{17}= -3.2, p =0.006), 4 (t_{17}= -2.5, p =0.02), 5 (t_{17}= -2.5, p =0.02), 7 (t_{17}= -3.2, p =0.005) and 8 (t_{17}= -3.4, p =0.003). There was also a significant effect of time point on water content (Figure 2.4; Table 2.4), with significant interactions between time point and habitat and time point and depth. Water content rose in the early part of the experiment in both habitats and at both depths (Figure 2.4).

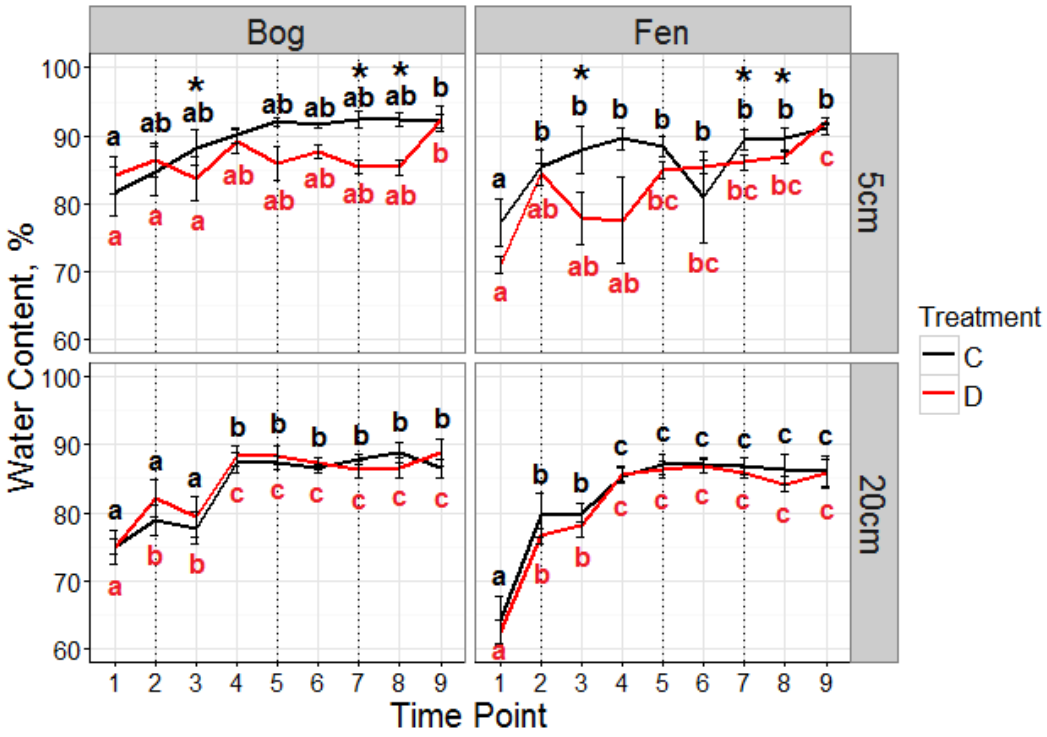

Figure 2.4: Mean water content as a percentage of total wet mass. Significant differences between the two treatments are marked with *, while significant differences between time points are marked with different letters (red = drought, black = control). Error bars show standard errors.

Table 2.4 Results of minimal adequate linear mixed-effect models, with percentage water content (arcsine-transformed) and redox potential as response variables. Degrees of freedom reported are reported in the form: $d.f._{denominator}$, $d.f._{numerator}$.

Variable	Effect	F	d.f.	p
Water Content	Habitat	26.0	1,17	<0.001
	Depth	43.5	1,306	<0.001
	Time Point	4.7	8,306	<0.001
	Treatment	2.8	1,17	0.110
	Time Point: Treatment	2.01	8,306	0.04
	Time Point: Habitat	2.8	8,306	0.006
	Time Point: Depth	4.6	8,306	<0.001
	Treatment: Depth	13.3	1,306	<0.001
Redox	Habitat	179.1	1,17	<0.001
	Treatment	6.4	1,17	0.022
	Time Point	3.7	5,198	0.034
	Time Point: Treatment	5.0	5,198	<0.001
	Depth: Time Point: Treatment	3.1	5,198	0.011

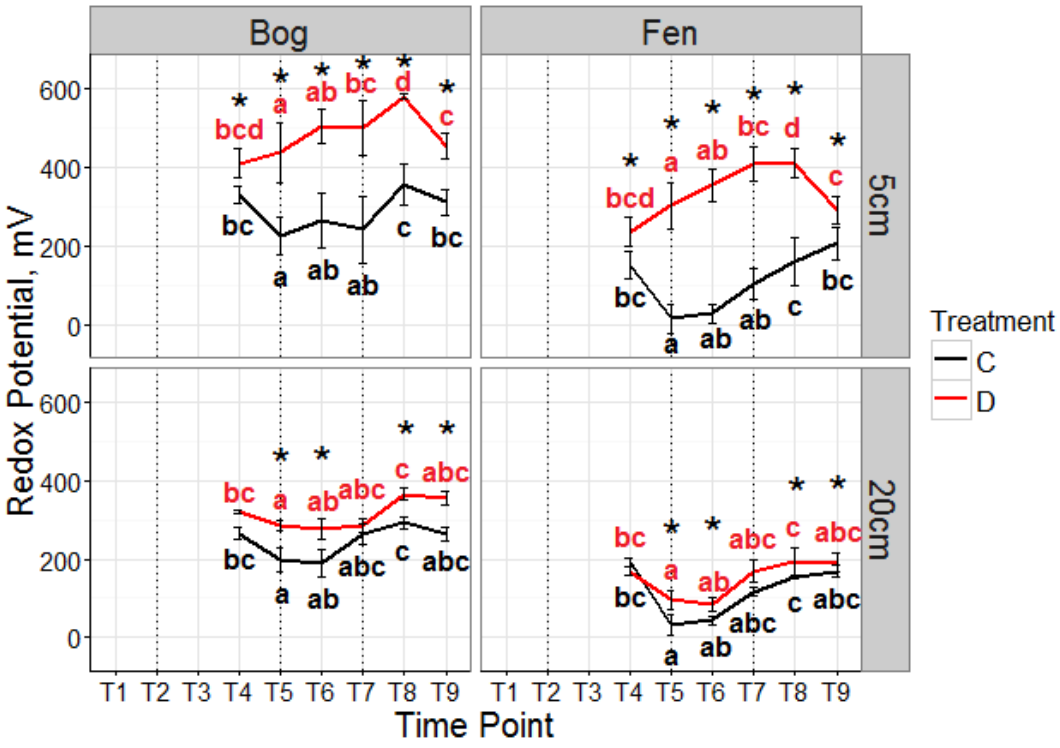

Figure 2.5: Mean redox potential (mV). Due to difficulties with equipment, redox potential was not measured prior to T4. Significant differences between the two treatments are marked with *, while significant differences between time points are marked with different letters (red = drought, black = control). Post-hoc tests were carried out across both habitats, as there were no significant interactions between habitat and other factors. Error bars show standard errors. Dotted lines represent transition between four stages of water table manipulation: pre-drought, drying, minimum water table, and rewetting (in that order).

Redox potential was significantly higher in bog than fen mesocosm cores and in drought cores compared to control cores (Table 2.4). In addition, there was a significant interaction between time point and treatment, as redox potential rose during drought (Figure 2.5). At 5 cm depth, redox potential was significantly higher in drought than control cores at every time point at which redox potential was measured: time points 4 ($z=2.53$, $p=0.01$), 5 ($z=4.52$, $p<0.001$), 6 ($z=6.02$, $p<0.001$), 7 ($z=4.81$, $p<0.0001$), 8 ($z=5.42$, $p<0.0001$) and 9 ($z=3.43$, $p=0.0006$). At 20 cm depth, redox potential was only significantly higher in drought than control cores at time points 5 ($z=3.06$, $p=0.002$), 6 ($z=2.56$, $p=0.01$), 8 ($z=3.10$, $p=0.002$) and 9 ($z=2.59$, $p=0.0095$). There was a significant effect of time point on redox potential in both droughted and control cores: in control cores at both depths and in droughted cores at 20 cm, the redox potential fell between time points 4 and 5, before rising again towards the end of the experiment (Figure 2.5). In the droughted cores at 5 cm, redox potential rose between time points 4 and 8 before falling again at the final time point (Figure 2.5).

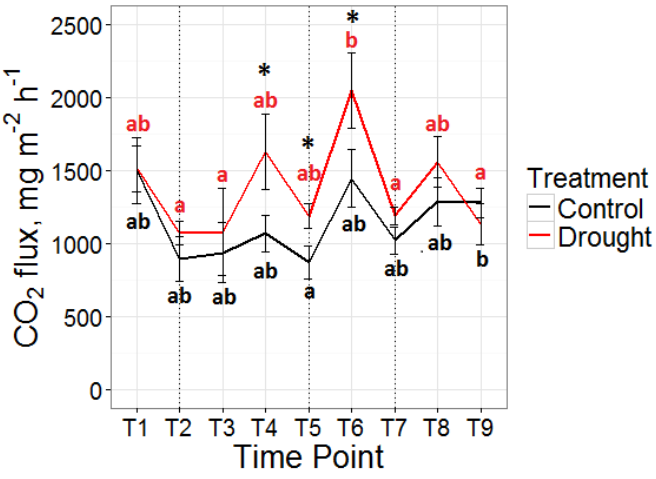

Figure 2.6: Mean CO_2 flux (mg m^{-2} h^{-1}) in droughted and control mesocosm cores. There was no significant effect of habitat on CO_2 flux, so averages were calculated across both habitats. Significant differences between the two treatments are marked with *, while significant differences between time points are marked with different letters (red = drought, black = control). Error bars show standard errors. Dotted lines represent transition between four stages of water table manipulation: pre-drought, drying, minimum water table, and rewetting (in that order).

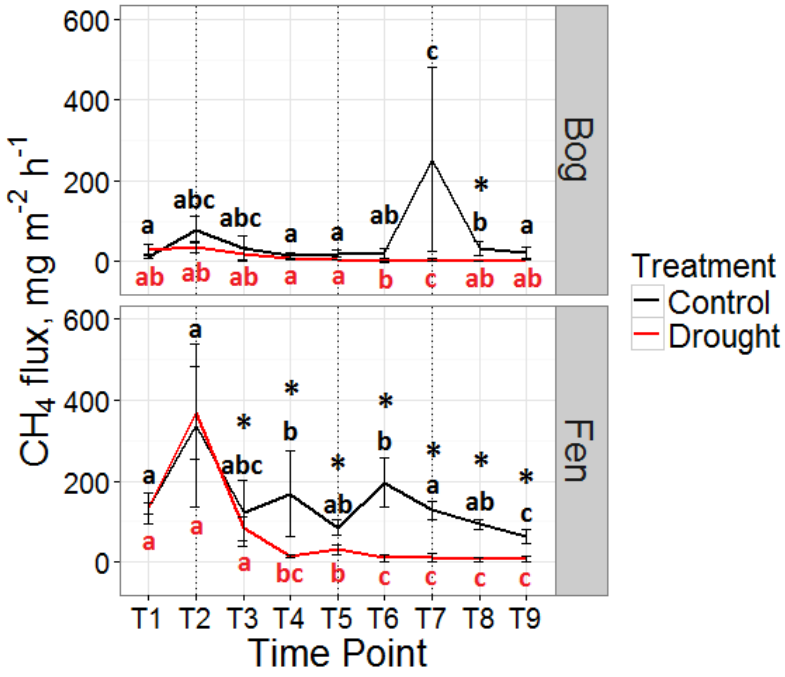

Figure 2.7: Mean methane flux (mg m^{-2} h^{-1}) in droughted and control mesocosm cores, separated by habitat. Significant differences between the two treatments are marked with *, while significant differences between time points are marked with different letters (red = drought, black = control). Error bars show standard errors. Dotted lines represent transition between four stages of water table manipulation: pre-drought, drying, minimum water table, and rewetting (in that order).

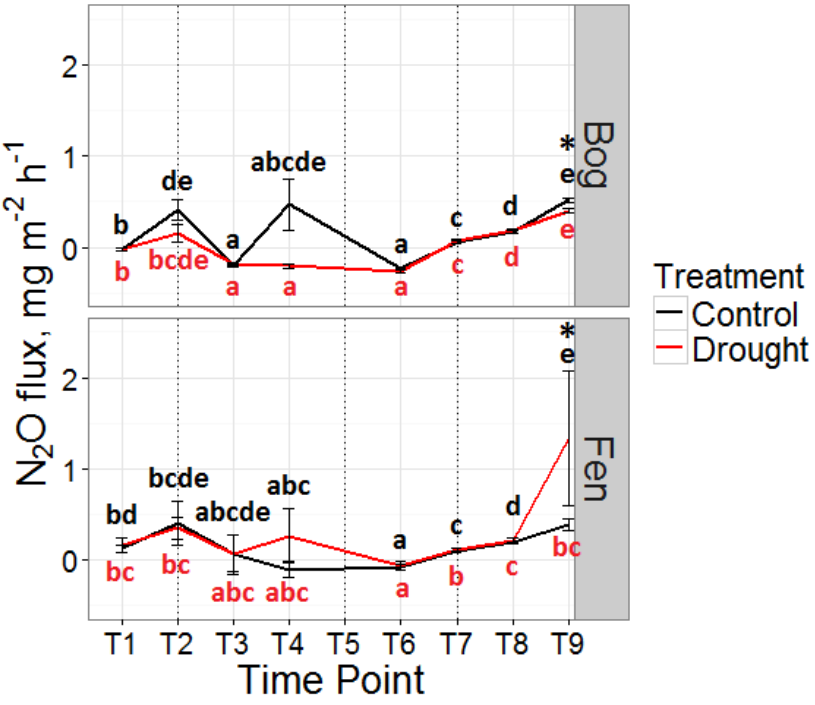

Figure 2.8: Mean flux of nitrous oxide (mg m^{-2} h^{-1}) in droughted and control mesocosm cores, separated by habitat. Significant differences between the two treatments are marked with *, while significant differences between time points are marked with different letters (red = drought, black = control). Error bars show standard errors. Dotted lines represent transition between four stages of water table manipulation: pre-drought, drying, minimum water table, and rewetting (in that order).

2.3.2 Gas Fluxes

There was a significant interaction effect between time point and treatment on carbon dioxide fluxes, with a significant main effect of time point (Table 2.5). As illustrated in Figure 2.6, CO_2 fluxes were significantly higher in drought than control cores at time points 4 (z=2.09, p=0.04), 5 (z=2.06, p=0.04) and 6 (z=2.28, p=0.02). In control cores, the carbon dioxide fluxes were significantly lower at time point 5 than time point 9, with a trend towards fluxes decreasing and then increasing over the course of the experiment, while in the droughted cores carbon dioxide fluxes peaked at time point 6, corresponding to minimum water table (Table 2.2).

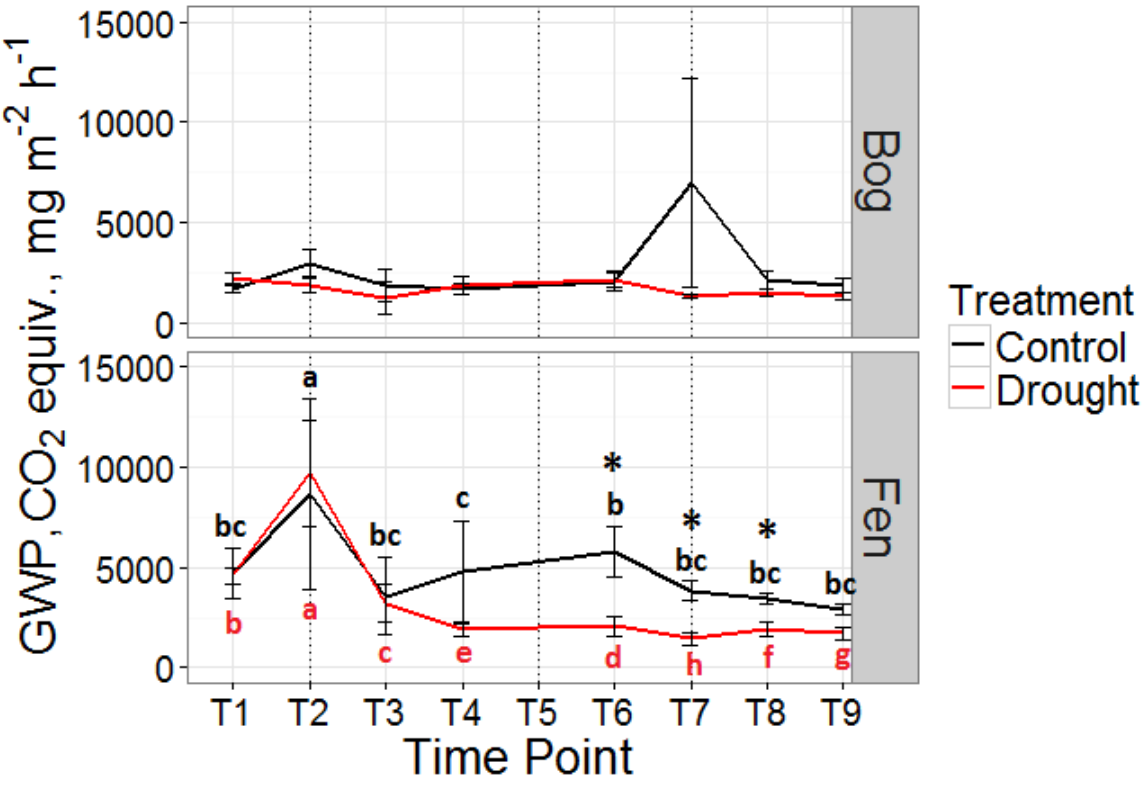

Figure 2.9: Global warming potential (GWP, mg of CO_2 equivalent m^{-2} h^{-1}) in droughted and control mesocosm cores, separated by habitat. Significant differences between the two treatments are marked with *, while significant differences between time points are marked with different letters (red = drought, black = control). Error bars show standard errors. Dotted lines represent transition between four stages of water table manipulation: pre-drought, drying, minimum water table, and rewetting (in that order).

Methane flux was likewise significantly affected by the interaction between time point and treatment (Table 2.5), with drought leading to lower methane emissions (Figure 2.7). In the fen, methane emissions were significantly lower in drought than control cores at time points 2 ($z=-2.36$, $p=0.02$), 3 ($z=-2.03$, $p=0.04$), 4 ($z=-4.11$, $p<0.0001$), 5 ($z=-4.65$, $p<.0001$), 6 ($z=-4.97$, $p<0.0001$), 7 ($z=-7.06$, $p<0.0001$), 8 ($z=-7.42$, $p<0.0001$) and 9 ($z=-4.61$, $p<0.0001$). In the bog, methane emissions were significantly lower in drought than control cores at time points 7 ($z=-3.99$, $p=0.0001$) and 8 ($z=-3.07$, $p=0.0022$). Methane flux was also significantly different between habitats, with a much higher methane flux in the fen (Table 2.5; Figure 2.7). Methane flux was also significantly affected by time point, with methane flux from control cores decreasing over time (Figure 2.7). When the analysis was repeated with a single very large outlier removed, all effects remained significant (results not shown).

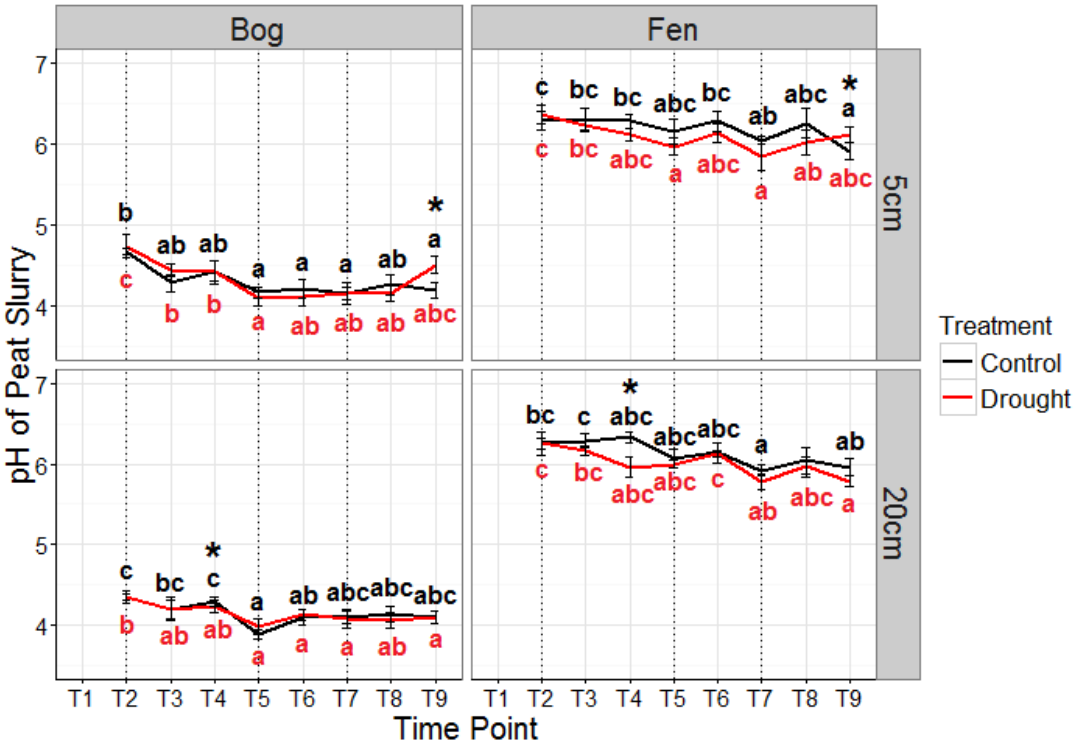

Figure 2.10: Mean pH of peat slurry (1 cm^3 peat suspended in 9 cm^3 ultrapure water). Significant differences between the two treatments are marked with *, while significant differences between time points are marked with different letters (red = drought, black = control). Error bars show standard errors. Dotted lines represent transition between four stages of water table manipulation: pre-drought, drying, minimum water table, and rewetting (in that order).

Nitrous oxide fluxes were significantly affected by the interaction between time point and treatment (Table 2.5): fluxes were significantly higher in drought than control cores at time point 9 only (z=-3.16, p=0.0016; (Figure 2.8). However, nitrous oxide fluxes at all time points were small and variable and the significant effect of treatment was primarily due to outlier effects. Nitrous oxide fluxes were significantly higher from bog mesocosm cores (Table 2.5; Figure 2.8), and were also significantly affected by time point, with a trend towards falling N_2O fluxes in the early time points followed by rising fluxes from time point 6 onwards (Figure 2.8).

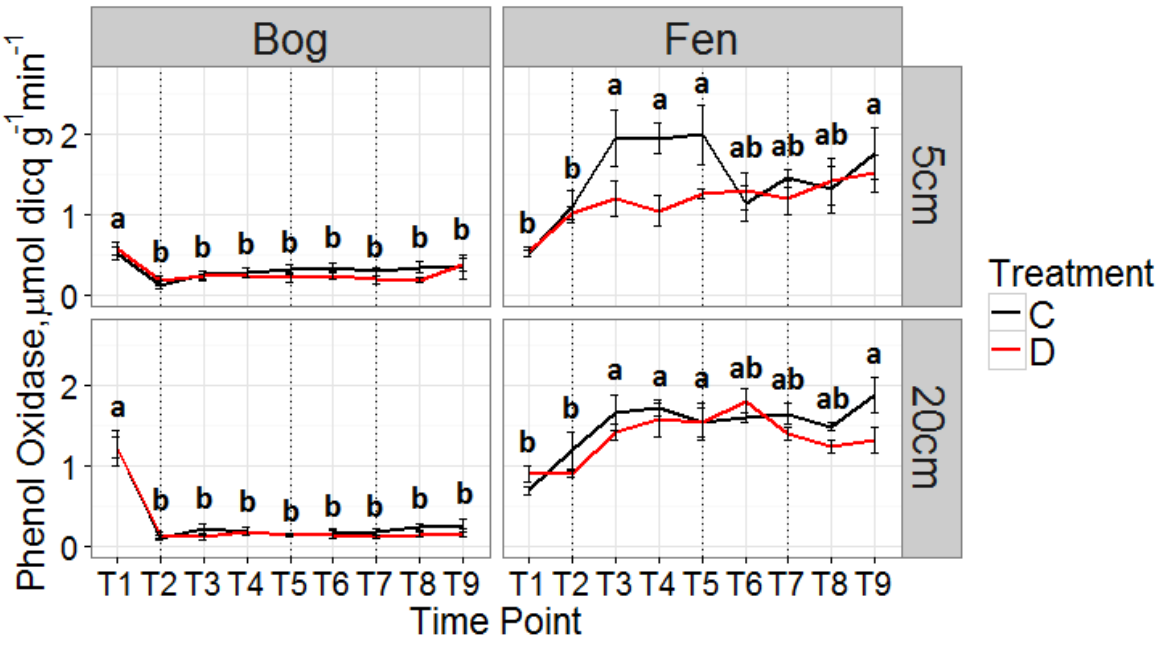

Figure 2.11: Mean phenol oxidase activity of peat (µmol dicq produced per gram of dry peat per minute), separated by habitat and depth. There was no significant effect of treatment so means were calculated across both treatments. Significant differences between time points are marked with different letters. Error bars show standard errors. Dotted lines represent transition between four stages of water table manipulation: pre-drought, drying, minimum water table, and rewetting (in that order).

Global warming potential (GWP) was significantly affected by the three-way interaction between time point, treatment and habitat (Table 2.5): GWP was significantly lower in droughted than control mesocosm cores from the fen between time points T6 and T8. GWP was also significantly affected by habitat, and by two-way interactions between habitat and time point and between treatment and time point.

2.3.3 Soil Biogeochemistry

Fen mesocosm cores had significantly higher slurry pH values than bog mesocosm cores (Figure 2.10). There were significant interaction effects between time point and treatment and between time point, treatment and depth: pH was significantly different between the treatment and control at 5 cm at time point 9 ($z=2.89$, $p=0.004$), and at 20 cm at time point 4 ($z=-2.10$, $p=0.036$). A significant interaction was also found between time point and habitat. There was a significant effect of time point on pH, with pH falling over time in control cores from both habitats.

Soil phenol oxidase activity was significantly higher in the fen than the bog (Figure 2.11; Table 2.6) and significantly differed between depths, with a significant interaction between depth and habitat. In the fen, activity was higher at 20 cm depth, while in the bog, activity was highest at 5 cm. There was also a significant effect of time point, and a significant interaction between time point and treatment (Table 2.6). In the bog, activity fell sharply between the first and second time points and remained low until the end of the experiment, while in the fen activity rose between time points 1 and 2 and the remaining time points (Figure 2.11). There was also a significant effect of depth, and a significant interaction between depth and habitat (Table 2.6). Phenol oxidase activity was modelled using a general linear model rather than a mixed effect model, because adding the random term did not significantly affect the model.

Table 2.5 Results of minimal adequate linear mixed-effect models, with fluxes of important greenhouse gases as response variables. Degrees of freedom reported are reported in the form: $d.f._{denominator}$, $d.f._{numerator}$.

Variable	Factor	F	d.f.	p
CO_2 Flux	Time Point	2.1	4,144	0.04
	Time Point:Treatment	3.8	4,144	<0.001
CH_4 Flux	Habitat	51.6	1,16	<0.001
	Time Point	4.7	8,136	<0.001
	Time Point:Habitat	9.3	8,136	<0.002
	Habitat:Treatment	7	1,16	0.02
	Time Point:Treatment	6.7	8,136	<0.001
N_2O flux	Time Point	167.3	7,119	<0.001
	Habitat	15.3	1,17	0.001
	Time Point:Habitat	6.9	7,119	<0.001
	Time Point:Treatment	2.8	7,119	0.01
GWP	Habitat	5.7	1,16	0.03
	Habitat:Time Point	8.2	7,112	<0.0001
	Treatment:Time Point	2.1	7,112	0.048
	Habitat:Treatment:Time Point	3.4	7,112	0.0025

The concentration of soluble phenolic compounds in soil was significantly higher in bog than fen mesocosm cores and at 5 cm than 20 cm (Figure 2.12; Table 2.6). There was a significant effect of time point on concentration of phenolic compounds, with a significant interaction between time point and habitat: in the bog, phenolic compounds rose sharply at time point six before falling slightly and remaining constant until the end of the experiment (Figure 2.12). In

the fen, there was a more gradual rise in the concentration of phenolic compounds (Figure 2.12). However, there was no significant effect of treatment ($F_{1,17}$= 2.61, p=0.12), and no significant interaction between time point and treatment ($F_{8,306}$= 1.45, p=0.18).

The activity of β-glucosidase was significantly higher at 5 cm than 20 cm depth, and was highest in the bog mesocosm cores (Figure 2.13; Table 2.6). There was a significant interaction between time point and treatment (Table 2.6), although visualisation of β-glucosidase activity in each treatment suggests that actual differences were minimal and significance may have resulted from random variation. In addition, there was a significant three-way interaction between time point, depth and treatment and a significant main effect of time point, with a fall in β-glucosidase activity between time points 1 and 5 at 5 cm and between time points 1 and 2 at 20 cm (Figure 2.13).

Full outputs (F, d.f. and p-values) from statistical tests on soil biogeochemical variables are shown in Table 2.6.

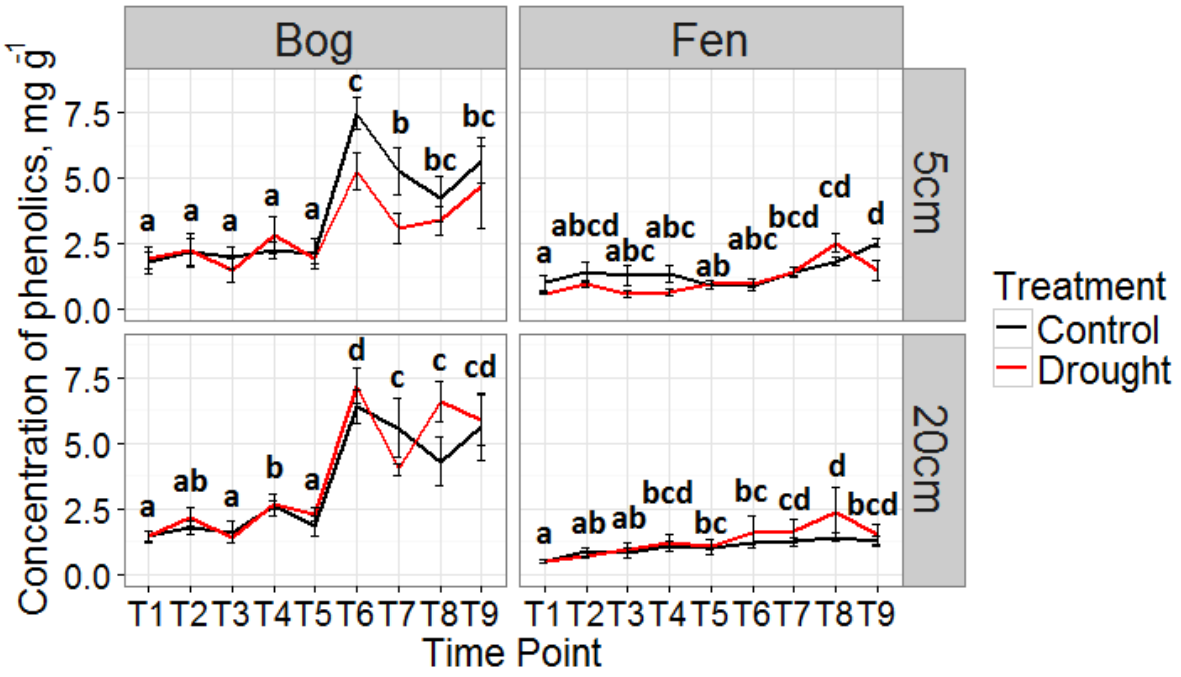

Figure 2.12: Quantity of soluble phenolics (mg of phenol per gram dry weight of peat) in peat, separated by habitat and depth. There was no significant effect of treatment so means were calculated across both treatments. Significant differences between time points are marked with different letters. Error bars show standard errors.

Table 2.6: Results of minimal adequate linear mixed-effect models, with pH, activity of β-glucosidase and phenol oxidase and concentration of water-soluble phenolics as response variables. Degrees of freedom reported are reported in the form: $d.f._{denominator}$, $d.f._{numerator}$.

Variable	Effect	F	d.f.	p
pH	Habitat	421.4	1,17	<0.001
	Time Point	4.6	7,263	<0.001
	Depth	3.9	1,263	0.049
	Time Point:Treatment	3.6	7,263	0.0025
	Time Point:Habitat	4.7	7,263	<0.001
	Time Point: Depth:Treatment	2.6	7,263	0.024
β-glucosidase	Time Point	7.1	8, 305	<0.0001
	Habitat	34.2	1, 17	<0.0001
	Depth	304.8	1, 305	<0.0001
	Treatment	0.3	1, 17	0.6
	Time Point: Depth	3.7	8, 305	0.0004
	Habitat: Depth	15.5	1, 305	0.0001
	Time Point: Treatment	1.4	8, 305	0.2
	Depth: Treatment	0.4	1, 305	0.5
	Time Point: Depth: Treatment	2.0	8, 305	0.04
Phenol Oxidase	Habitat	1.32	1,331	<0.25
	Depth	95.23	1, 331	<0.001
	Time Point	52.25	8, 331	<0.001
	Treatment	2.8	1,331	0.09
	Habitat: Time Point	23.08	8, 331	<0.001
	Depth: Time Point	13.39	8, 331	<0.0001
	Habitat: Treatment	6.74	1, 331	0.01
Phenolics	Habitat	22.2	1,17	<0.001
	Depth	11.1	1,306	0.001
	Time Point	28.7	8,306	<0.001
	Time Point: Habitat	22.9	8,306	<0.001
	Habitat: Depth	2.1	8,306	0.032
	Depth: Treatment	9.6	1,306	0.002

2.3.4. Water Chemistry

The pH of pore water was significantly higher in fen than bog mesocosm cores and significantly higher in control than drought cores (Figure 2.14). There was a significant interaction between depth and treatment, with pH showing a greater decrease during drought at 5 cm depth (Table 2.7). There was a significant effect of time point on porewater pH although the response to

time point differed between the two habitats: in the fen, porewater pH fell slowly throughout, while in the bog pH fell sharply at W5 and peaked at W6 (Figure 2.14).

The concentration of dissolved phenolic compounds was significantly lower in droughted than control mesocosm cores (Figure 2.15), with a significant interaction between time point and treatment (Table 2.7): the concentration of phenolics was significantly lower in droughted than control cores at time point W3 (t_{174}=-4.1, p=0.0001) in the bog, and at time points W1 (t_{174}=3.3, p=0.0014) and W4 (t_{174}=-3.5, p=0.0006) in the fen. There was also a significant main effect of time point (Table 2.7): particularly in the fen habitat, the concentration of dissolved phenolics was highest at time points W1 and W4 (Figure 2.15). The concentration of dissolved phenolics was modelled using a general linear model rather than a mixed effect model, because adding the random term did not significantly affect the model.

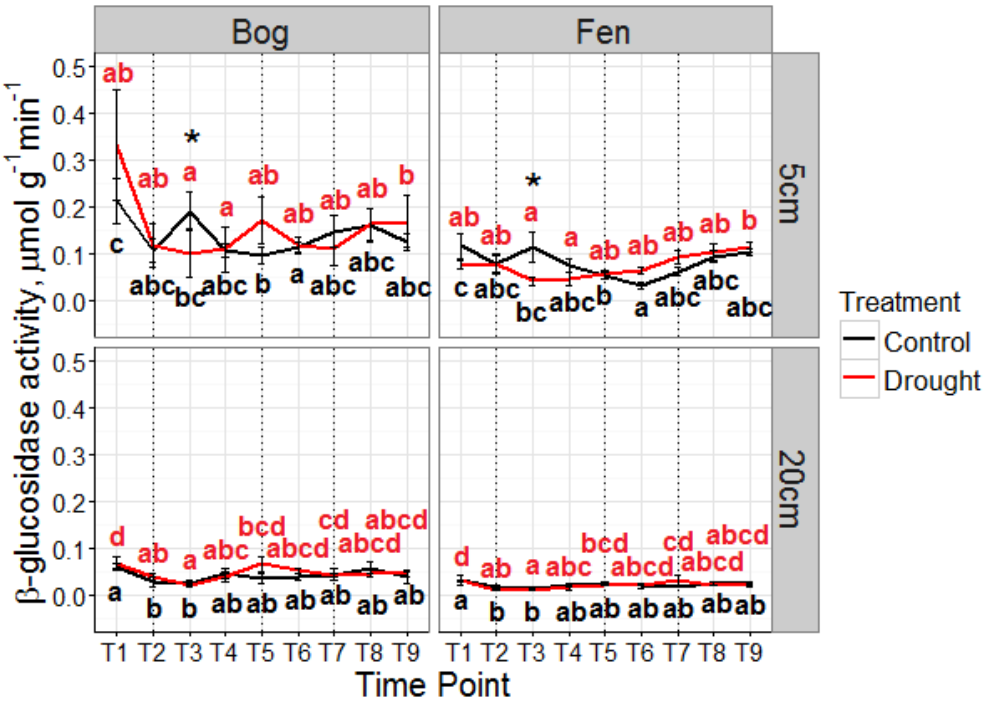

Figure 2.13: Mean β-glucosidase activity (µmol MUF released per gram dry peat per minute), separated by habitat, depth and treatment. Significant differences between the two treatments are marked with *, while significant differences between time points are marked with different letters (red = drought, black = control). Error bars show standard errors. Dotted lines represent transition between four stages of water table manipulation: pre-drought, drying, minimum water table, and rewetting (in that order).

Table 2.7: Results of minimal adequate linear mixed-effect models, with water chemistry measurements as response variables: porewater pH and concentrations of phenolic compounds, dissolved organic carbon (DOC), nitrate and sulfate in pore water. Degrees of freedom reported are reported in the form: $d.f._{denominator}$, $d.f._{numerator}$. Non-significant terms and interactions were removed during model fitting, but remained within the model where they were part of a significant interaction term: however, this are omitted from the table for clarity.

Variable	Effect	F	d.f.	p
pH	Habitat	563.1	1,17	<0.001
	Treatment	8.7	1,17	0.009
	Time Point	14.0	1, 174	<0.001
	Time Point: Habitat	10.2	5,174	<0.001
	Depth: Treatment	11.5	5,174	<0.001
Phenolics	Time Point	14.3	5, 174	<0.001
	Habitat	25.6	1, 174	<0.001
	Treatment	9.1	1,174	<0.001
	Time Point: Habitat	3.4	5,174	0.006
	Time Point: Depth	2.8	5, 174	0.02
	Time Point: Treatment	2.5	5,174	0.03
	Depth: Treatment	6.8	1,174	0.001
	Time Point: Habitat: Depth	2.7	5,174	0.002
	Time Point: Habitat: Treatment	3.8	5,174	0.003
DOC	Treatment	4.7	1,17	0.04
	Habitat	32.8	1,17	<0.001
	Time Point	6.0	5,181	0.014
Nitrate	Habitat	8.5	1,203	0.004
	Depth	10.1	1, 203	0.002
	Treatment	27.7	1,203	<0.001
	Time Point	11.8	2,203	<0.001
	Time Point:Habitat	3.0	5,203	0.01
	Time Point: Depth	4.5	5, 203	<0.001
	Depth: Treatment	13.6	1,203	<0.001
	Time Point: Treatment	4.1	5, 203	0.001
	Time Point: Habitat: Depth	2.4	5,203	0.04
	Time Point: Depth: Treatment	2.7	5,203	0.02
Sulfate	Treatment	6.9	1,173	0.01
	Time Point	2.6	1,173	0.03
	Time Point: Treatment	4.0	5,173	0.002
	Habitat: Depth	4.4	1,173	0.04
	Depth: Time Point: Treatment	2.9	5,173	0.02

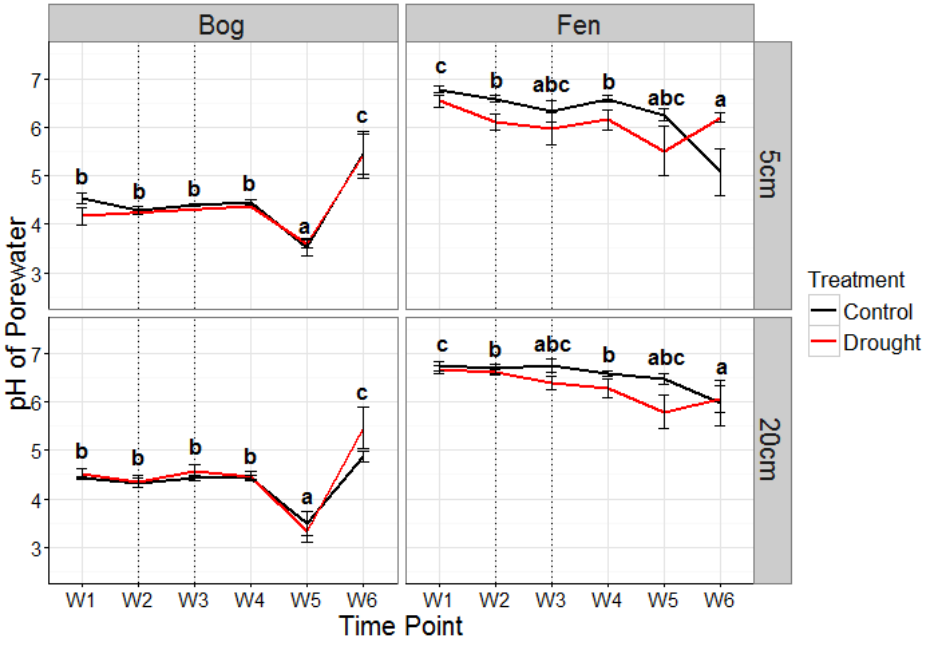

Figure 2.14: Mean pH of porewater, separated by habitat, depth and treatment. Significant differences between time points are marked with different letters. Error bars show standard errors. Dotted lines represent transition between three stages of water table manipulation: drying, minimum water table, and rewetting (in that order).

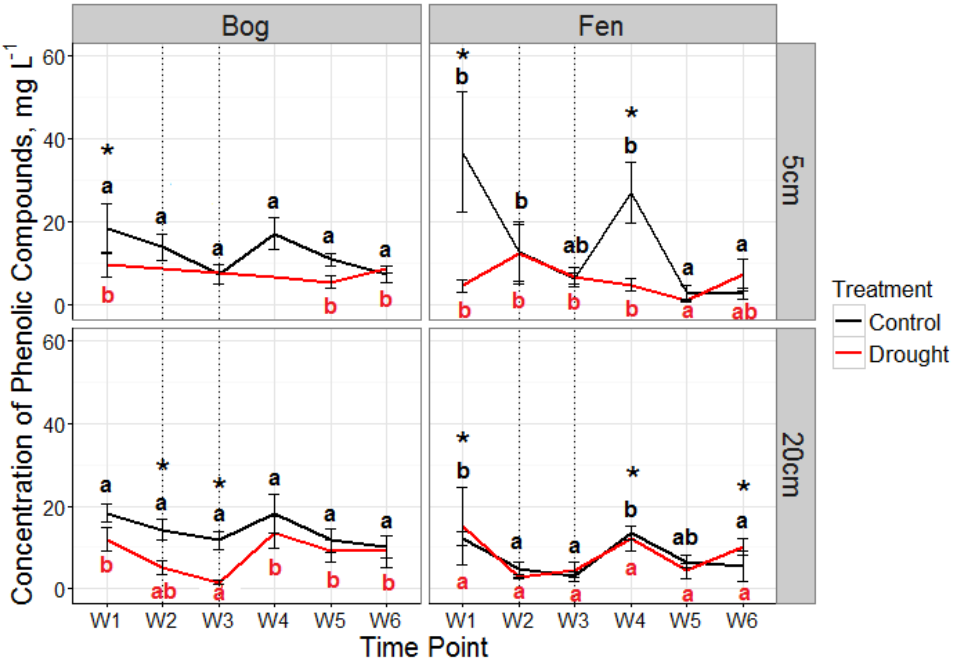

Figure 2.15: Mean concentration of phenolic compounds in porewater (mg L^{-1}), separated by habitat, depth and treatment. Significant differences between the two treatments are marked with *, while significant differences between time points are marked with different letters (red = drought, black = control). Error bars show standard errors.

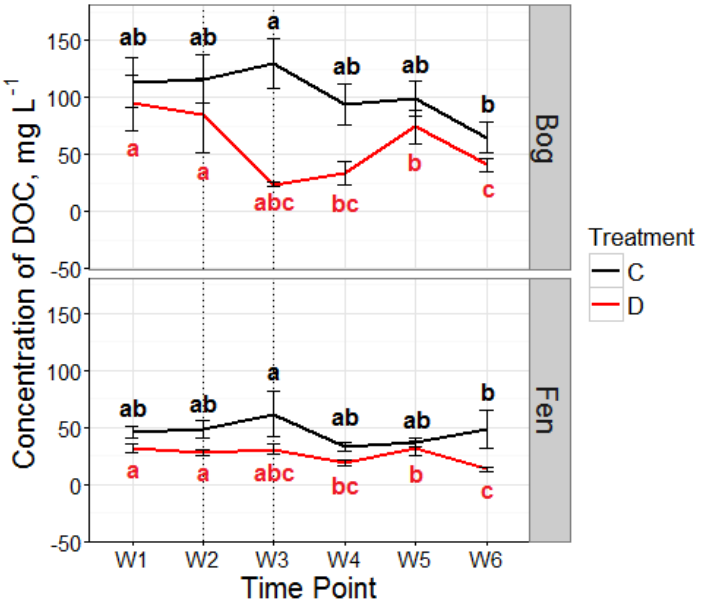

Figure 2.16: Mean concentration of dissolved organic carbon (DOC; mg L^{-1}) in porewater, separated by habitat, depth and treatment. Significant differences between time points are marked with different letters (red = drought, black = control). Error bars show standard errors. Dotted lines represent transition between three stages of water table manipulation: drying, minimum water table, and rewetting (in that order).

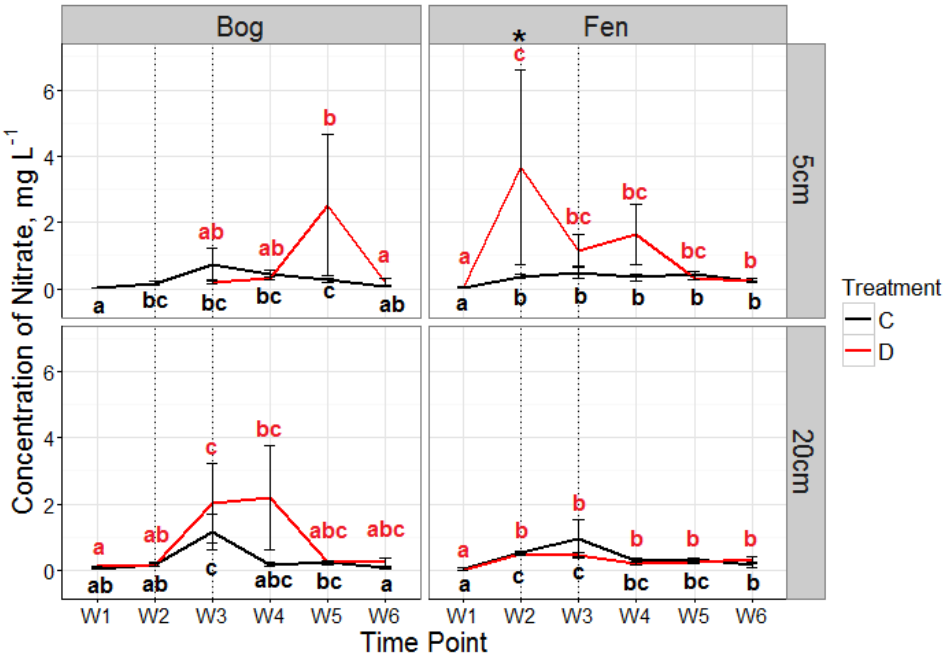

Figure 2.17: Mean concentration of nitrate (mg L^{-1}) in porewater, separated by habitat, depth and treatment. Significant differences between the two treatments are marked with *, while significant differences between time points are marked with different letters (red = drought, black = control). Error bars show standard errors.

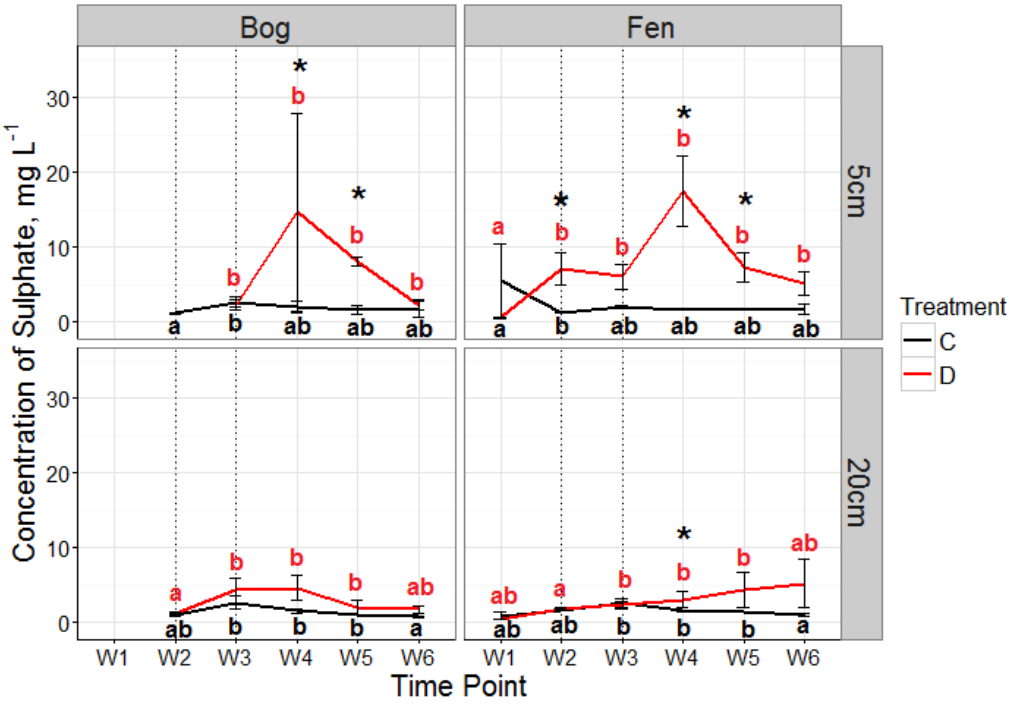

Figure 2.18: Mean concentration of sulfate (mg L^{-1}) in porewater, separated by habitat, depth and treatment. Significant differences between the two treatments are marked with *, while significant differences between time points are marked with different letters (red = drought, black = control). Error bars show standard errors. Dotted lines represent transition between three stages of water table manipulation: drying, minimum water table, and rewetting (in that order).

Concentration of dissolved organic carbon (DOC) was significantly lower in fen than bog mesocosm cores and in drought than control cores (Table 2.7; Figure 2.16). There was also a significant effect of time point (Table 2.7), with the concentration of DOC highest at W3 and lowest at W6 in both habitats (Figure 2.16).

Nitrate concentration was significantly higher in bog than fen mesocosm cores and in drought than control cores, with a significant effect of time (Figure 2.17). There was a significant interaction between time point and treatment (Table 2.7). The concentration of nitrate was modelled using a general linear model rather than a mixed effect model, because adding the random term did not significantly affect the model.

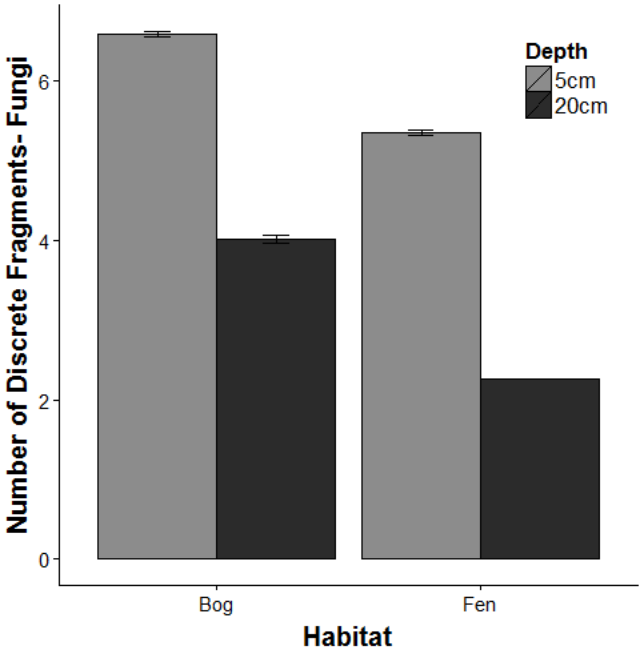

Figure 2.19: Mean number of bands obtained by ARISA fingerprinting of fungal communities in bog and fen mesocosm cores at two depths. Error bars represent standard errors.

Sulfate concentration was significantly higher in the drought treatment (Figure 2.18), and was significantly affected by time point (Table 2.7), with a slight increase in sulfate concentration over time (with the exception of the fen at 5 cm, where there was a decrease in sulfate concentration between W1 and W2). There was also a significant interaction between time point and treatment (Table 2.7). In the bog at 5 cm, sulfate concentration was significantly higher in drought cores at time points W2 ($z=2.6$, $p=0.009$), W4 ($z=3.3$,$p=0.0009$), and W5 ($z=4.0$, $p=0.0001$); while in the fen at 5 cm sulfate concentration was higher at time points W2 ($z = 2.8$, $p=0.005$), W4 ($z= 4.0$, $p= 0.0007$) and W5 ($z=4.2$, $p <0.0001$). At 20 cm, sulfate concentration was significantly higher in the drought treatment at time point W4 in both the bog ($z = 2.4$, $p =0.015$) and the fen ($z = 2.3$, $p =0.02$). There was a significant interaction between depth and treatment, as drought led to a greater increase in sulfate concentrations at 5 cm depth than at 20 cm.

Full outputs (F, d.f. and p-values) from statistical tests on water chemistry variables are shown in Table 2.7.

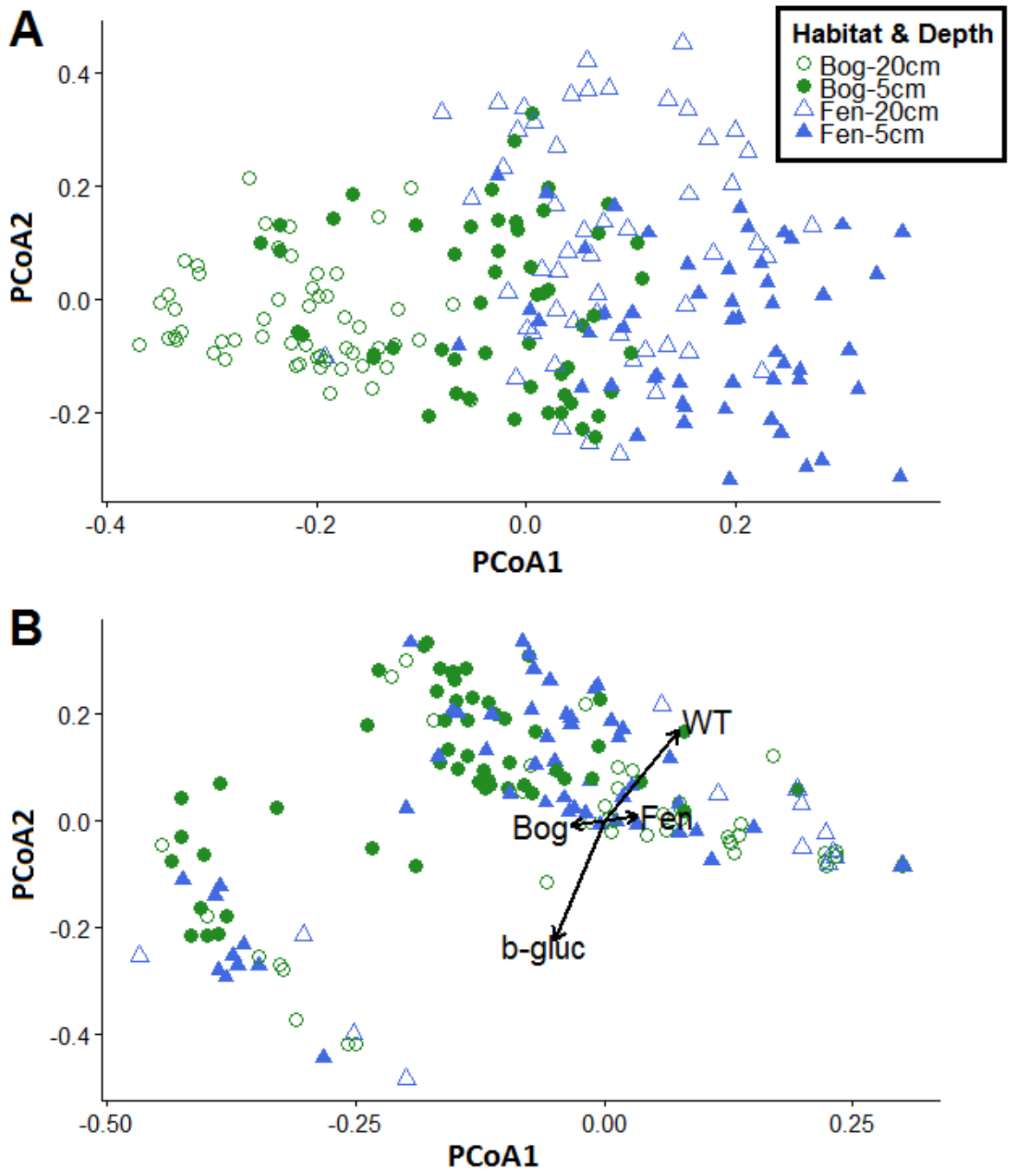

Figure 2.20: PCoA plots, based on Jaccard distances, depicting bacterial (A) and fungal (B) community composition. Arrows on the fungal plot (B) represent environmental variables plotted using the 'envfit' function: only variables which were significant ($p<0.05$) are shown. WT= water table depth; b-gluc= β-glucosidase activity. Dim1 and Dim2 represent the first two axes ('dimensions') of the ordination result.

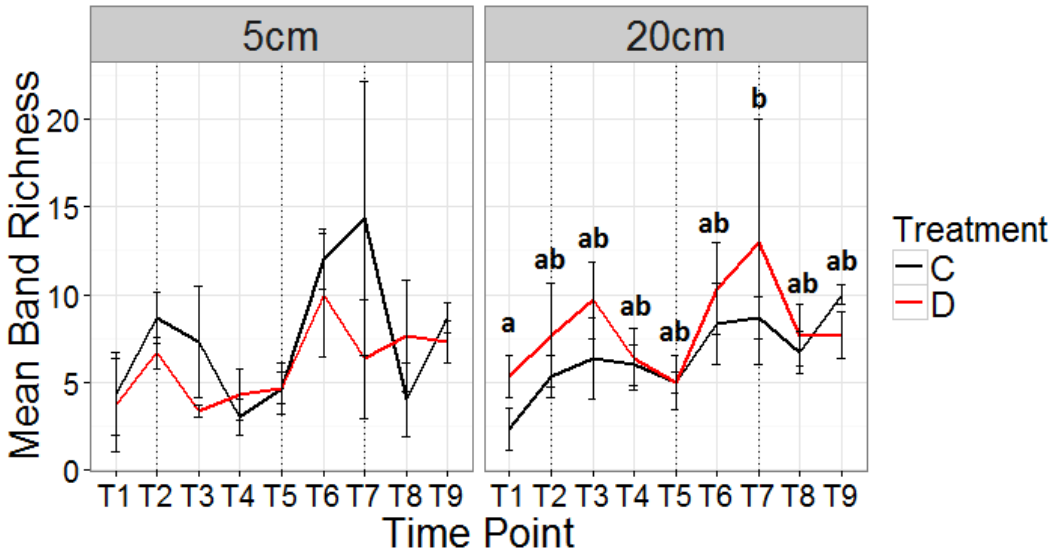

Figure 2.21: Effect of time on mean bacterial richness in the fen at both depths (i.e. the number of bands detected by ARISA fingerprinting). Significant differences between time points are marked with different letters: significant differences were only found at 20 cm depth, and were the same for both treatments. Error bars show standard errors. Dotted lines represent transition between three stages of water table manipulation: drying, minimum water table, and rewetting (in that order).

Table 2.8: Test statistics resulting from generalised linear models in which fungal band richness was the dependent variable. Significant p-values are denoted by * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). Marginal significance ($p < 0.1$) is denoted by '.'.

Data Subset	Variable	χ^2	d.f.	p
All Data	**Habitat**	26.8	1,214	<0.001***
	Depth	96.8	1,213	<0.001***
	Habitat:Depth	7.1	1,212	<0.001***
Bog-5cm	**Time Point**	1.5	8	1
	Treatment	0.9	1	0.3
	Time Point:Treatment	2.1	8	1
Bog-20cm	**Time Point**	13.0	8	0.1
	Treatment	<0.1	1	0.8
	Time Point:Treatment	2.7	8	1
Fen-5cm	**Time Point**	2.7	8	1
	Treatment	<0.1	1	0.8
	Time Point:Treatment	5.8	8	0.7
Fen-20cm	**Time Point**	1.9	8	1
	Treatment	<0.1	1	0.9
	Time Point:Treatment	2.5	8	1

2.3.5 ARISA Fingerprinting

There was a significant effect of habitat and depth on fungal band richness, as well as a significant interaction between habitat and depth (Table 2.8; Figure 2.19). Fungal band richness was higher at 5 cm than 20 cm depth and in the bog rather than fen habitat. Bacterial band richness was not significantly different between habitats or depths (Table 2.9).

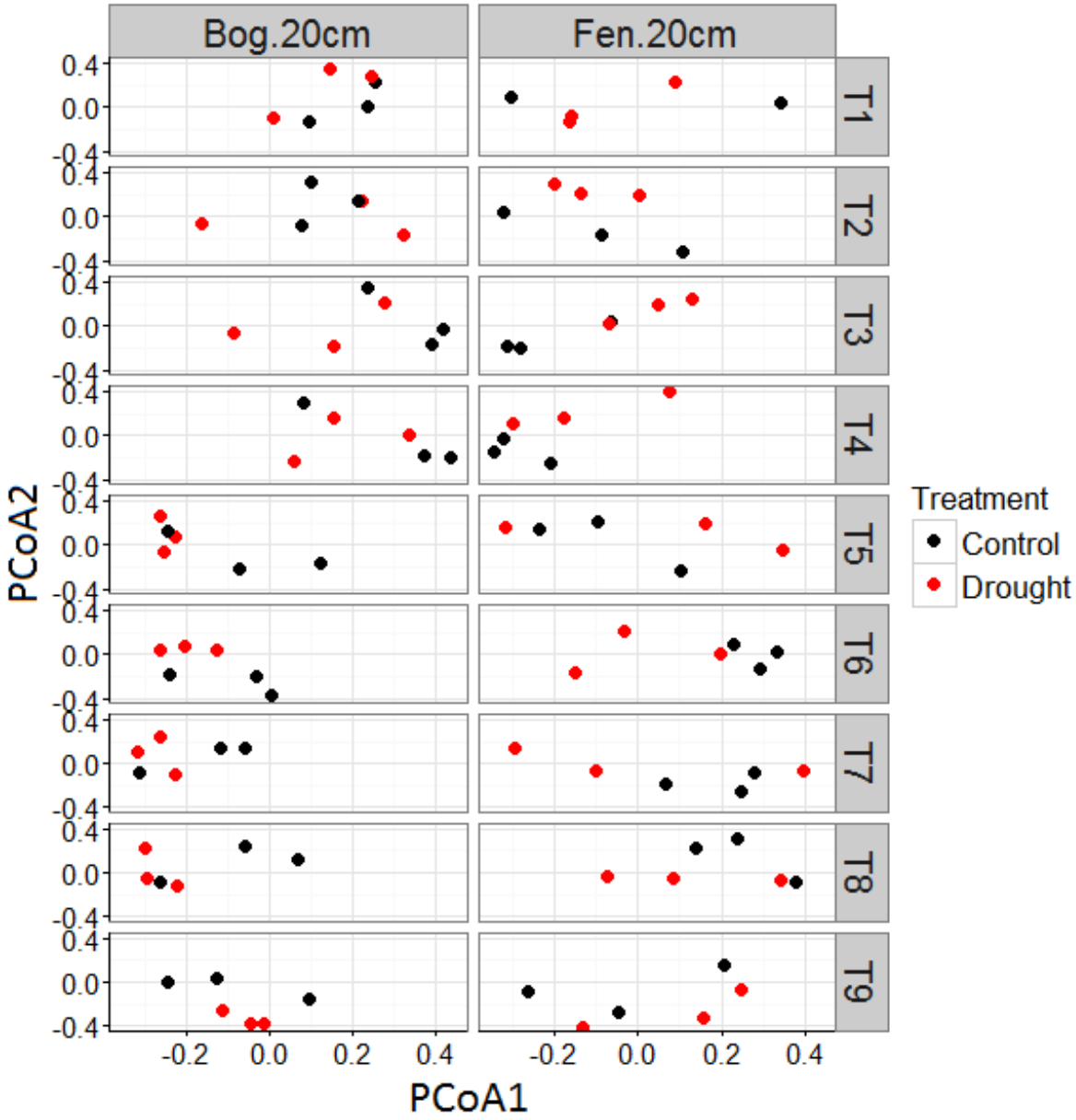

Figure 2.22: NMDS ordination of bacterial communities at 20 cm depth, separated by habitat and time point. There was a significant effect of time point on bacterial communities in the bog at 20 cm ($p = 0.004$) and fen at 20 cm ($p = 0.01$). Full results of PERMANOVA tests are shown in Table 2.11.

The composition of both bacterial and fungal communities was significantly different between the two habitats and depths, with a significant interaction effect between habitat and depth, although effect sizes (R^2 values) were small (Table 2.10). A PCoA plot of the bacterial communities shows the bog samples from the 20 cm depth to be distinct from the other samples, with other habitat-depth combinations clustering weakly with some overlap (Figure 2.20A). A PCoA plot of the fungal communities shows that fungal communities form two clusters which are not linked to habitat or depth, with the two depths showing a degree of separation within each cluster (Figure 2.20B). Correlations between the community composition and environmental variables (calculated using 'envfit' in package 'VEGAN') found that both water table depth and β-glucosidase activity are strongly correlated to the differences between the two clusters.

Table 2.9 Test statistics resulting from generalised linear models in which bacterial band richness was the dependent variable. Significant p-values are denoted by * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). Marginal significance ($p < 0.1$) is denoted by '**.**'.

Data Subset	Variable	χ^2	d.f.	p
All Data	**Habitat**	0.7	1,214	0.52
	Depth	1.9	1,213	0.31
	Habitat:Depth	0.1	1,212	0.86
Bog-5cm	**Time Point**	3.4	8	0.90
	Treatment	0.4	1	0.50
	Time Point:Treatment	4.7	8	0.79
Bog-20cm	**Time Point**	1.2	8	1.00
	Treatment	0.8	1	0.40
	Time Point:Treatment	1.9	8	1.00
Fen-5cm	**Time Point**	18.0	8	0.02*
	Treatment	1.5	1	0.20
	Time Point:Treatment	5.8	8	0.70
Fen-20cm	**Time Point**	19.7	8	0.01*
	Treatment	2.7	1	0.10
	Time Point:Treatment	3.8	8	0.90

Table 2.10: Test statistics resulting from PERMANOVA in which bacterial and fungal community composition were the dependent variables and habitat, depth and the habitat:depth interaction term were independent variables. Significant p-values are denoted by * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). Marginal significance ($p < 0.1$) is denoted by '.'.

Community	Variable	F	d.f.	R^2	p
Bacteria	**Habitat**	10.0682	1,210	0.04357	0.001**
	Depth	6.3164	1,210	0.02733	0.001**
	Habitat:Depth	4.7193	1,210	0.02042	0.001**
Fungi	**Habitat**	7.2602	1	0.02988	0.001**
	Depth	22.3763	1	0.0921	0.001**
	Habitat:Depth	2.331	1	0.00959	0.01*

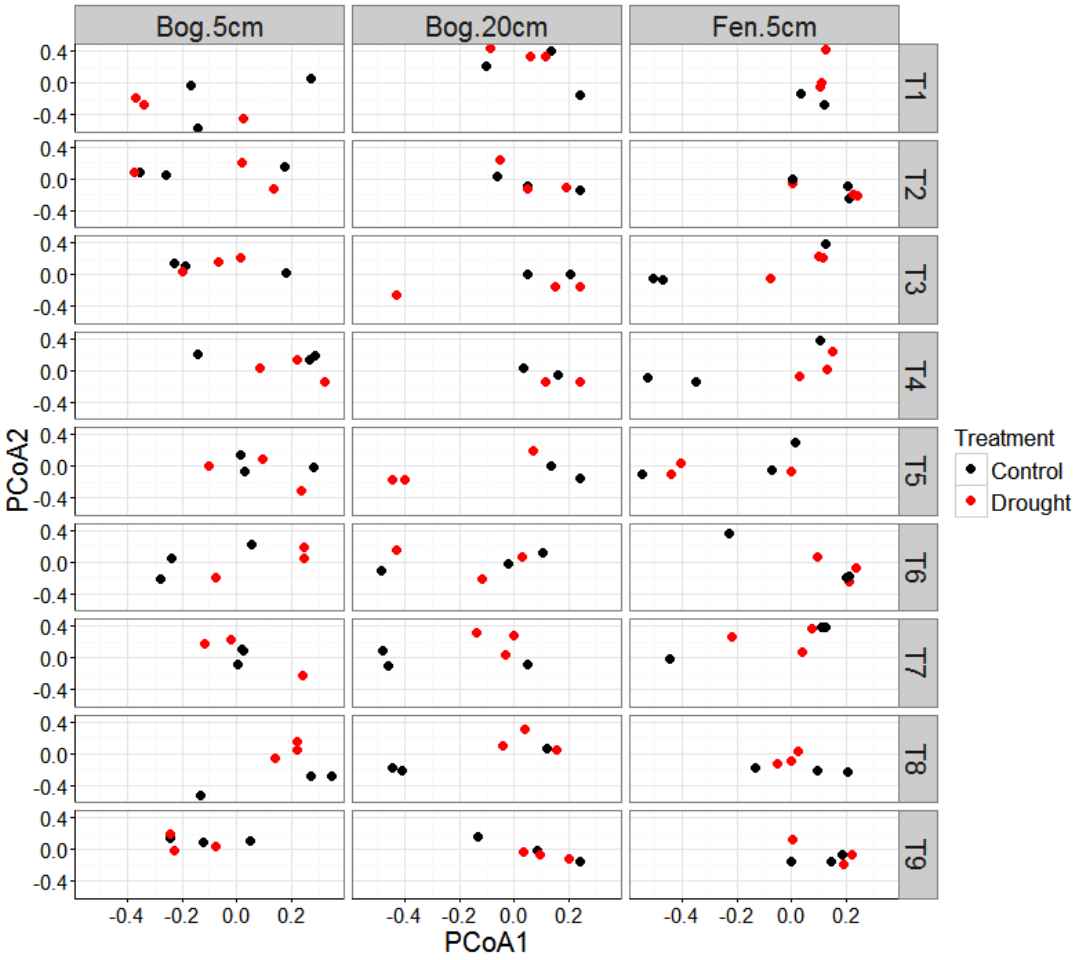

Figure 2.23: NMDS ordination of fungal communities in the bog at both depths, and in the fen at 5 cm, separated by habitat, depth and time point. There was a significant effect of time point on fungal community composition in each of these habitat depth combinations: bog-5cm ($p=0.001$), bog-20cm ($p = 0.001$) and fen-5cm ($p=0.01$). Full results of PERMANOVA tests are shown in Table 2.11.

Richness was not significantly different between treatments for either bacterial or fungal communities, nor was there a significant effect of the interaction between time point and treatment (Table 2.8; Table 2.9). However, there was a significant effect of time point on bacterial band richness in the fen at both depths (Table 2.9; Figure 2.21). However, post-hoc tests failed to find any significant pairwise differences between time points in the fen at 5 cm. In the fen at 20 cm, significantly more bands were present at time point 7 than time point 1, and there was a trend towards increasing fungal band richness with time at both depths (although richness fell at T5 in both cases).

There was a significant effect of treatment on bacterial community composition in the bog at both depths and in the fen at 20 cm (Table 2.11), while the effect of treatment on the fungal community was only significant in the fen at 5 cm. There was a significant two-way interaction effect between time point and treatment on fungal community composition in the fen at 20 cm. In addition, there was a significant effect of time point on bacterial communities at 20 cm in both habitats (Table 2.11; Figure 2.22) and on fungal communities in the bog at both depths and in the fen at 20 cm (Table 2.11; Figure 2.23). Bacterial communities show a clear shift along the first axis of a PCoA plot at time point 5 in the bog at 20 cm, and time point 4 in the fen at 20 cm. Conversely, fungal communities at 5 cm depth in both habitats appear to show changes at the third time point, while fungal communities at 20 cm responded at time point 5.

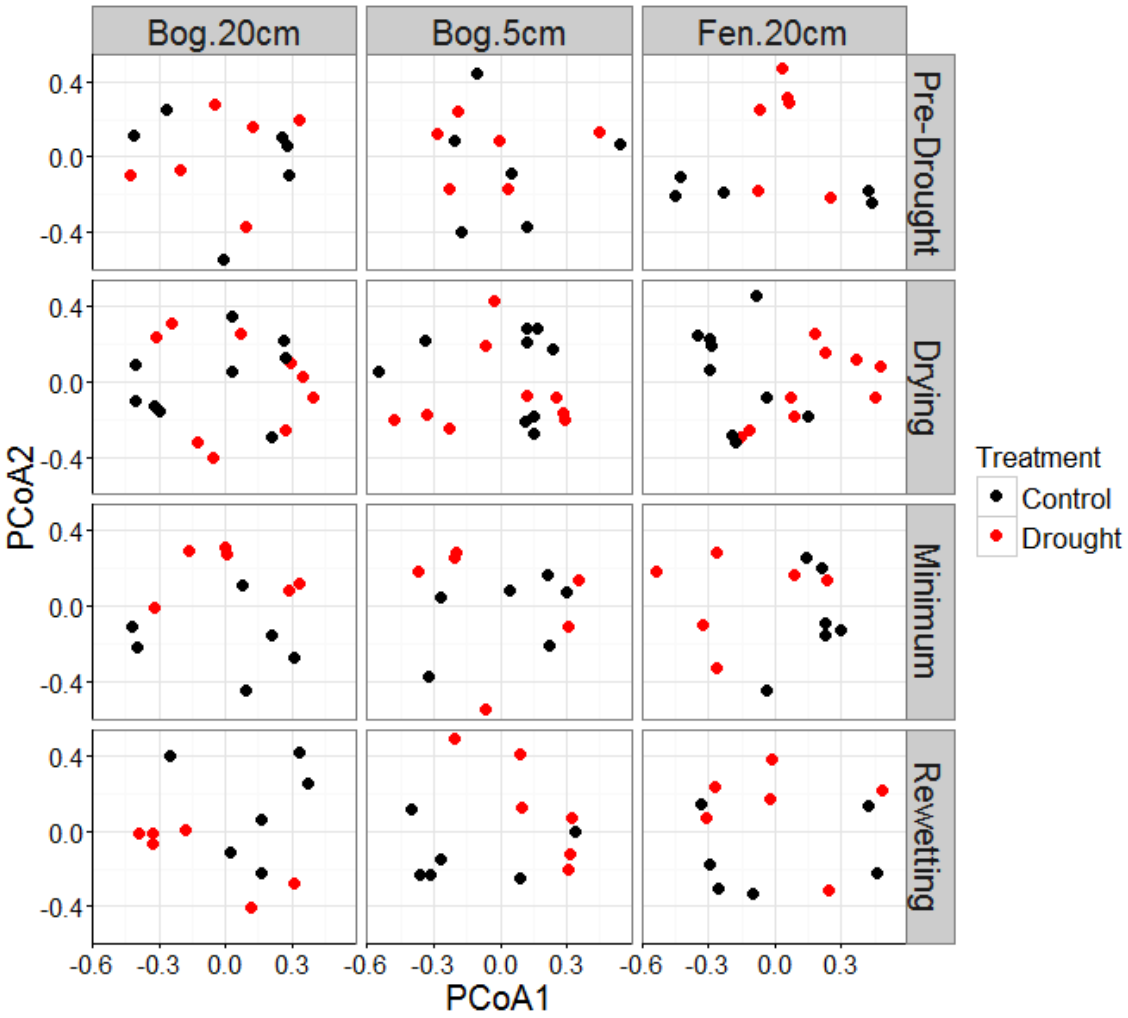

Figure 2.24: NMDS ordination of bacterial communities within the bog at 20 cm, bog at 5 cm and fen at 20 cm depth, separated by stage of the drought manipulation. Treatment differences were significant in the bog at 5 cm during rewetting only ($p = 0.02$); in the bog at 20 cm during minimum water table ($p = 0.04$) and rewetting ($p = 0.02$); and in the fen at 20 cm during both drying ($p=0.004$) and minimum water table ($p=0.04$). Full results of PERMANOVA tests are shown in Table 2.11.

Table 2.11: Test statistics resulting from PERMANOVA in which bacterial and fungal community composition were the dependent variables and time, treatment and the time:treatment interaction term were independent variables. Significant p-values are denoted by * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). Marginal significance ($p < 0.1$) is denoted by '**.**'.

Community	Data Subset	Variable	F	d.f.	R^2	p
Bacteria	**Bog-5cm**	**Treatment**	1.5	1	0.03	0.001**
		Time Point	1.1	8	0.16	0.1
		Treatment: Time Point	0.9	8	0.13	0.9
	Bog-20cm	**Treatment**	1.5	1	0.03	0.04*
		Time Point	1.3	8	0.19	0.004**
		Treatment: Time Point	1	8	0.14	0.6
	Fen-5cm	**Treatment**	1.4	1	0.03	0.1
		Time Point	1.1	8	0.16	0.3
		Treatment: Time Point	0.9	8	0.14	0.8
	Fen-20cm	**Treatment**	1.7	1	0.03	0.02*
		Time Point	1.3	8	0.18	0.01*
		Treatment: Time Point	1.1	8	0.13	0.2
Fungi	**Bog-5cm**	**Treatment**	1.0	1	0.02	0.4
		Time Point	2.0	8	0.26	0.001**
		Treatment: Time Point	1.0	8	0.13	0.4
	Bog-20cm	**Treatment**	0.9	1	0.01	0.6
		Time Point	1.8	8	0.24	0.001**
		Treatment: Time Point	1.1	8	0.14	0.3
	Fen-5cm	**Treatment**	1.9	1	0.03	0.04*
		Time Point	1.5	8	0.20	0.01*
		Treatment: Time Point	1.1	8	0.15	0.3
	Fen-20cm	**Treatment**	2.2	1	0.04	0.09.
		Time Point	1.0	8	0.14	0.4
		Treatment: Time Point	1.5	8	0.20	0.09.

Where either treatment or the interaction between time point and treatment had a significant effect on microbial communities, PERMANOVA tests were applied to subsets based on the phase of drought (pre-drought, drying, minimum water table and rewetting). Bacterial

communities were significantly different between the two treatments during rewetting in the bog at 5 cm; during drying and minimum water table periods in the bog at 20 cm; and during minimum water table and rewetting in the fen at 20 cm (Table 2.12; Figure 2.24). Fungal communities were significantly different between treatments in the fen at 5 cm during rewetting (Table 2.12; Figure 2.25), and in the fen at 20 cm during both pre-drought and rewetting. The low fungal richness in the fen at 20 cm meant that very little variation in community composition was detected in this subset of the data, and so PCoA plots of these results were not plotted. Results of these tests are reported in Table 2.12.

Table 2.12: Test statistics resulting from PERMANOVA in which bacterial and fungal community composition were the dependent variables, and treatment was a dependent variable. Where a significant treatment effect was found (Table 2.11), data was further divided subsets groups according to the phase of drought (pre-drought indicates time points 1 and 2, drying indicates time points 3-5, minimum water table indicates time points 6 and 7, and rewetting indicates time points 8 and 9). It was not possible to subset individual time points due to insufficient replication. Significant p-values are denoted by * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). Marginal significance ($p < 0.1$) is denoted by '**.**'.

Community	Data Subset	Time Subset	F	d.f.	R^2	p
Bacteria	**Bog-5cm**	**Pre-drought**	1.2	1,10	0.11	0.1
		Drying	1.3	1,16	0.07	0.2
		Minimum	0.9	1,10	0.09	0.6
		Rewet	1.5	1,10	0.13	0.02*
	Bog-20cm	**Pre-drought**	0.9	1,10	0.08	0.7
		Drying	1.3	1,16	0.07	0.1
		Minimum	1.6	1,10	0.14	0.04*
		Rewet	1.5	1,10	0.13	0.02*
	Fen-20cm	**Pre-drought**	1.4	1,9	0.13	0.2
		Drying	2.0	1,16	0.11	0.004**
		Minimum	1.6	1,10	0.14	0.04*
		Rewet	1.1	1,10	0.10	0.3
Fungi	**Fen-5cm**	**Pre-drought**	0.8	1,10	0.08	0.727
		Drying	1.5	1,16	0.08	0.155
		Minimum	1.1	1,10	0.10	0.255
		Rewet	1.6	1,10	0.14	0.03*
	Fen-20cm	**Pre-drought**	1.0	1,10	0.09	0.001**
		Drying	3.0	1,16	0.16	0.047*
		Minimum	2.0	1,10	0.17	0.076 **.**
		Rewet	1.7	1,10	0.14	0.001**

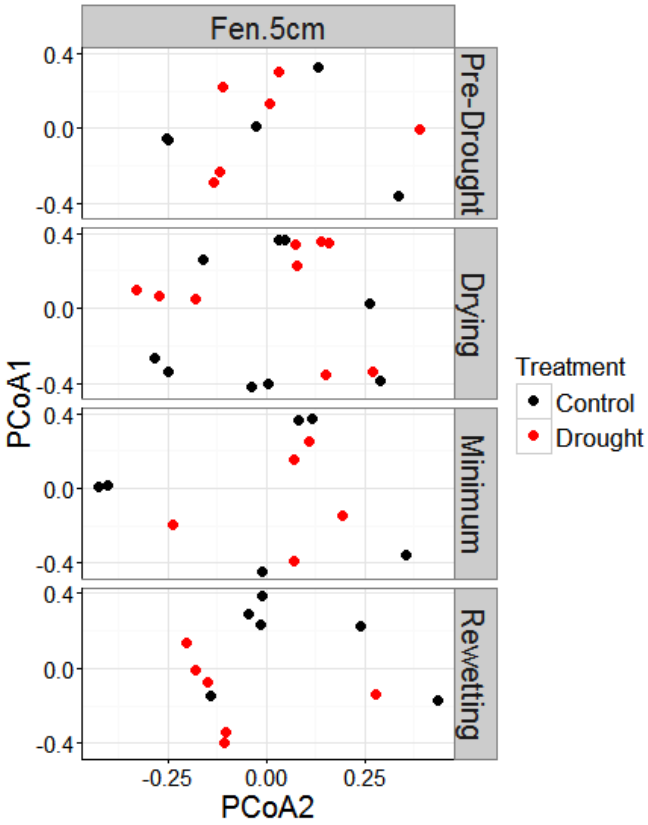

Figure 2.25: NMDS ordination of fungal communities in the fen at 5 cm, separated by stage of drought manipulation. Significant differences were found between communities in the control and droughted mesocosm cores during rewetting only (p=0.03). Full results of PERMANOVA tests are shown in Table 2.12.

2.4 Discussion

As expected, drought led to a rise in carbon dioxide emissions, although this was only significant at minimum water table and emissions from droughted cores fell to the same level as those from control cores as soon as rewetting began. Given that carbon dioxide emissions returned to control levels before the concentration of dissolved phenolic compounds did, it appears that in the current study phenolic compounds were not the primary factor responsible for the suppression of carbon dioxide emissions from water-saturated peat. Instead, it is suggested that increased carbon dioxide emissions from peat were driven by the aeration of the peat, which potentially enabled increased activity of aerobic micro-organisms or root expansion into deeper peat. Changes in microbial communities occurred sooner at 20 cm depth than at 5 cm, and the timing of changes in microbial communities at 20 cm corresponded to the

timing of maximum carbon dioxide efflux. Therefore, it is possible that drought allows increased growth of aerobic micro-organisms at 20 cm depth, and that the associated increase in microbial respiration leads to the release of carbon dioxide.

2.4.1 Water Content and Redox Potential

Alongside water table depth, two variables were considered to act as indicators of the drought status of each mesocosm core: redox potential and water content. Redox potential was significantly affected by the drought treatment at both depths: droughted mesocosm cores had considerably higher redox potentials than control cores, demonstrating that the water table manipulation caused conditions to become more oxidising throughout the peat profile. At 5 cm depth, redox potential was higher in droughted than control mesocosm cores at all time points measured (i.e. time point 4 onwards); however, at 20 cm the effect of treatment on redox potential only became significant when the water table reached 20 cm depth, suggesting that drought only affected the redox potential of peat which was at or above the water table. Water content, the second indicator of drought status, was also affected by treatment: the mean water content of droughted cores was lower than that of control cores during the droughted period, demonstrating that peat became less water-saturated during drought. The drought treatment did not affect water content at the 20 cm depth, probably because this depth represented minimum water table.

Redox potential was lower in the fen than the bog mesocosm cores. There are a number of possible explanations for the lower redox potential of the fen. Firstly, the higher bulk density of fen peat (Hill *et al.* 2014) leads to smaller pore sizes and slower hydraulic conductivity, which in turn may prevent electron acceptors from travelling between layers. However, the lack of a significant difference in redox potential between depths in either habitat suggests that this was not the case. Therefore, differences in redox potential can likely be attributed to differences in the vegetation between habitats: despite a large amount of intra-habitat variation, fen cores were typically graminoid-dominated while the bog mesocosm cores were dominated by mosses (Figure 2.26). Differences in vegetation type are likely to have a strong effect on carbon availability: root-exudates from vascular plants are an important source of carbon in deeper peat (Corbett *et al.* 2012), while *Sphagnum* mosses are known to be very slow to decompose (Lang *et al.* 2009). In turn, the availability of labile carbon sources may lead to increased rates of respiration, and thus to rapid use of oxygen and other electron acceptors.

It should be noted that a number of problems exist with the use of redox probes: in complex systems such as soils, many redox couples co-exist but are combined into a single value by the redox probe, which may not be truly representative of any couple present. This problem is compounded by the fact that members of several important redox couples (including oxygen, nitrate and nitrogen gas) are not well measured by platinum electrodes (McBride 1994). Despite the drawbacks of this method, the design of this experiment imposed severe limitations on the amount of material that could be removed from the peat cores at each time point, meaning that dye-based methods (Vepraskas and Faulkner 2001) were not feasible. Nevertheless, strong and consistent patterns were observed which are consistent with theory, suggesting that the methodology was robust enough to draw conclusions about the overall effect of drought on redox potential.

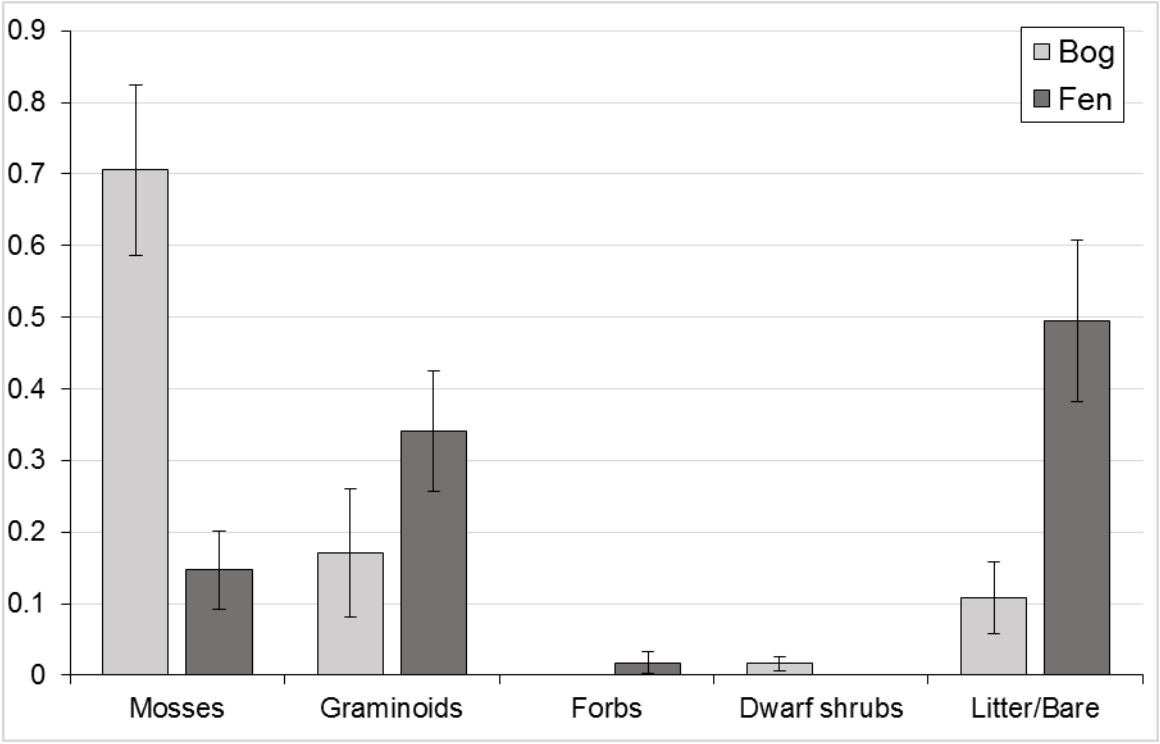

Figure 2.26: Mean proportion of area covered of plant functional groups in the bog and fen mesocosm cores. Error bars represent standard error. The cover of each plant functional group was assessed by taking a photo of each core from above, drawing a grid over it in Microsoft Paint, and assessing the number of squares taken up by each functional group.

Unexpectedly, despite the actual droughting experimental manipulation, water content did increase with time in both treatments during the early part of the experiment. Three possible explanations for this observation can be suggested: (i) mesocosm cores were drier than

expected on collection, possibly due to seasonal effects, and slowly rewetting after being placed indoors; (ii) mesocosm cores became drier during the acclimatisation period; and/or (iii) sample collection led to water ingress. However, each of these explanations seems unlikely. While the months preceding collection of cores experienced below average precipitation (http://www.metoffice.gov.uk/climate/uk/summaries/2013/spring), on collection of fen mesocosm cores the holes from which cores were taken filled rapidly with water, suggesting that peat was saturated on collection from the field. On collection, cores were transferred to bins of water within hours, and small holes drilled in each core to allow water transfer. Finally, holes were plugged with tightly-fitting rubber plugs following sample collection, meaning that a large ingress of water would be unlikely. It is not possible to confidently determine how the water content changed prior to the beginning of the experiment: no samples were collected during the acclimatisation period due to limitations on the amount of peat material which could be removed from each mesocosm core. Redox potential was also affected by time point, despite the fact that it was only measured from time point 4 onwards, suggesting that the rise in water content during the earlier time points may have had a lasting effect on redox potential. The effect of time on redox potential was treatment-dependent, but in the control cores the redox potential fell (i.e. conditions became more reduced) between time points T5 and T6 before rising again towards the end of the experiment (Figure 2.5). Although the rising water content at the beginning of the experiment may mean that the two treatments represent differential rates of wetting, rather than 'saturated control' and 'drought' conditions, the fact that the water content fell and redox potential rose in the cores exposed to water-level drawdown means that these cores will hereafter continue to be referred to as 'drought' cores.

2.4.2 Gas Fluxes

Fluxes of all three greenhouse gases measured were significantly affected by the drought treatment (Table 2.5). Each greenhouse gas was also affected by time point, and changes with time point were likely related to the increase in water content which was observed at the beginning of the experiment (Figure 2.4). Together, these findings highlight the potential danger of feedback responses if global climate change increases the severity or regularity of drought in regions containing bogs and fens (Bates 2008).

Carbon dioxide (CO_2)

Carbon dioxide fluxes were strongly elevated during drought, corresponding with the results of previous studies (Moore and Knowles 1989; Freeman *et al.* 1993; Moore and Dalva 1993; Fenner and Freeman 2011; Chen *et al.* 2012). However, as soon as rewetting began, carbon dioxide emissions in the drought cores fell to the same level as emissions from control cores. This is in contrast to previous drought manipulations in which carbon dioxide emissions rose higher during rewetting than during drought itself (Fenner and Freeman 2011; Kuiper *et al.* 2014), although other studies have similarly found carbon dioxide emissions to fall on rewetting (Freeman *et al.* 1993; Chen *et al.* 2012). Surprisingly, the return of carbon dioxide fluxes to control levels occurred while many biogeochemical factors (e.g. pH and redox potential) remained significantly different to controls and while the water table remained below the level at which carbon dioxide fluxes initially rose. The concentration of dissolved phenolic compounds, thought to be a key inhibitor of carbon release in peatlands (Freeman *et al.* 2001), likewise remained supressed until after time point T8 in the fen mesocosm cores at 5 cm. Although water samples could not be taken from droughted bog mesocosm cores at 5 cm until the final two time points, the concentration of phenolic compounds in the bog porewater at 20 cm likewise remained supressed at time point W3 (corresponding to T7; Figure 2.15). This result suggests that phenolic compounds may not be the key driver of microbial degradation and carbon cycling in the current experiment: while phenolic compounds act as an 'enzymatic latch' restricting degradation of organic carbon in many peatlands (Freeman *et al.* 2001; Fenner and Freeman 2011), this is not universally the case (Williams *et al.* 2000; Romanowicz *et al.* 2015).

There are several possible explanations for the weak relationship between CO_2 fluxes and phenolic compounds in the current experiment. Firstly, the drought treatment may not have been severe enough to impose long-lasting biogeochemical changes in the peat, as enzyme activities were not significantly affected despite the changes in carbon fluxes. However, the drought treatment *was* severe enough to induce a fall in the concentration of phenolic compounds, suggesting that a reduced concentration of dissolved phenolic compounds was not sufficient to elevate CO_2 fluxes and lead to increased enzyme activity. Secondly, it is possible that methodological differences between the current study and previous ones reduced the influence of phenolic compounds: in particular, mesocosm cores in the current study were held

in a growth room at a relatively low temperature, potentially meaning that temperature inhibited microbial activity even when the concentration of phenolic compounds fell. Finally, it is possible that in some wetlands the concentration of phenolic compounds is too low to initiate the 'enzymatic latch' mechanism, and other mechanisms limit decomposition. In both the current study and that of Romanowicz *et al.* (2015), neither of which found phenolic compounds to be related to enzyme activities, the concentration of phenolic compounds in the porewater was low in comparison to the concentrations in the control condition of Fenner and Freeman (2011). These results add to a growing body of evidence (Williams *et al.* 2000; Xiang *et al.* 2013; Brouns *et al.* 2014; Romanowicz *et al.* 2015) that phenolic compounds are not the only factor governing drought-driven carbon release from peatlands.

The coupling of water table and carbon dioxide fluxes in the current study suggests that oxygen availability may be the most important factor, potentially regulating microbial respiration and thus carbon fluxes. Previous comprehensive studies of the biogeochemistry of peatlands (Fenner and Freeman 2011) and humic-rich lakes (Fenner and Freeman 2013) suggest that carbon release on oxygenation is a result of a cascade, of which the first step is the stimulation of microbial growth and activity in response to increased oxygen availability. In the current study, therefore, it appears likely that this direct effect of oxygenation occurs but is not accompanied by the downstream effects of oxygenation (e.g. degradation of phenolic compounds and increased nutrient availability), allowing a fall in CO_2 fluxes following rewetting. Alternatively, the fall in carbon dioxide flux as the water table rises may be a result of carbon dioxide dissolving in the porewater rather than being released at the surface of the peat: the concentration of dissolved inorganic carbon (DIC) increases rapidly on rewetting (Knorr and Blodau 2009), suggesting the potential for porewater to absorb the gases released by microbial metabolism.

Another explanation for the rise in carbon dioxide emissions under droughted conditions is that aerobic conditions allowed increased root growth coupled with an increase in root respiration. Plant respiration makes up over half of total respiration in peat, and has been shown to increase during water table drawdown (Knorr *et al.* 2008). Knorr *et al.* (2008) suggest that the increase in root respiration following water table drawdown is primarily as a result of plant species which are poorly adapted for anaerobic conditions, while in the current experiment saturated conditions at the sites where mesocosm cores were selected would be expected to select for a

plant community which is more resistant to anaerobic conditions, potentially reducing the effect of drought on root respiration. Root expansion following water table drawdown could also lead to changes in the distribution of root exudates, potentially driving changes in microbial respiration. A second mechanism by which plants may alter carbon dioxide fluxes is by altered rates of photosynthesis: *Sphagnum* mosses may decrease their rate of photosynthesis when desiccated, causing a reduction in carbon dioxide uptake (Kuiper *et al.* 2014). There was no significant effect of habitat on carbon emissions in either droughted or control conditions despite the very different plant communities in the two habitats. Nonetheless, the current data does not rule out an effect of increased root respiration or decreased photosynthesis on carbon dioxide fluxes during drought, and this represents a weakness of the study.

Methane (CH_4)

Methane fluxes were much higher in the fen than in the bog, reflecting lower redox potentials in the fen (Figure 2.7). Methanogenesis occurs at a measured redox potential of -200 mV (McBride 1994), which is lower than any redox potential measurement obtained in the current study. However, known problems exist with redox probe measurements (Section 2.4.1), which are compounded by the high degree of spatial variation in redox potential (Vepraskas and Faulkner 2001). Methanogenesis likely occurs in small pockets of peat with especially low redox potentials (Knorr *et al.* 2009; Askaer *et al.* 2010). Previous studies have also reported higher methane emissions from fens than from bogs (Moore and Knowles 1989; Drewer *et al.* 2010), likely reflecting better conditions for methanogenesis in fen habitats: for example, the release of root exudates by the graminoid-dominated plant communities in fens may result in higher concentrations of suitable substrates for methanogenesis (Hornibrook 2009). Interestingly, fens have distinct assemblages of methanogens compared to bogs (Kim *et al.* 2008) and contain more diverse methanogens at the order level (Lin *et al.* 2012), but it is currently unknown exactly what impact this has on methane fluxes from different habitats.

Methane emissions fell during drought, especially in the fen mesocosm cores (methane fluxes in bog mesocosm cores were very low in both treatments, making treatment differences difficult to detect). There is a strong link between water table and methane emissions (Moore and Knowles 1989; Freeman *et al.* 1993; Blodau *et al.* 2004), resulting from decreased methane production rather than increased methane oxidation (Freeman *et al.* 2002). Falling methane fluxes during drought are unsurprising: methanogenesis only proceeds at very low redox

potentials (Vepraskas and Faulkner 2001) and drought leads to a higher redox potential (Figure 2.5) as well as a rise in the concentration of nitrate (Figure 2.17) and sulfate (Figure 2.18), both of which can inhibit methanogenesis (Dowrick *et al.* 2006).

There was also an effect of time point on methane emissions, likely linked to changes in water content (Figure 2.4) and redox potential (Figure 2.5) over time. In the fen, methane fluxes were highest at the first two time points (Figure 2.7), corresponding to low water content. High methane emissions at this time point are surprising given the link between methane and waterlogged (reduced) conditions (Moore and Knowles 1989; Freeman *et al.* 1993; Blodau *et al.* 2004). A number of biogeochemical variables differed between the first time points and the remainder of the experiment: for example, β-glucosidase activity was at the highest values observed. It is possible that the high β-glucosidase activities led to higher levels of substrate for methanogenesis, as the breakdown of polymeric organic matter such as cellulose is the first step in the multi-organism process leading to methanogenesis (Drake *et al.* 2009). Conversely, in the bog methane fluxes peaked at time points T7 and T8, approximately three months after the initially low water content but only three weeks after the minimum redox potential was reached in control cores (Figure 2.5). The higher redox potential in the bog than the fen may mean that redox potential is a more important inhibitor of methanogenesis than substrate availability: methane fluxes were very low in the bog in comparison to the fen (Figure 2.7).

Nitrous oxide (N_2O)

Nitrous oxide emissions were small and variable, meaning that conclusions about fluxes of this gas should be regarded with caution. N_2O fluxes were significantly higher from the bog than the fen cores, with mean fluxes of 0.134 and 0.097 mg m^{-2} d^{-1} respectively (values calculated for control cores at time points 2-9). This is surprising given that N_2O fluxes show a strong positive correlation to Ca^{2+} ions and to pH (Regina *et al.* 1996), both of which were higher in the fen. Indeed, previous studies have found nitrous oxide fluxes in bogs to be either negligible or negative (Regina *et al.* 1996; Drewer *et al.* 2010), although nitrous oxide fluxes from fens are often below detection as well (Aerts and de Caluwe 1997; Palmer and Horn 2015). Nitrous oxide is a product of the reduction of nitrate, which is generally only found at low concentrations in peatlands due to highly reducing conditions (Knorr *et al.* 2009). In this study, redox potentials in the bog at or slightly lower than the range expected for nitrate reduction

(McBride 1994). Conversely, the lower redox potential in the fen suggests that redox couples with lower redox potentials, such as sulfate, will dominate.

Nitrous oxide fluxes were significantly higher in droughted than control mesocosm cores at the final time point (T9). Previous studies have likewise found elevated nitrous oxide emissions during water table drawdown (Freeman *et al.* 1993; Regina *et al.* 1996; Dowrick *et al.* 1999; Goldberg *et al.* 2010). As with methane, changes in nitrous oxide fluxes during drought are closely linked to changes in redox potential: the higher redox potentials observed during drought allow oxidation of ammonium to nitrate (nitrification). During rewetting, nitrate is reduced to nitrogen gas via a series of intermediates which include N_2O (Ponnamperuma 1972; Knorr *et al.* 2009). However, in the current study nitrous oxide fluxes were small and variable, and the rise in the N_2O flux of droughted mesocosm cores at the final time point was primarily due to outlier effects.

Global Warming Potential (GWP)

Despite the increased flux of carbon dioxide from droughted cores, global warming potential (GWP) actually fell in droughted cores due to the fall in methane emissions. The fall in GWP was particularly pronounced in the fen, where methane emissions were highest, and reflects the fact that the overall change in carbon dioxide emissions during drought was fairly small as well as the much larger global warming potential of methane.

2.4.3 Soil and Water Biogeochemistry

pH

Samples from the fen mesocosm cores had a much higher pH than those from the bog, both in peat slurry and pore water. This is unsurprising as pH differences are one of the main divisions between the two habitat types, and may occur due to relatively high concentrations of basic cations in groundwater (the main source of water in fens) compared to rainwater (Bridgham *et al.* 2000).

The pH of porewater in the fen mesocosm cores responded strongly to the drought treatment, with drought leading to lower porewater pH in the fen, but not the bog, habitat (Figure 2.14). A fall in pH during drought is consistent with theory (Ponnamperuma 1972; McBride 1994) and with previous experiments (Fenner and Freeman 2011; Clark *et al.* 2012): drought causes a rise in sulfate concentrations due to the oxidation of sulfur-containing species under oxic

conditions (Freeman *et al.* 1998; Clark *et al.* 2012), and potentially also leads to an increase in dissolved CO_2 as microbial respiration increases (Ponnamperuma 1972). However, the effect of drought on the pH of peat extract was much weaker than the effect on the pH of porewater (Figure 2.10). Measurement of pH from peat extracts may be more accurate than pH measurements from porewater due to the risk of degassing from porewater: in particular, degassing of CO_2 can cause the pH to rise as CO_2 is acidic (Argo *et al.* 1997). Unexpectedly, the pH of porewater from the bog did not respond to drought in either porewater or extracts (Figure 2.14). This may be as a result of the higher redox potential in the bog, meaning that sulfate reduction in this environment was minimal even under saturated conditions.

There was also a sharp fall and then rise in the pH of porewater from the bog at time points W5 and W6, but the magnitude of the change (a rise of over 1 pH unit in a single week, representing a tenfold increase in H^+ ion concentration) suggests that this change represented a methodological error rather than a genuine change in pH.

Phenol oxidase

Phenol oxidase activity was far higher in the fen than the bog (Figure 2.11; Table 2.6), corresponding to earlier results (Williams *et al.* 2000; Lin *et al.* 2012; c.f. Hill *et al.* 2014). The higher phenol oxidase activity in the fen was likely related to higher pH, as phenol oxidase activity is inhibited at low pH (Pind *et al.* 1994; Williams *et al.* 2000). In addition, it has been suggested that tannins (a type of polyphenolic compound) may inhibit phenol oxidase activity in bogs (Williams *et al.* 2000): condensed tannins are absent from graminoids, which dominate fens, but are present in woody plants (e.g. dwarf shrubs) and potentially in *Sphagnum* mosses (Wilson *et al.* 1989). However, the evidence for condensed tannins in mosses is contradictory (Erickson and Miksche 1974). Nonetheless, it seems clear that compounds within *Sphagnum* moss inhibits microbial and enzymatic activity (Painter 1991; Borsheim *et al.* 2001; Lang *et al.* 2009) and that phenolic compounds play a role in this inhibition (Freeman *et al.* 2001). Somewhat unexpectedly, phenol oxidase activity in the fen was higher at 20 cm than 5 cm: this may be a result of changes in the litter composition with depth. Lignin-degrading fungi are expected to become more abundant as decomposition proceeds and more labile substrates are degraded (Bengtsson 1992; Thormann *et al.* 2003), potentially leading to an increase in phenol oxidase activity as peat becomes more degraded along the depth of the profile. Under normal conditions the low redox potential in fen peat would halt phenol oxidase activity, but peat was

stored under aerobic conditions following sample collection and so this may suggest rapid production of enzymes occurred in this period.

Phenol oxidase activity was not affected by the drought treatment, but was significantly affected by sampling time, suggesting that the rise in water potential at the beginning of the experiment may have affected the activities of this enzyme. In the bog, phenol oxidase activity fell rapidly between the first and second time points, suggesting that rising water content during the early part of the experiment may have enabled increased phenol oxidase activity. Phenol oxidases require bimolecular oxygen for their functionality (Sinsabaugh 2010) and oxygen is rapidly depleted following submergence (Chen *et al.* 2012), and so the fall in phenol oxidase activity following the initial increase in water content is expected. However, phenol oxidase activity in the fen showed the opposite pattern, and actually rose with time despite the rise in water content. Given that redox potentials in fen mesocosm cores indicated anoxic conditions during this period, it is unlikely that phenol oxidases were active *in situ*, but it is possible that rapid enzyme synthesis occurred following sample collection. Ferrous iron (Fe^{2+}) is known to increase phenol oxidase activity (Bodegom *et al.* 2005), and so it is possible that anoxic conditions allowed reduction of ferric iron (Fe^{3+}) to ferrous iron, stimulating phenol oxidase activity under assay conditions. Ion concentrations are typically higher in fens than bogs, potentially explaining why this effect was not observed in the fen (Bridgham *et al.* 2000). It should also be noted that the temperature at which enzyme assays were carried out (4 °C) was lower than the temperature in the controlled temperature room in which peat cores were kept: it is possible that this reduced the ability of enzyme assays to detect changes in enzyme activity during drought, although this is unlikely given that the assays were sensitive enough to detect differences in enzyme activities between habitats, depths and time points.

Phenolics

The concentration of both porewater and soil phenolic compounds was higher in the bog than the fen. Therefore, the concentration of phenolic compounds showed the opposite pattern to phenol oxidase activities, demonstrating that phenol oxidase production in the bog is not limited by substrate availability and suggesting that additional constraints on the degradation of phenolics exist in bogs, such as pH (Pind *et al.* 1994; Williams *et al.* 2000). Similarly, Tfaily *et al*. (2013) observed that the concentration of phenolic compounds and aromatics decreased with depth in a fen but not in a bog, supporting the suggestion that the degradation of phenolics

is inhibited in bogs. Despite the differences in the concentration of phenolic compounds between habitats, there were no significant differences in carbon dioxide release between the two habitats: this suggests that the 'enzymatic latch' mechanism suggested by Freeman *et al.* (2001) does not control habitat differences in carbon release.

The quantity of water-extractable phenolic compounds in peat soil was unaffected by the drought treatment, but the concentration of phenolic compounds in porewater was lower during drought, consistent with previous work (Fenner and Freeman 2011). The weak effect of drought on the quantity of phenolic compounds in peat extracts is similar to previous findings (Chris Freeman, pers. comm.), and may be due to the larger pool of phenolic compounds in the soil making changes hard to detect or to the fact that soil phenolic compounds are often held within larger humic complexes with very low turnover rates (Zaccone *et al.* 2008). Dissolved phenolic compounds represent a smaller pool and are potentially more accessible to degradation. The fall in the concentration of dissolved phenolic compounds is somewhat surprising as phenol oxidase activities did not show a measurable response to drought. However, previous studies have likewise only found a weak correlation between phenol oxidase activity and the concentration of phenolics (Romanowicz *et al.* 2015). It is likely that use of L-DOPA as a substrate does not detect all phenol oxidases present in the soil: phenol oxidase is a broad term encompassing a wide variety of enzymes with strongly differing in substrate specificities (Sinsabaugh *et al.* 1994). Additionally, peroxidase enzymes were not measured. Peroxidases often play a role in degradation of phenolic compounds (Sinsabaugh *et al.* 1994) and in some cases have been found to be even more important than phenol oxidases (Jassey *et al.* 2012). Alternatively, the fall in concentrations of dissolved phenolics may be due in part to a reduction in DOC solubility as sulfate concentration rises (Clark *et al.* 2012).

While the quantity of water-extractable phenolic compounds in the peat was not affected by the drought treatment, it was significantly different between sampling time points. In the fen, there was a slow increase in the quantity of water-extractable soil phenolics with time. This rise could represent the build-up of phenolic compounds in the soil after water content rose at the beginning of the experiment (Figure 2.4), inhibiting aerobic phenol oxidases and peroxidases. However, the activity of phenol oxidase enzymes actually rose during this period. It is possible that alongside rising phenol oxidase activity, increased activity of degradative enzymes led to the breakdown of large structural polymeric phenolic compounds and resulted

in the release of monomeric phenol or smaller, water soluble phenolic polymers. Alternatively, peroxidase enzymes might play an important role in the degradation of water-extractable phenolics (Jassey *et al.* 2012), as these enzymes are functional at higher redox potentials than phenol oxidases (Sinsabaugh 2010). In the bog there was a sharp increase in the quantity of water-extractable phenolics at T5 followed by a slight fall: as with the rising concentration of phenolic compounds in the fen, this could have been driven by the rise in water content at the beginning of the experiment. However, given that the concentration of dissolved phenolic compounds increased in both control and drought mesocosm cores, care should be taken when interpreting these results as they could have been caused by a change in the environmental conditions. For example, between W1 and W2 (the first two water sampling dates, roughly corresponding to time points T4 and T7 for soil sampling), the concentration of chloride ions rose markedly across all habitats, depths and treatments from a mean of 51.6 to a mean of 1263.7 mg L^{-1} (data not shown). It is not clear what caused this rise in the concentration of chloride ions: at time point 5 all cores were watered using distilled water from the same source, so it is possible that a problem developed with the still used in the preparation of distilled water (although no problems were noted at the time).

β-glucosidase

Unlike phenol oxidase, β-glucosidase activity was higher in the bog than the fen (Figure 2.13), corresponding to a previous study (Hill *et al.* 2014). The higher β-glucosidase activity could suggest C-limitation (Hill *et al.* 2014): although the carbon content of peat is extremely high (Hill *et al.* 2014), much of the carbon in peat is contained within recalcitrant compounds and therefore may be difficult for many micro-organisms to access (Tfaily *et al.* 2013). Alternatively, higher β-glucosidase activity in the bog could reflect higher redox potential in this habitat, providing better growth conditions for many enzyme-producing micro-organisms. β-glucosidase activity decreased with depth, potentially due to changes in litter composition as decomposition proceeded with increasing depth down the peat profile. Models of litter decomposition typically divide this process into three phases characterised by different microbial guilds: firstly, opportunists break down labile soluble substrates; next, 'decomposers' break down holocellulose; and finally, 'miners' break down lignocellulose (Moorhead and Sinsabaugh 2006). As an important enzyme in the degradation of cellulose, β-glucosidase is important in the second phase of this model but will become less relevant later in decomposition when lignin makes up the majority of remaining litter (Moorhead and

Sinsabaugh 2006). This model is a highly simplified version of the process of litter decomposition (Cotrufo *et al.* 2015), however, and there is little evidence for a succession of guilds in the decomposition of peatland litters (Thormann *et al.* 2003).

Although the difference between β-glucosidase in control and droughted cores was statistically significant at 5 cm depth at the third time point (T3), with lower activities of this enzyme in droughted cores, visualisation of the data suggests that this difference was minimal (Figure 2.13). While it is possible that drought initially suppressed hydrolase enzyme activities at 5 cm depth, inspection of the data suggests that it is more likely this represents a spurious *p*-value and that hydrolase enzymes were unaffected by drought treatment.

DOC

The concentration of dissolved organic carbon was higher in the bog than in the fen, consistent with the literature (Lin *et al.* 2012; Tfaily *et al.* 2013). DOC in bogs is more resistant to degradation than DOC in fens (Corbett *et al.* 2013; Tfaily *et al.* 2013), possibly due to vegetation differences, as *Sphagnum* moss is especially resistant to decomposition (Lang *et al.* 2009), or to higher concentrations of phenolic compounds found in bogs (Figure 2.12; Figure 2.15). It is also possible that the transport of carbon into deep peat in the form of root exudates which is observed in fens (Corbett *et al.* 2013) results in a priming effect and causes more rapid consumption of fen DOC (Blagodatskaya *et al.* 2014). Additionally, higher sulfate concentrations in the fen (Figure 2.18) may have decreased DOC solubility (Clark *et al.* 2012) and thus led to lower DOC concentrations.

The concentration of dissolved organic carbon fell during drought (Figure 2.16), although treatment differences were only significant in the fen. However, the sample size in the bog during drought was very small due to difficulties with collection of water samples during drought (Table 2.3) and there was a non-significant trend towards lower concentrations of DOC in both habitats, so it is possible that a larger sample size would have revealed significant changes in DOC in the bog during drought. Previous studies have also observed a decrease in dissolved organic carbon during drought (Freeman *et al.* 2004; Ellis *et al.* 2009; Fenner and Freeman 2011; Clark *et al.* 2012), either as a result of complete mineralisation to carbon dioxide (Acharya 1935) or as a result of acidification leading to decreased solubility of DOC (Clark *et al.* 2012).

Dissolved organic carbon concentration was also significantly affected by time point in the control cores: it was significantly higher at W3 than W6, representing a small peak in DOC concentration followed by a fall. This may be linked to unexplained changes in porewater ion concentrations which occurred between W1 and W2 (Figure 2.17; Figure 2.18), as sulfate in particular has a well-known effect on DOC solubility in peatland porewater (Clark *et al.* 2012; Evans *et al.* 2012).

Nitrate and Sulfate Concentrations

Porewater concentrations of nitrate and sulfate were higher in drought than control cores (Figure 2.17; Figure 2.18). Both nitrate and sulfate are used as electron acceptors in microbial metabolism under anoxic conditions, but may be re-oxidised under oxic conditions. Previous studies have almost invariably found sulfate concentrations to rise during drought (Clark *et al.* 2005; Knorr *et al.* 2009; Clark *et al.* 2012; Brouns *et al.* 2014; Juckers and Watmough 2014), while the effect of drought on nitrate concentrations is more variable. Drought usually leads to an increase in nitrate concentrations (Knorr *et al.* 2009; Kane *et al.* 2013; Juckers and Watmough 2014), but decreasing (Hughes *et al.* 1999) or nonresponsive (Dowrick *et al.* 1999; Goldberg *et al.* 2010; Brouns *et al.* 2014) nitrate concentrations during drought have also been reported. Nitrate levels in peatlands are often very close to the limit of detection (Knorr *et al.* 2009; Palmer and Horn 2015), making accurate quantification of changes difficult. It is also possible that differences in plant communities affect drought-driven changes in nitrate concentration: for example, the presence of plants can control whether nitrate is converted to ammonium or into gaseous form under anoxic conditions (Matheson *et al.* 2002), potentially influencing whether nitrogen is available to be re-oxidised when conditions are aerobic. Plant nitrate uptake may also significantly affect concentrations in the soil (Rückauf *et al.* 2004). In this study, changes in nitrate and sulfate concentrations were highly variable between cores and time points (Figure 2.17; Figure 2.18). Variability in the concentration of these ions potentially represents spatial and temporal variability in redox potential (Askaer *et al.* 2010), but also makes it difficult to distinguish genuine changes in nitrate and sulfate concentrations from outlier effects and random variability.

The concentration of both nitrate and sulfate rose between time points W1 and W2 (Figure 2.17; Figure 2.18), approximately corresponding to time points T4 and T7 for soil samples, accompanied by a sharp rise in chloride concentration (data not shown). The reasons for these

changes in ion concentrations are unclear, as given that they are observed in both treatments and habitats the effect of experimental problems (such as a problem with temperature regulation in the controlled temperature room or the still used to prepare distilled water) cannot be discounted.

The concentrations of nitrate and sulfate were both significantly different between bog and fen mesocosm cores. Nitrate concentration was higher in the bog than the fen, reflecting the higher redox potential in the former habitat, while sulfate concentration was higher in the fen. It is unclear why sulfate concentration was higher in the fen, as inputs to the two habitats were expected to be similar both before and after collection of mesocosm cores: sulfate concentration was similar in artificial rainwater and groundwater, as well as in groundwater at Cors Erddreiniog (Table 2.1). Atmospheric sulfate deposition has a large impact on sulfate concentration in peatlands (Clark *et al.* 2012) but is similar for both sites (http://www.apis.ac.uk/search-by-location, accessed 14/09/15). Therefore, the most likely explanation is that sulfate run-off from agricultural land surrounding Cors Erddreiniog (e.g. from ammonium sulfate fertilisers) may have acted as an additional source of sulfate (Kaown *et al.* 2009).

2.4.4 ARISA Fingerprinting

Both bacterial and fungal communities differed between the two habitats and depths (Table 2.10), matching the results of recent next-generation sequencing studies (Lin *et al.* 2012; 2014a; 2014b). However, a PCoA plot of bacterial communities showed a significant degree of overlap between habitats and depths (Figure 2.20A). This overlap was likely a result of the limitations of ARISA fingerprinting: in diverse communities, multiple species may share the same intergenic spacer length, causing fragment numbers to become 'saturated' (Kovacs *et al.* 2010). Binning fragments, as was done here, minimises inaccuracy in measuring fragment length but at the cost of increasing the likelihood that fragment sizes will overlap. Nonetheless, the PCoA plot of bacterial communities demonstrates a clear tendency of samples from the same habitat to cluster together, with the first axis showing a gradient from the bog to the fen samples and separating the two depths within the bog samples. Strong habitat differences were likely driven by pH differences between the bog and the fen, as pH is one of the main determinants of bacterial community composition (Fierer and Jackson 2006; Rousk *et al.* 2010). Within the bog, bacterial communities differed between depths, reflecting differences

between a number of environmental parameters. For example, the texture of peat differed strongly between depths: samples from the 5 cm depth consisted of partially-decomposed *Sphagnum* moss while samples from 20 cm consisted of fully humified material (pers. obs.), potentially affecting bacterial communities both directly and indirectly (e.g. through differences in pore size or carbon availability). Previous studies have shown nutrient availability, particularly of phosphorus, to likewise differ between depths (Hill *et al.* 2014; Lin *et al.* 2014b). The second axis on the PCoA plot separates samples from the fen at 20 cm from all other samples. This reflects a pattern observed for dissolved organic matter properties (Tfaily *et al.* 2013), which are known to be related peatland microbial community composition (Lin *et al.* 2012).

Fungal communities also differed between the two habitats and depths, both in species richness (Figure 2.19; Table 2.8) and community composition (Figure 2.20B; Table 2.10). Fungal communities were significantly more diverse in the bog habitat and at 5 cm, potentially reflecting the higher redox potential and larger pore sizes in the bog, both of which are known to be beneficial to fungal growth (Boer *et al.* 2005; Seo and DeLaune 2010). In many peatlands fungal abundance decreases rapidly with depth (Lin *et al.* 2012; 2014b), likely leading to lower diversity in deeper layers of peat. Intriguingly, a PCoA plot of fungal communities' showed two distinct clusters which were not defined by either habitat or depth and may instead have been linked to water table depth or β-glucosidase activity (Figure 2.20B). Within each of the two clusters, fungal communities were more strongly affected by depth within the peat profile than by habitat, corresponding to previous studies of peatland fungal communities (Lin *et al.* 2012).

Bacterial band richness increased over time in the fen at both depths (Table 2.9; Figure 2.21), although post-hoc tests were only significant in the fen at 20 cm. The rise in diversity potentially resulted from a delayed response to the rise in water content observed at the beginning of the experiment. Conditions following a rise in water content may allow high levels of bacterial growth (Fenner and Freeman 2011) and thus facilitate an increase in diversity.

Despite a large degree of heterogeneity between communities in different mesocosm cores and at different time points, treatment significantly affected microbial community composition in the peat (Table 2.11; Table 2.12). In addition, time point had a significant effect on both fungal

and bacterial communities. To further examine the timing of treatment effects, time points were separated into four categories: pre-drought, drying, minimum water table, and rewetting. While the effect of drought on microbial community composition was weak, a number of interesting patterns emerged from analysis of changes in microbial communities during drought.

The time at which treatment effects were significant varies greatly between the two habitats and depths, as well as between bacteria and fungi. For example, at 5 cm depth significant changes occurred only during the rewetting period (Table 2.12), and were only significant for fungi in the fen and for bacteria in the bog. This is surprising given that abiotic variables at 5 cm changed quickly following the commencement of drought. However, many micro-organisms may have survived the early stages of drought by entering a dormant state: previous research shows that analyses based on community RNA (e.g. metatranscriptomics) find stronger responses to drought than DNA-based analyses (Barnard *et al.* 2013; Barnard *et al.* 2015), suggesting that many bacteria may survive droughts by persisting in an inactive state (mRNA has a much faster rate of turnover than DNA (Moran *et al.* 2013)). During rewetting, some previously dormant bacteria undergo a pulse of fast growth while other bacteria may be lysed by the change in osmotic pressure (Manzoni *et al.* 2014), leading to a shift in microbial community composition.

At 20 cm depth, bacterial community composition in both habitats was significantly different between treatments when the water table was at its minimum level, as well as during drying in the fen and during rewetting in the bog. Earlier changes in community composition at 20 cm than at 5 cm depth could suggest that micro-organisms living at this depth are less resistant to changes in water content and redox potential as drying occurs more rarely in peat at this depth. Notably, drought only affected fluxes of carbon dioxide during minimum water table, potentially suggesting that microbial communities at 20 cm may play a key role in drought-driven CO_2 release. Despite lower enzyme activities in deeper peat, it has been shown previously that carbon dioxide release from peat at 20 cm depth increases significantly with drought (Blodau *et al.* 2004).

Nonetheless, where significant effects occurred NMDS plots show only small differences between the community composition in each treatment, as well as a great deal of variability in community composition (Figure 2.24; Figure 2.25). This means that it is extremely difficult to distinguish random variation in community composition from genuine changes

caused by the drought treatment. This difficulty was likely caused in part by the low resolution of ARISA fingerprinting, which meant that even community differences between different habitats were weak (Figure 2.20), as well as the small number of samples at each time point. It should also be noted that by analysing microbial communities in each time subset separately, a larger number of statistical tests were carried out and thus the likelihood of false positives was increased. However, tests were only carried out on time subsets in cases where the effect of treatment on community composition was significant overall, suggesting that a real effect is likely in these cases. Therefore, while it appears that drought has some impact on the bacterial and fungal communities in peatlands, further work is required to confirm this result using higher resolution methods such as high-throughput sequencing.

2.5 Conclusions

1. Drought had a large effect on gas fluxes: carbon dioxide fluxes increased during the period of minimum water table, but fell back to control levels during rewetting. Given that the concentration of dissolved phenolic compounds in porewater remained supressed during the early stages of rewetting, this suggests that factors other than the concentration of phenolic compounds played a role in suppressing carbon dioxide emissions from submerged peat. The tight coupling of water table and carbon dioxide suggests that oxygen availability may be important, potentially by regulating the activity of aerobic micro-organisms. However, an increase in root expansion and respiration cannot be ruled out as a factor influencing carbon dioxide fluxes.
2. Both habitat and depth had a large impact on bacterial and fungal communities.
3. Changes in bacterial communities in the deeper peat (20 cm depth) occurred during minimum water table while bacterial communities at 5 cm depth did not respond to drought until rewetting. Given that carbon dioxide efflux from the peat was only elevated from the droughted peat during minimum water table, this suggests a potential role of microbial communities at 20 cm depth in the release of stored carbon during water table drawdown.
4. Microbial communities at 5 cm depth only responded to during the rewetting period. Both fungi and bacteria responded to rewetting, but each in only a single habitat: bacteria in the bog, and fungi in the fen. It is unclear whether the opposing responses

of the two groups were due to methodological issues, different environmental drivers or biotic interactions between the two groups.

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Chapter 3

The effects of simulated drought and rewetting on the taxonomic composition of eukaryotic and prokaryotic communities

3.1 Introduction

Marker gene analysis (MGA) is here defined as the sequencing of a single marker gene (or several genes in combination) to study the composition of a community. Commonly, the markers used are chosen to be taxonomically informative, such as ribosomal RNA genes (rRNA genes). However, in some cases key genes for a function of interest may be chosen, such as genes for denitrification (Palmer and Horn 2015) or *mcrA* for methanogenesis (Kim *et al.* 2008). Marker gene analysis (MGA)-based studies have played a huge role in our understanding of microbial diversity and taxonomy. An early example is the use of 16S rRNA sequencing in the revolutionary discovery of Archaea as a third domain of life (Woese and Fox 1977) and in the development of a taxonomically meaningful system of classification for bacteria (Fox *et al.* 1980). Later studies used 16S rRNA genes to investigate bacterial communities in natural ecosystems, uncovering numerous uncultured species (Giovannoni *et al.* 1990; Ward *et al.* 1990) and even candidate phyla (Hugenholtz *et al.* 1998). However, initial DNA-based microbial ecology studies were limited by the low-throughput of existing sequencing technologies. The introduction of second-generation sequencing platforms immediately decreased the cost per base pair of sequencing datasets, enabling 'metagenomics' and marker gene analysis (MGA) to be used on much broader scales to test hypotheses about microbial distributions and the interactions between micro-organisms and the environment (Tringe and Hugenholtz 2008). Earlier studies of microbial ecology relied on microscopy or culturing of isolates, limiting the amount of information which could be obtained on bacterial taxonomy and ecology and introducing bias towards species which grow well in culture. The use of ribosomal genes as markers in MGA was later expanded to include eukaryotic taxa (Blaxter *et al.* 2005), including fungi (Epp *et al.* 2012) and protozoa (Pawlowski *et al.* 2012).

It is therefore unsurprising that since the advent of next-generation sequencing, the number of published metagenomic and MGA studies has risen steadily (Figure 3.1). Despite the recent explosion in popularity of marker gene analyses, terminology has not yet fully converged among all practitioners: current terms include 'metabarcoding' (Taberlet *et al.* 2012), 'metagenetics' (Creer *et al.* 2010), 'amplicon sequencing' and 'metasystematics' (Hajibabaei 2012). Additionally, MGA studies are sometimes referred to as 'metagenomics', although this is incorrect: metagenomics refers to sequencing of a random sample of all DNA present in the environment (see Chapter 4). Figure 3.1 shows a year-on-year increase in the number of papers containing each of these terms, reflecting increasingly powerful sequencing technology

(Loman *et al.* 2012) and user-friendly bioinformatic workflows such as QIIME (Caporaso *et al.* 2010) or MOTHUR (Schloss *et al.* 2009), both of which have made marker gene analyses ever more accessible. In this work, the term MGA will be used because 'metabarcoding' and 'metagenetics' most commonly refer to macrofaunal studies, and 'amplicon sequencing' is ambiguous, referring to any study in which a gene is PCR-amplified prior to sequencing.

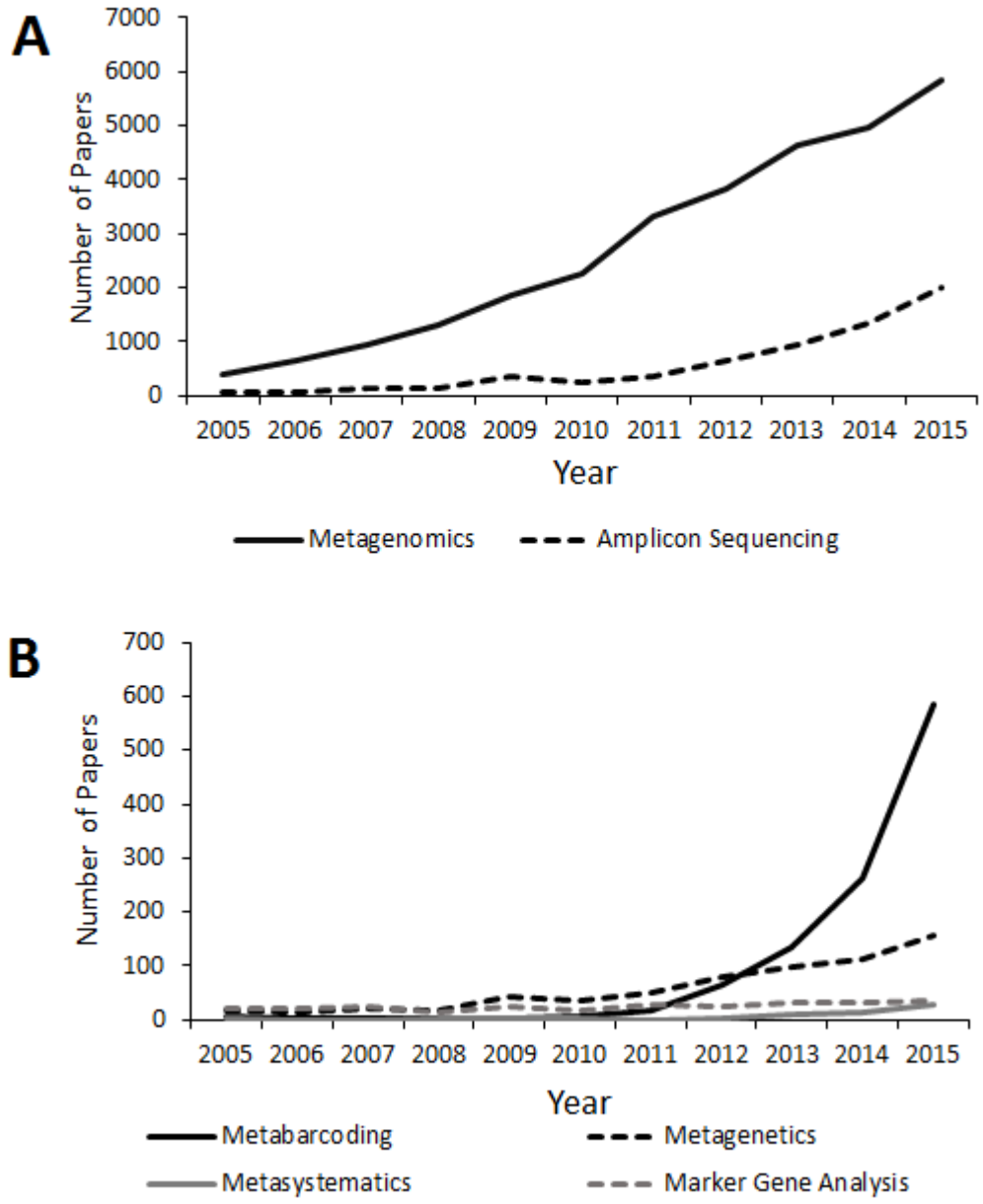

Figure 3.1: Number of papers published containing the words 'metabarcoding', 'metagenetics' and 'metagenomics', by year. Number of papers denotes the number of hits obtained for each word on Google Scholar, filtered by year.

Two recent studies have applied MGA to the study of bacterial, archaeal and fungal communities in peatlands (Lin *et al.* 2012; 2014b) and both have revealed strong differentiation between the microbial communities present in bogs and fens as well as large changes in the community along the peat profile. Peat bacterial communities are dominated by Acidobacteria and Proteobacteria, while the abundances of other bacterial phyla are more variable: one study found high abundances of Firmicutes in a fen (Lin *et al.* 2012), while a second study by the same authors found much lower abundances of Firmicutes in a different fen (Lin *et al.* 2014b), reflecting site or regional differences. Fungal communities in peat are dominated by Ascomycota and Basidiomycota (Lin *et al.* 2012; 2014b).

Both bacterial and fungal community composition in peat is driven by environmental factors, including dissolved carbon and nitrogen, pH, C:N ratio and vegetation (Lin *et al.* 2012; 2014a). Drought affects many of the aforementioned variables, for example leading to an increase in dissolved organic carbon and a decrease in pH (Clark *et al.* 2005; Fenner and Freeman 2011), and would thus be expected to have a large effect on peatland microbial communities. Microbial community fingerprinting methods have been used to study the effect of drought on peatland microbial communities: these have found that drought leads to a decrease in denitrifiers and methanogens (Kim *et al.* 2008) and an increase in the diversity of phenol oxidase genes (Fenner *et al.* 2005). In addition, Chapter 2 of the present work demonstrated that ARISA fingerprinting of peatland bacterial and fungal communities detected a significant, yet weak, effect of drought. MGA has a number of advantages over community fingerprinting: it avoids problems inherent in community fingerprinting methodologies such as the potential for multiple species to produce the same 'fingerprint' (Kovacs *et al.* 2010). In addition, MGA allows the identification of the taxa responsible for community changes. However, despite indications that peat microbial communities will respond to climate change and that this will in turn affect carbon release (Jassey *et al.* 2015; Peltoniemi *et al.* 2015; Wang *et al.* 2015), no study has yet applied second-generation sequencing to study the effects of drought on microbial communities in the active layers of peat.

As discussed in Chapter 2, it is possible that the changes in carbon dioxide emissions observed during drought in the current study were driven by plants: in particular, water table drawdown could allow root expansion into newly aerated parts of the peat profile, and thus lead to increased root respiration (Knorr *et al.* 2008). However, in the current study micro-organisms

were chosen for further study rather than plants for several reasons. Firstly, previous studies have shown that drought influences carbon dioxide fluxes regardless of the effect of plants: for example, Brouns *et al*. (2014) found oxygenation to increase carbon dioxide release from peat incubations without plants. Kuiper *et al.* (2014) found that while plant functional group removal did affect net ecosystem exchange of CO_2, it did not affect ecosystem resilience and resistance to drought. Therefore, it is clear that plant respiration is not the only mechanism underpinning drought-driven CO_2 release from peatlands. Secondly, ARISA fingerprinting found a significant, albeit weak, effect of water table drawdown on the peat microbial community in the current study (Chapter 2). It was hypothesised that MGA would be able to capture the same differences with better resolution. Finally, changes in global warming potential (GWP) during drought were primarily due to the fall in methane emissions, and both methane production and consumption are microbial processes. Therefore, further analysis of the microbial community was considered justified. Given that knowledge of the microbial mechanisms underlying drought-driven changes to the carbon cycle is currently lacking, high-throughput sequencing was used to analyse changes in the microbial community as a whole in order to generate more detailed hypotheses about which microbial taxa are most affected by drought.

Aims and Objectives of Chapter

The primary objective of this chapter was to identify differences in both prokaryotic and eukaryotic communities between habitats and depths as well as changes in communities during a period of drought and rewetting. Marker gene analysis (MGA) was chosen as a tool for this purpose, using 16S rRNA genes as markers for prokaryotes and 18S rRNA genes as markers for eukaryotes. The aims of this chapter were as follows:

1. To determine the taxonomic composition of a Welsh bog and fen at two depths (5 cm and 20 cm)
2. To identify differences in the taxonomic composition and taxon richness of microbial communities between habitats and depths
3. To identify changes in the taxonomic composition of microbial communities and species richness of bogs and fens during simulated drought

3.2 Methods

3.2.1 Sequencing and Quality Control

Experimental design and DNA extraction methodologies are described in full in Chapter 2. Sequencing was carried out by the Earth Microbiome Project (Gilbert *et al.* 2014) following standard protocols (http://www.earthmicrobiome.org). Briefly, the V4 region of the 16S rRNA gene was amplified using primers 515f (GTGCCAGCMGCCGCGGTAA) and 806r (GGACTACHVGGGTWTCTAAT) (Caporaso *et al.* 2011a), which amplify both bacterial and archaeal sequences, and the V9 region of the 18S rRNA gene was amplified using Illumina_Euk_1391f (GTACACACCGCCCGTC) and Illumina_EukBr (TGATCCTTCTGCAGGTTCACCTAC) (Amaral-Zettler *et al.* 2009). Sequencing was carried out on an Illumina HiSeq in rapid run mode, giving paired-end reads of 150bp in length. Quality control and demultiplexing was carried out by the Earth Microbiome Project (Gilbert *et al.* 2014) in QIITA (http://qiita.microbio.me/), a QIIME-based repository and analysis platform for "-omics" data, and was equivalent to quality control in QIIME using default parameters. For 18S rRNA, forward and reverse reads were joined using the 'obijoinpairedend' command from the obitools suite (Boyer *et al.* 2015). The primers used for amplification of the 16S rRNA region gave amplicons of 300-350bp in size, meaning that it was not possible to consistently join the forward and reverse reads belonging to the 16S rRNA amplicons.

3.2.2 OTU Clustering and Taxonomy Assignment

Further quality control and OTU clustering was carried out in vsearch (https://github.com/torognes/vsearch), a method which has been found to output high quality OTUs (Westcott and Schloss 2015). Firstly, identical reads were merged and singletons removed ('vsearch --derep_fulllength'), before chimera removal was carried out on the dereplicated reads using uchime_denovo. Next, OTUs were clustered at 97% similarity ('vsearch --cluster_fast') and OTU centroids were extracted from each OTU generated in this way. An OTU table suitable for downstream analysis was created by mapping all initial reads (including singletons) to the OTU centroids. Inclusion of singletons at this final step meant that while singletons could not form OTUs on their own, they were included if they belonged to an OTU consisting of sequences that occurred multiple times. Finally, the results were converted to an OTU table suitable for further analysis using the script 'uc2otutab.py' (http://drive5.com/python/uc2otutab_py.html).

Taxonomy was assigned to each OTU centroid using the 'utax' command in usearch v8.12 (Edgar 2010). Taxonomy was assigned against the Greengenes database for 16S rRNA genes (DeSantis *et al.* 2006), and against the SILVA database for 18S rRNA genes (Quast *et al.* 2012).

3.2.3 Statistical Analysis

Prior to further analysis, read numbers were standardised in all samples using the 'rrarefy' command from the 'VEGAN' package (Oksanen *et al.* 2015). While the use of rarefying to normalise library size has been criticised (McMurdie and Holmes 2014), a more recent analysis combining simulated and real datasets suggests that rarefying may be the most appropriate normalisation technique to apply prior to clustering analyses (Weiss *et al.* 2015). In particular, Weiss *et al*. (2015) recommend the use of rarefying to normalise library size when clusters are subtle, as was expected to be the case for treatment effects in the current study.

Samples in the 16S rRNA gene dataset were standardised to contain 70,000 reads and samples in the 18S rRNA gene dataset were standardised to contain 20,000. The thresholds used for standardisation were chosen to include the majority of samples, but exclude samples where sequencing had failed. Samples which contained fewer reads than these thresholds were removed from the dataset: 10 samples were removed from the 16S rRNA gene dataset and 9 from the 18S rRNA dataset as they did not contain the requisite number of reads for the read number standardisation step. Once read numbers had been standardised, rarefaction analysis was carried out to assess coverage using the 'rarefy' command in the R package 'VEGAN'.

Both OTU richness and Simpson's Complement Index (hereafter Simpson's Index) were calculated for each sample using commands 'specnumber' and 'diversity' in package 'VEGAN' (Oksanen *et al.* 2015). For the 18S rRNA dataset, OTUs assigned to the phyla Holozoa, Metazoa, Chloroplastida and 'NA' (i.e. OTUs with no taxonomic assignment) at any confidence level were excluded from calculations of α-diversity as the experiment was designed to focus on micro-organisms. Next, linear mixed effects models were used to test for effects of habitat, depth, time point and treatment on α-diversity. Models were initially fitted with all main effects and two and three-way interactions included, and interaction effects were removed sequentially until only significant interactions remained (with the exception of the interaction between time point and treatment which was kept in all models due to the importance of this term to the chapter aims). As Simpson's Index is always a value between 0

and 1 and is thus not normally distributed, Simpson's Index values were logit transformed (logit(y) = log(y/[1-y])) prior to model fitting, following the recommendations of Warton and Hui (2011). 'Core' OTUs, defined as OTUs found in 95% of samples, were calculated for each sample using a custom R script.

Next, models were fitted to test for significant effects of habitat, depth, time point and treatment on the proportion of the community which was made up by each of the most abundant phyla (i.e. those phyla which made up a mean of >1% of the community in at least one habitat and depth). The proportion of each phyla was logit-transformed prior to fitting linear mixed-effects models with '~1|Core' as a random effect. Linear mixed-effects models were fitted using package 'lme' (Pinheiro *et al.* 2013). Models were initially fitted with all main effects and two and three-way interactions included, but interaction effects were removed sequentially until only significant interactions remained (with the exception of the interaction between time point and treatment, which was kept in all models due to the importance of this term to the chapter aims). Only OTUs which could be assigned at phylum level with an utax confidence value of 0.85 or higher were included in this part of the analysis.

To visualise differences in the community composition of prokaryotic and eukaryotic communities, NMDS plots were calculated using function 'metaMDS' in R package 'VEGAN' (Dixon and Palmer 2003). NMDS ordination was based on Bray-Curtis dissimilarities. NMDS plots were first calculated using all samples, and then using subsets corresponding to each combination of habitat and depth. In addition, PERMANOVA tests were carried out using function 'adonis' from the R package 'VEGAN'. In order to focus on the community composition of microbial eukaryotes, all OTUs assigned to phyla Holozoa, Chloroplastida, Metazoa and "NA" with any confidence level were excluded from NMDS ordination, CCA, and PERMANOVA.

Next, partial constrained correspondence analysis (also known as canonical correspondence analysis) was carried out on a filtered subset of OTUs (i.e. those which occurred in at least 20% of samples). Constrained correspondence analysis (CCA) first involves chi-square transformation of the species matrix, followed by multiple linear regression of the species matrix on the variables of interest (constraints). Therefore, CCA only displays variation which can be accounted for by the chosen constraints. Partial CCA (pCCA) additionally allows the inclusion of a 'conditioning' variable, the effect of which is removed prior to regression on the

constraints. In the current study, water table direction was used as the constraining variable: water table direction was always 'Steady' for control cores but for drought cores was either 'Steady' (T1&2), 'Drying' (T3, 4 & 5), 'Minimum' (T6 & 7) or 'Rewet' (T8 & 9). Core was included as a conditioning variable, to remove the effect that large between-core community differences had on the results. Following the fitting of the CCA, an ANOVA-like permutation test was calculated to test the significance of water table direction (function 'anova.cca').

To identify individual OTUs which showed significant responses to drought, OTUs were first filtered to include only those OTUs which were sufficiently abundant (at least 1 read per 1000 reads) and present in at least 20% of samples. This strict filtering was carried out in order to minimise spurious effects of rare OTUs or OTUs which were highly abundant in a few samples but otherwise absent; the high proportion of rare OTUs in the dataset was considered likely to generate these spurious results. Following filtering, OTU abundances were logit transformed (Warton and Hui 2011) and linear mixed effect models were fitted with '~1|Core' as a random effect and with time point, treatment and the interaction between time and treatment as fixed effects. Benjamini-Hochberg corrections were calculated to correct for the large number of comparisons. Where a significant effect of the time point: treatment interaction was found, OTU abundances were visually inspected and cases where the interaction effect was due to outlier effects were removed.

To investigate the underlying causes of community differences between different mesocosm cores, PERMANOVA tests were carried out using function 'adonis' (Dixon and Palmer 2003) to test whether the effect of core was significant for each habitat-depth subset. To determine the variables underlying the differences in prokaryotic and eukaryotic communities between different cores, a subset of likely variables were selected: pH and the percentage cover of important plant functional groups (mosses, dwarf shrubs and graminoids for the bog; mosses and graminoids in the fen). The cover of each plant functional group was assessed by taking a photo of each core from above, drawing a grid over it in Microsoft Paint, and assessing the number of squares taken up by each functional group. Constrained correspondence analysis (CCA) was then carried out within each habitat-depth subset, and the significance of each variable determined using ANOVA-based permutation tests (function 'anova.cca').

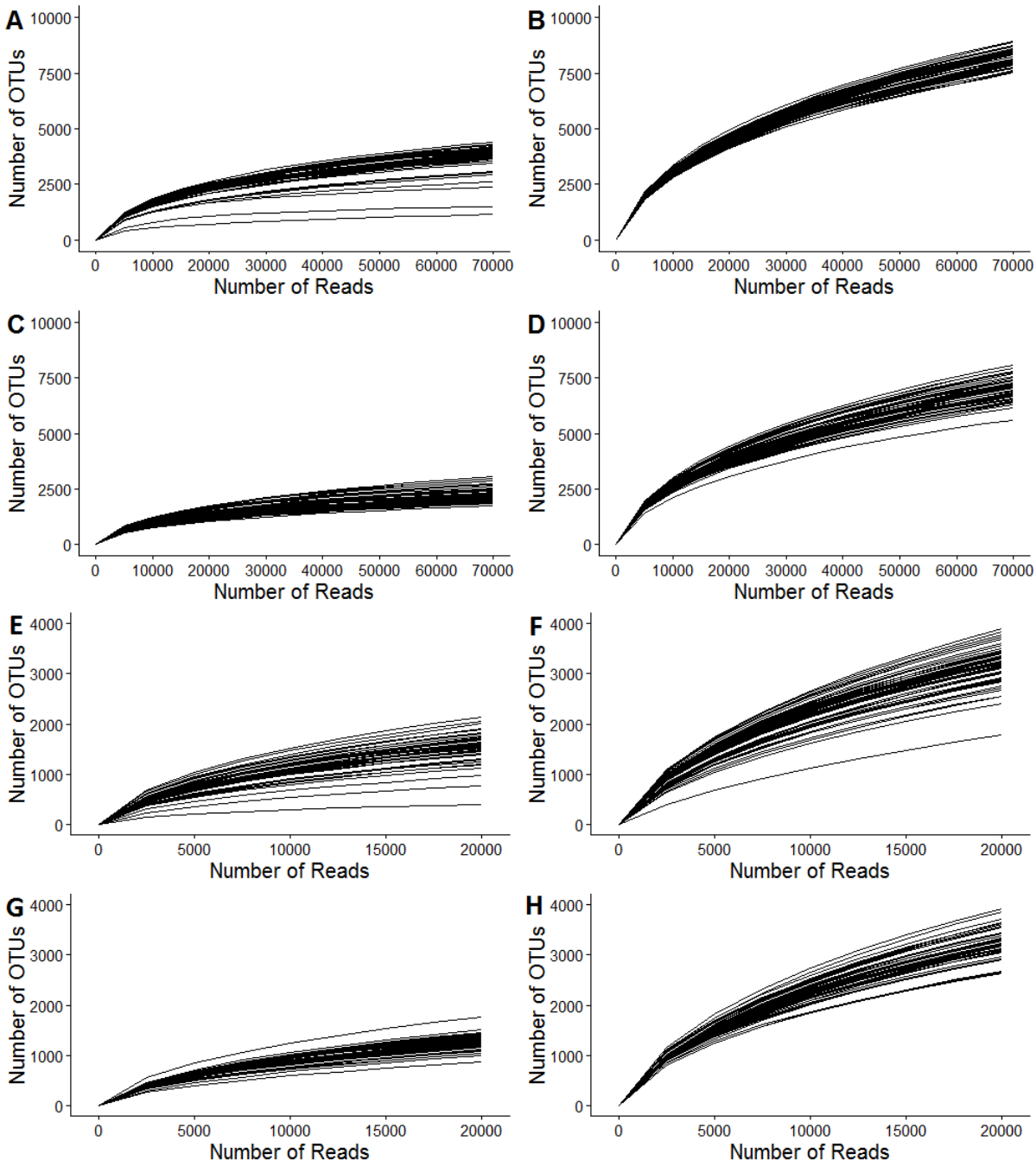

Figure 3.2: Rarefaction analysis of prokaryotic (A-D) and eukaryotic (E-H) communities, with each line representing the results of rarefaction analysis on a single sample. Results are plotted separately for each habitat-depth combination as follows: A and E, bog-5cm; B and F, fen-5cm; C and G, bog-20cm; D and H, fen-20cm. Rarefaction was carried out after standardisation of read numbers within each sample.

3.3 Results

3.3.1 Sequencing and Quality Control

A total of 102,439,895 and 104,156,662 paired-end reads were obtained for 16S and 18S rRNA marker genes, respectively. Of the 16S rRNA reads, 29,337,117 passed initial quality control steps (including 4,184,029 singletons) and were clustered into 49,892 OTUs. A total of 17,214,346 18S rRNA reads passed quality control and paired-end joining, of which 9,600,970 were singletons. The 18S rRNA genes were clustered into 43,058 OTUs. During standardisation of read counts, 10 samples were removed from the 16S rRNA gene dataset and 9 from the 18S rRNA gene dataset as they did not contain the requisite number of reads for the read number standardisation step.

Rarefaction analysis suggested that coverage was adequate in the bog samples at both depths, with rarefaction curves for bog samples reaching a near asymptote for both prokaryotic (Figure 3.2A; Figure 3.2C) and eukaryotic (Figure 3.2E; Figure 3.2G) OTUs. However, both prokaryotic and eukaryotic communities were considerably more diverse in the fen samples, and diversity did not come close to an asymptote in fen samples (Figure 3.2B; Figure 3.2D; Figure 3.2F; Figure 3.2H).

3.3.2 α-Diversity

Habitat and depth significantly affected the α-diversity of both prokaryotes and microbial eukaryotes (Table 3.1; Figure 3.3). The total number of OTUs was significantly higher in the fen than the bog habitat for both 16S and 18S rRNA genes (Figure 3.3). For prokaryotic communities, higher OTU richness was found at 5 cm than 20 cm depth in both habitats; for eukaryotic communities, OTU richness was higher at 5 cm than 20 cm in the bog, but was similar between both depths in the fen. Likewise, Simpson's Index was significantly different between habitats and depths (Table 3.1). The Simpson's Index of prokaryotic communities was lower in the bog at 20 cm than other habitats and depths, while the Simpson's Index of eukaryotic communities was lower in the bog than the fen (Figure 3.3). There was no significant effect of the drought treatment on OTU richness for either prokaryotes or microbial eukaryotes (Table 3.1). However, the Simpson's Index of eukaryotic communities was significantly affected by the interactions between depth and time point and between depth and treatment (Table 3.1). These significant interaction effects were caused by a rise in the

Simpson's Index of droughted mesocosm cores from the bog at 5 cm between time point 3 and time point 5 (Figure 3.4).

Table 3.1: Minimal adequate linear mixed effects model with alpha diversity as the response variables. Non-significant interactions are not shown, with the exception of the interaction between time point and treatment. Significant p-values are denoted by * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). Marginal significance ($p < 0.1$) is denoted by '**.**'. OTUs assigned to the following phyla were excluded from the 18S rRNA gene dataset prior to analysis: Holozoa, Metazoa, Chloroplastida and 'NA'.

Marker Gene	Response	Independent Variable	F	d.f.	p
16S	**OTU Richness**	**Habitat**	1190.7	1, 9	<0.0001***
		Depth	441.2	1, 177	<0.0001***
		Time Point	0.3	8, 177	1
		Treatment	0.4	1, 9	0.6
		Time Point: Treatment	0.5	8, 177	0.9
	Simpson's Index	**Habitat**	667.3	1, 9	<0.0001***
		Depth	582.7	1, 176	<0.0001***
		Time Point	0.3	8, 176	1
		Treatment	0.2	1, 9	0.7
		Habitat: Depth	183	1, 176	<0.0001***
		Time Point: Treatment	0.8	8, 176	0.7
18S	**OTU Richness**	**Habitat**	689.2	1, 9	<0.0001***
		Depth	13.2	1, 177	0.0004**
		Time Point	1.0	8, 177	0.4
		Treatment	0.1	1, 9	0.8
		Habitat: Depth	7.6	1, 177	0.0065**
		Time Point: Treatment	0.9	8, 177	0.6
	Simpson's Index	**Habitat**	189.7	1, 9	<0.0001***
		Depth	0.5	1, 168	0.5
		Time Point	0.4	8, 168	0.9
		Treatment	<0.1	1, 9	1
		Habitat: Depth	69.1	1, 168	<0.0001***
		Depth: Time Point	2.2	8, 168	0.03*
		Depth: Treatment	5.6	1, 168	0.02*
		Time Point: Treatment	0.5	8, 168	0.8

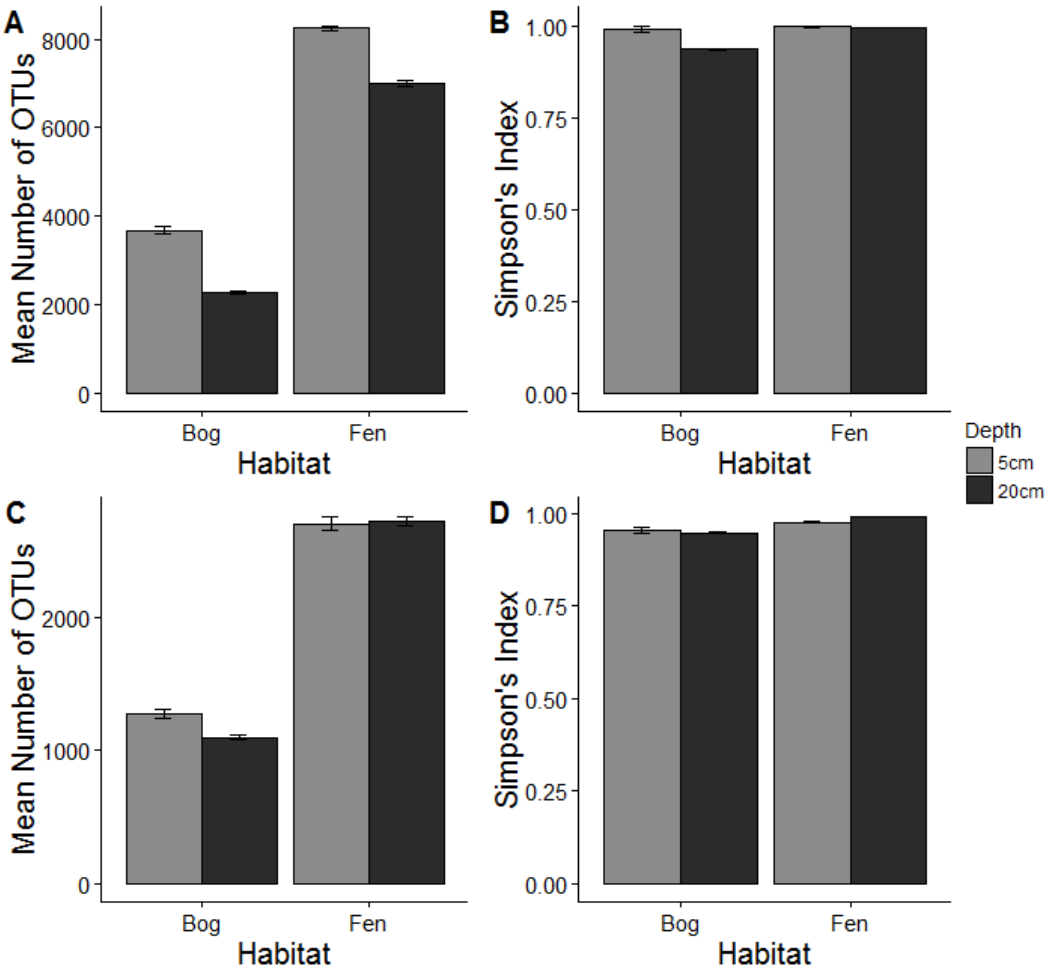

Figure 3.3: OTU richness of prokaryotic (A) and eukaryotic (C) organisms, and Simpson's Index of prokaryotic (B) and eukaryotic (D) organisms. OTUs assigned to the following phyla were excluded from the 18S rRNA gene dataset prior to analysis: Holozoa, Metazoa, Chloroplastida and 'NA'.

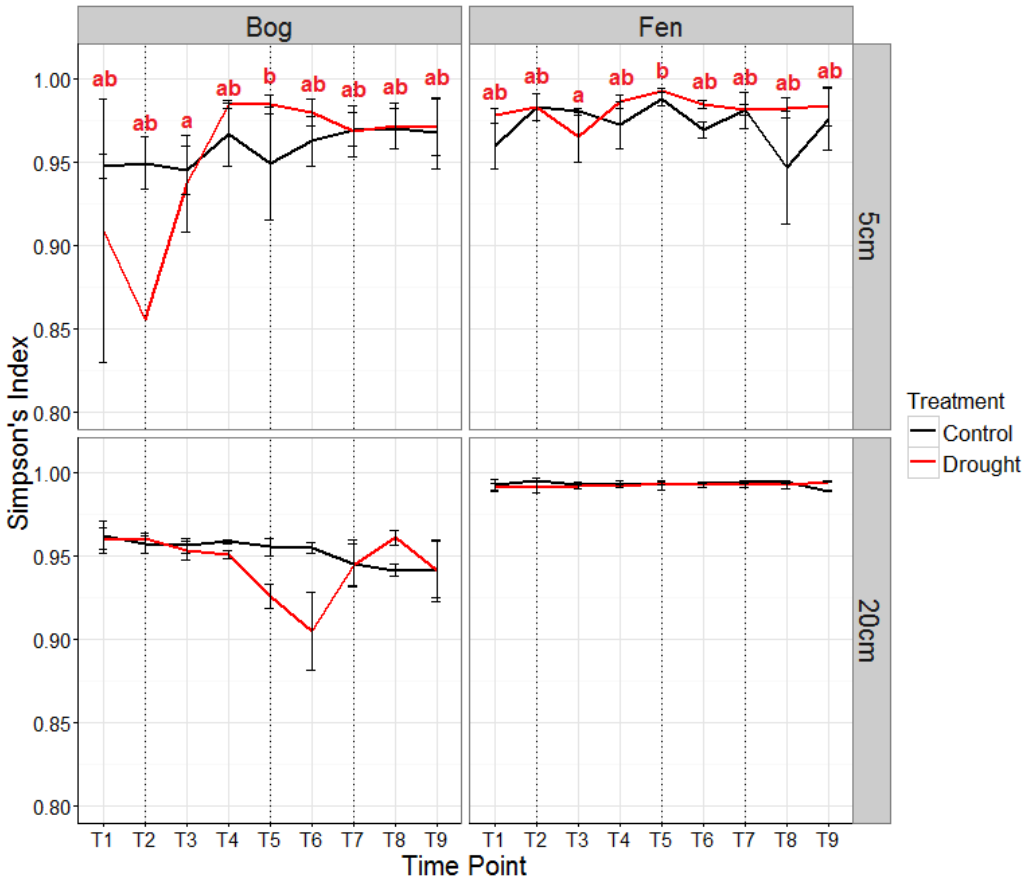

Figure 3.4: Mean Simpson's Index of microbial eukaryote communities by time and treatment. Error bars represent standard errors. Different letters represent time points with significantly different means (Simpson's Index was not significantly different between time points at 20 cm depth, or in the control mesocosm cores).

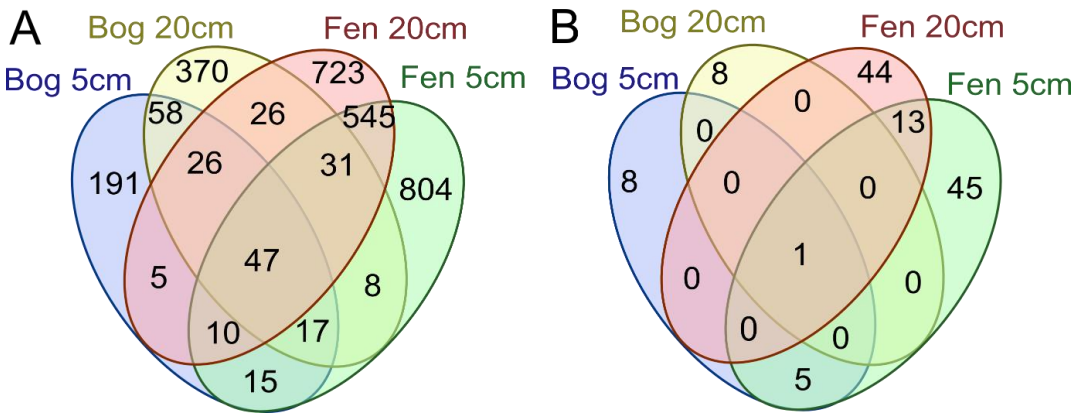

Figure 3.5: Venn diagram depicting the number of (A) prokaryotic and (B) eukaryotic 'core' OTUs (i.e. OTUs present in 95% of samples for a given habitat) shared between the four different habitat-depth combinations. OTUs assigned to the following phyla were excluded from the 18S gene rRNA dataset prior to analysis: Holozoa, Metazoa, Chloroplastida and 'NA'.

Table 3.2: Minimal adequate linear mixed effects model with the proportion of reads assigned to Archaea with a confidence value of 0.85 as the response variable. Non-significant interactions are not shown, with the exception of the interaction between time point and treatment. Significant p-values are denoted by * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). Marginal significance ($p < 0.1$) is denoted by '**.**'.

Factor	d.f.	F	p
Time Point	8, 174	1.0	0.4
Treatment	1, 8	6.8	0.03*
Habitat	1, 8	16.1	0.004**
Depth	1, 174	20.2	<0.0001***
Time Point: Treatment	8, 174	0.8	0.6
Treatment: Depth	1, 174	66.8	<0.0001***
Habitat: Depth	1, 174	32.9	<0.0001***
Treatment: Habitat: Depth	1, 174	30.6	<0.0001***

Corresponding to the higher diversity in the fen habitat (Figure 3.3), the fen had higher numbers of both eukaryotic and prokaryotic 'core' OTUs (cOTUs; OTUs shared by 95% of samples belonging to a given habitat-depth combination) than the bog (Figure 3.5). In the fen more prokaryotic cOTUs were found at 5 cm than 20 cm, matching the patterns of overall species richness, but the opposite pattern was seen in the bog (more cOTUs at 20 cm than 5cm despite higher overall species richness at 5 cm). The number of eukaryotic cOTUs was similar at both depths within both the bog and fen, but considerably more cOTUs were present in the fen than the bog. Relatively few cOTUs were shared between habitats (even when the sampling depth was the same), while a larger proportion were shared between depths in each habitat.

3.3.3 Relative Abundances of Domains & Abundant Phyla

The relative abundance of Archaea was low in all habitats, but was significantly higher in the fen than in the bog (Figure 3. 6A; Table 3.2). There was a significant effect of treatment on the relative abundance of Archaea, as well as a significant two-way interaction between treatment and depth and a significant three-way interaction between treatment, habitat and depth (Table 3.2). However, if differences between treatments were due to the effect of drought then a significant interaction effect between time point and treatment would be expected, but this was not the case and so differences between treatments were likely due to pre-existing community differences between mesocosm cores.

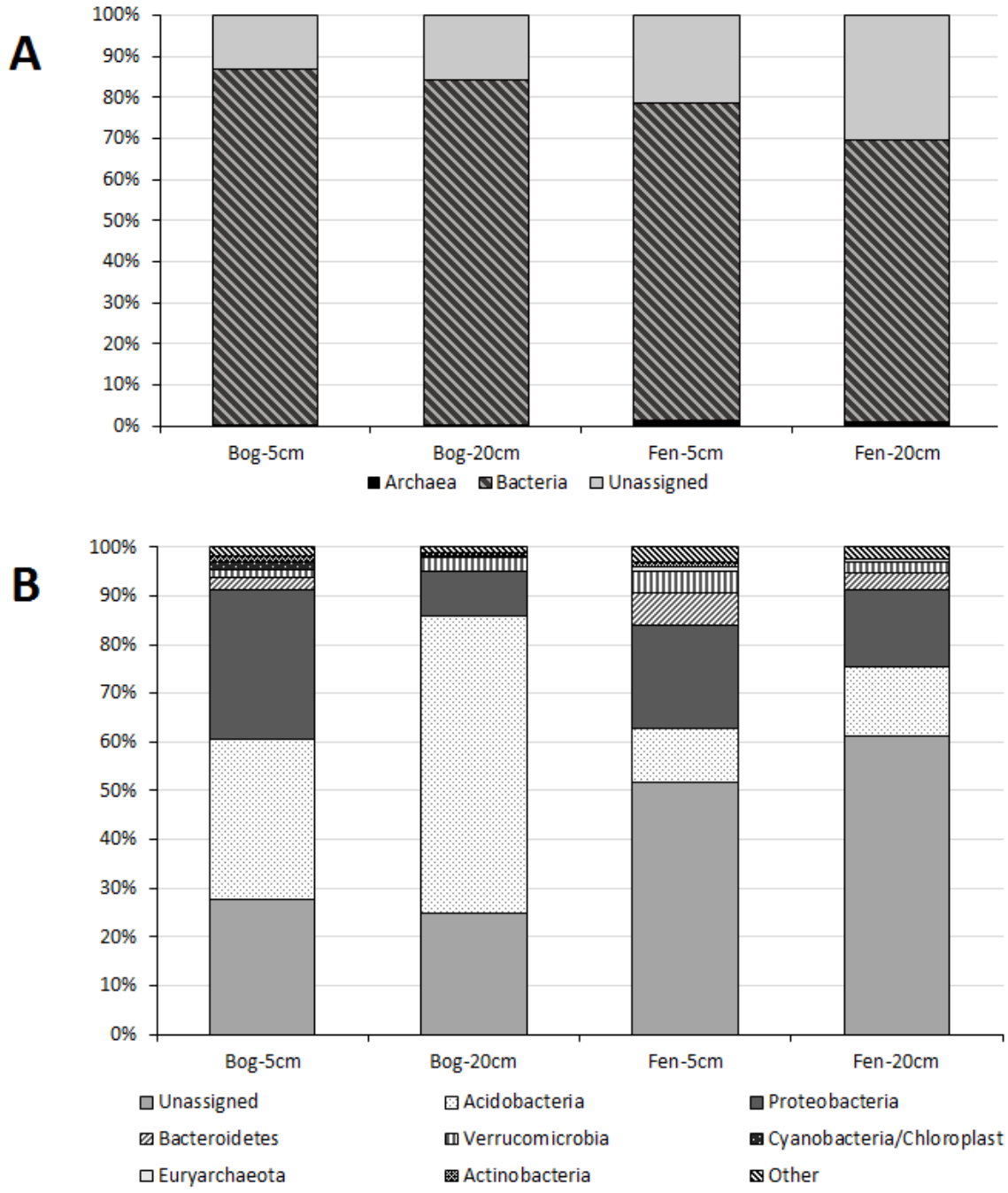

Figure 3.6: Taxonomic assignment of 16S reads, at (A) domain level and (B) phylum level. OTUs which were assigned with a confidence value of <0.85 in utax were classed as 'unassigned'. To remove potential confounding effects of time point and treatment, only samples from time point 2 are included.

Table 3.3: Minimal adequate linear mixed effects model with the proportion of reads assigned to each of the most abundant prokaryotic phyla with a confidence value of 0.85 as the response variable. Non-significant interactions are not shown, with the exception of the interaction between time point and treatment. Significant p-values are denoted by * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). Marginal significance ($p < 0.1$) is denoted by '**.**'.

Phylum	Factor	d.f.	F	p
Acidobacteria	**Habitat**	1, 9	170.3	<0.0001***
	Depth	1, 168	402.8	<0.0001***
	Time Point	8, 168	1.2	0.3
	Treatment	1, 9	<0.1	1
	Habitat:Depth	1, 168	107.0	<0.0001***
	Habitat:Time Point	8, 168	2.0	0.045*
	Time Point: Treatment	8, 168	0.3	1
Actinobacteria	**Habitat**	1, 8	10.2	0.01*
	Depth	1, 166	28.8	<0.0001***
	Time Point	8, 166	0.7	0.7
	Treatment	1, 8	8.3	0.02*
	Habitat:Depth	1, 166	14.9	0.0002***
	Habitat: Treatment	1, 8	5.6	0.045*
	Depth: Treatment	1, 166	34.1	<0.0001***
	Habitat: Depth: Treatment	1, 166	27.7	<0.0001***
Bacteroidetes	**Habitat**	1,8	147.4	<0.0001***
	Depth	1, 174	464.3	<0.0001***
	Time Point	8, 174	0.5	0.9
	Treatment	1, 8	3.0	0.1
	Habitat: Depth	1, 174	130.9	<0.0001***
	Habitat: Treatment	1, 8	5.1	0.055
	Depth: Treatment	1, 174	8.7	0.004**
	Time Point: Treatment	8, 174	0.8	0.6
	Habitat:Depth:Treatment	1, 174	7.6	0.007**
Euryarchaeota	**Habitat**	1, 8	7.9	0.02*
	Depth	1, 174	19.6	<0.0001***
	Time Point	8, 174	1.0	0.4
	Treatment	1, 8	6.8	0.03*
	Habitat: Depth	1, 174	41.7	<0.0001***
	Depth: Treatment	1, 174	60.8	<0.0001***
	Time Point: Treatment	8, 174	0.7	0.7
	Habitat: Depth: Treatment	1, 174	28.1	<0.0001***
Proteobacteria	**Habitat**	1, 9	76.8	<0.0001***
	Depth	1, 176	350.0	<0.0001***
	Time Point	8, 176	0.3	1
	Treatment	1, 9	0.3	0.6
	Habitat: Depth	1, 176	78.2	<0.0001***
	Time Point: Treatment	8, 176	1.0	0.4
Cyanobacteria/ Chloroplast	**Habitat**	1, 9	7.7	0.02*
	Depth	1, 168	293.4	<0.0001***
	Time Point	8, 168	2.2	0.03*
	Treatment	1, 9	2.4	0.2
	Habitat:Depth	1, 168	40.5	<0.0001***
	Habitat: Time Point	8, 168	2.9	0.005**
	Time Point: Treatment	8, 168	0.5	0.8
Verrucomicrobia	**Habitat**	1, 9	1.2	0.3

Depth	1, 173	3.4	0.07.
Time Point	8, 173	0.9	0.5
Treatment	1, 9	2.6	0.1
Habitat: Depth	1, 173	52.6	<0.0001***
Depth: Treatment	1, 173	8.6	0.004**
Time Point: Treatment	8, 173	0.6	0.8
Habitat: Depth: Treatment	2, 173	4.5	0.01*

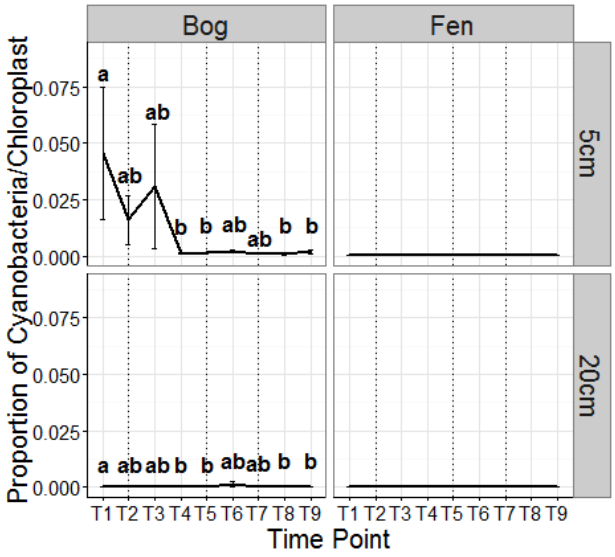

Figure 3.7: Mean proportion of 16S rRNA genes assigned to Cyanobacteria/Chloroplast DNA. Error bars represent standard errors. Different letters represent time points where Cyanobacteria/Chloroplasts make up significantly different proportions of the community.

The most abundant prokaryotic phyla by far were Acidobacteria and Proteobacteria (Figure 3.6B). Four other bacterial phyla were present at relative abundances >1%: Bacteroidetes, Actinobacteria, Verrucomicrobia and Cyanobacteria/Chloroplasts. Euryarchaota were the only phylum within the Archaea to make up >1% of prokaryotic reads. However, many reads (25-61%) could not be assigned to phylum level with a confidence value of 0.85 or greater. Both the main effects of habitat and depth and the interaction between habitat and depth significantly affected the proportion of almost all of the seven most abundant bacterial phyla: the only exception was Verrucomicrobia, the proportion of which was significantly affected by the interaction term but not the main effects of habitat and depth (Table 3.3). In particular, Acidobacteria was highest in the bog at 20 cm; Bacteroidetes were most abundant in the fen at 5 cm; Actinobacteria were more abundant in the bog than the fen; and Proteobacteria were most abundant in the fen at 5 cm (Figure 3.6B). Treatment had a significant effect on the

relative abundances of most of the abundant bacterial phyla, as did interactions between treatment and habitat or treatment and depth (Table 3.3). However, the interaction between time point and treatment was not significant for any of the phyla tested, suggesting that the treatment effect was an artefact of pre-existing community differences between mesocosm cores (Section 3.3.6). Time point had a significant effect on the proportion of reads assigned to Cyanobacteria/Chloroplasts (Table 3.3), with a sharp fall in the abundance of Cyanobacteria occurring after time point three in the bog at 5 cm (Figure 3.7).

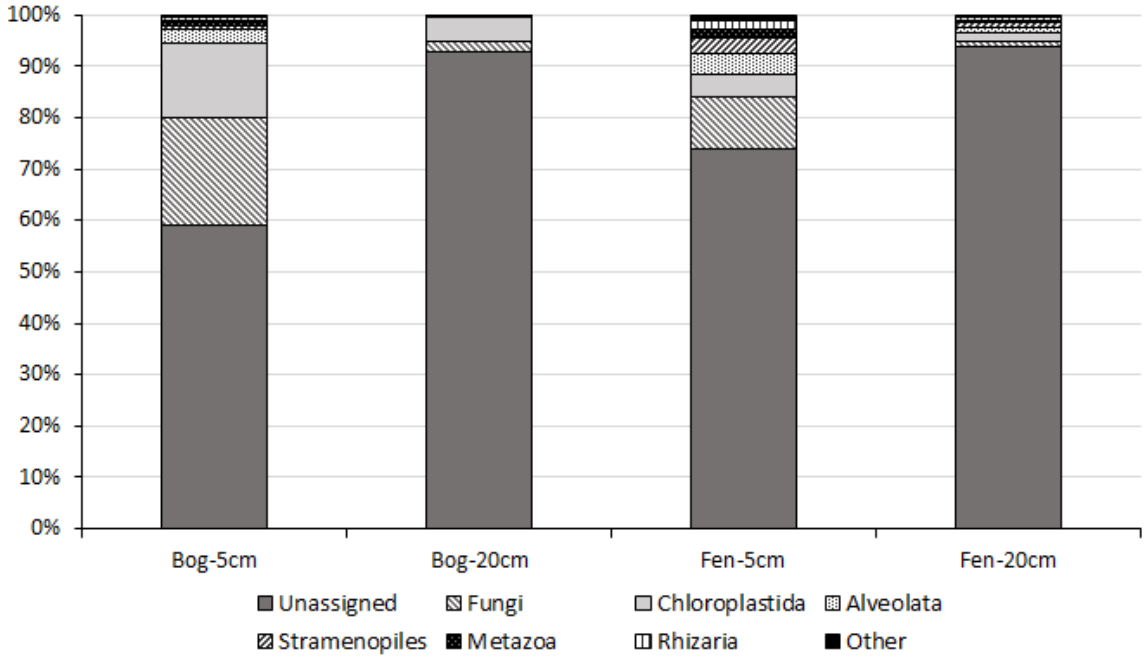

Figure 3.8: Taxonomic assignment of 18S reads at phylum level. OTUs which were assigned with a confidence value of <0.85 in utax were classed as 'unassigned'. To remove potential confounding effects of time point and treatment, only samples from time point 2 are included.

The majority of eukaryotic reads belonged to OTUs which could not be assigned to a phylum level with a confidence value of 0.85 or greater, particularly at 20 cm depth (Figure 3.8). Within the OTUs which could confidently be assigned to a phylum, the two most abundant phyla were Fungi and Chloroplastida. Alveolata, Stramenopiles, Metazoa and Rhizaria also made up a mean of >1% of the community in at least one habitat type. Four of the six eukaryotic phyla tested were significantly more abundant at 5 cm than 20 cm: these were Fungi, Alveolata,

Metazoa and Rhizaria (Figure 3.8; Table 3.4). The effect of habitat on the abundances of eukaryotic phyla was weaker than the effect of depth, although Alveolata, Stramenopiles and Rhizaria had significantly higher relative abundances in the fen than the bog (Figure 3.8; Table 3.4). The relative abundances of four of the six eukaryotic phyla were significantly affected by interactions involving treatment and other factors (Table 3.4). Only in the case of Rhizaria was the interaction between time point and treatment significant: this represented a large increase in the relative abundance of Rhizaria at minimum water table in the fen at 5 cm (Figure 3.9).

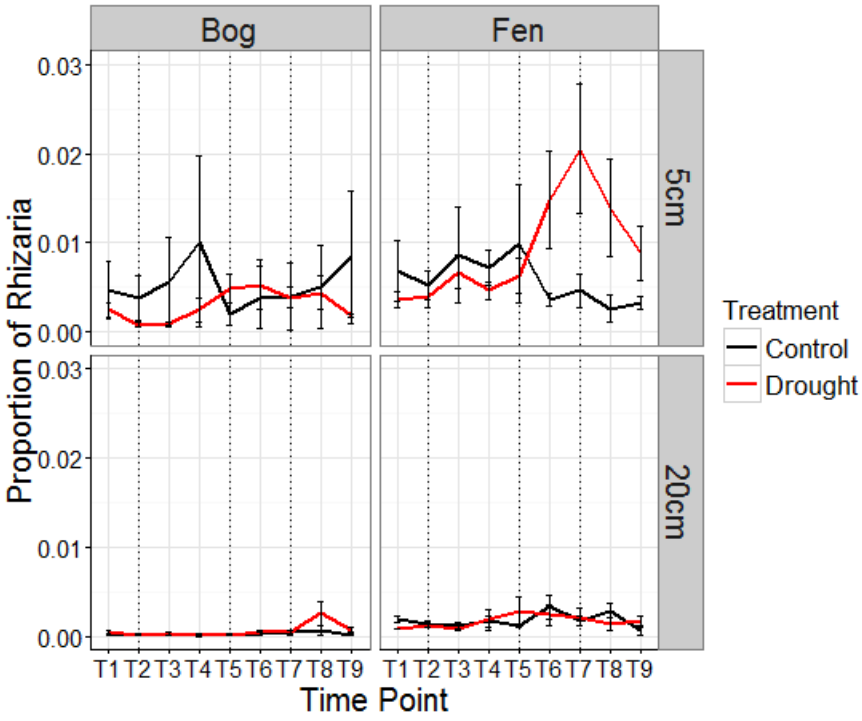

Figure 3.9: Mean proportion of 18S rRNA genes belonging to OTUs which were assigned to phylum Rhizaria. Error bars represent standard errors.

Table 3.4: Minimal adequate linear mixed effects model with the proportion of reads assigned to the most abundant eukaryotic phyla with a confidence value of 0.85 as the response variable. Non-significant interactions are not shown, with the exception of the interaction between time point and treatment. Significant p-values are denoted by * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). Marginal significance ($p < 0.1$) is denoted by '**.**'.

Phylum	Factor	d.f.	F	p
Fungi	**Habitat**	1, 8	3.4	0.1
	Depth	1, 175	149.0	<0.0001***
	Time Point	8, 175	0.9	0.5
	Treatment	1, 8	0.1	0.8
	Habitat: Depth	1, 175	5.7	0.02*
	Habitat: Treatment	1, 8	4.2	0.07.
	Depth: Treatment	1, 175	14.9	0.0002***
	Time Point: Treatment	8, 175	1.0	0.5
	Habitat: Depth: Treatment	1, 175	17.0	0.0001***
Chloroplastida	**Habitat**	1, 8	2.4	0.2
	Depth	1, 175	0.6	0.4
	Time Point	8, 175	0.5	0.8
	Treatment	1, 8	<0.1	0.8
	Depth:Treatment	1, 175	18.9	<0.0001***
	Time Point: Treatment	8, 175	0.7	0.7
	Habitat: Depth: Treatment	1, 175	24.4	<0.0001***
Alveolata	**Habitat**	1, 9	100.1	<0.0001***
	Depth	1, 177	330.7	<0.0001***
	Time Point	8, 177	0.9	0.6
	Treatment	1, 9	1.5	0.3
	Habitat: Depth	1, 177	58.8	<0.0001***
	Time Point: Treatment	8, 177	0.4	0.9
Stramenopiles	**Habitat**	1, 8	23.1	0.001**
	Depth	1, 175	2.9	0.09.
	Time Point	8, 175	0.9	0.5
	Treatment	1, 8	2.4	0.2
	Habitat: Depth	1, 175	15.5	0.0001***
	Depth: Treatment	1, 175	9.7	0.002***
	Time Point: Treatment	8, 175	1.1	0.4
	Habitat: Depth: Treatment	1, 175	8.6	0.004**
Metazoa	**Habitat**	1, 9	2.7	0.1
	Depth	1, 178	439.2	<0.0001***
	Time Point	8, 178	0.8	0.6
	Treatment	1, 9	0.1	0.8
	Time Point: Treatment	8, 178	0.9	0.5
Rhizaria	**Habitat**	1, 9	24.1	0.0008***
	Depth	1, 177	217.1	<0.0001***
	Time Point	8, 177	0.4	0.9
	Treatment	1, 9	0.1	0.8
	Habitat: Depth	1, 177	18.5	<0.0001***

Time Point: Treatment	8, 177	2.6	0.009**

Table 3.5: PERMANOVA tests for the effect of habitat, depth and the interaction between habitat and depth on the community composition of prokaryotes (16S rRNA genes) and eukaryotes (18S rRNA genes). OTUs assigned to the following phyla were excluded from the 18S rRNA gene dataset prior to analysis: Holozoa, Metazoa, Chloroplastida and 'NA'.

Marker	Factor	d.f.	F	R^2	p
16S rRNA gene	**Habitat**	1	228.0	0.38	0.001***
	Depth	1	94.6	0.16	0.001***
	Habitat: Depth	1	82.5	0.14	0.001***
18S rRNA	**Habitat**	1	25.8	0.10	0.001***
	Depth	1	13.7	0.05	0.001***
	Habitat: Depth	1	11.3	0.04	0.001***

Table 3.6: PERMDISP tests for the effect of habitat and depth on the multivariate homogeneity of group dispersions, implemented using the 'betadisper' function from R package 'VEGAN'. OTUs assigned to the following phyla were excluded from the 18S rRNA gene dataset prior to analysis: Holozoa, Metazoa, Chloroplastida and 'NA'.

Marker	Factor	d.f.	F	p
16S rRNA gene	**Habitat**	1	29.0	<0.0001***
	Depth	1	4.5	0.04*
18S rRNA gene	**Habitat**	1	109.3	<0.0001***
	Depth	1	33.6	<0.0001***

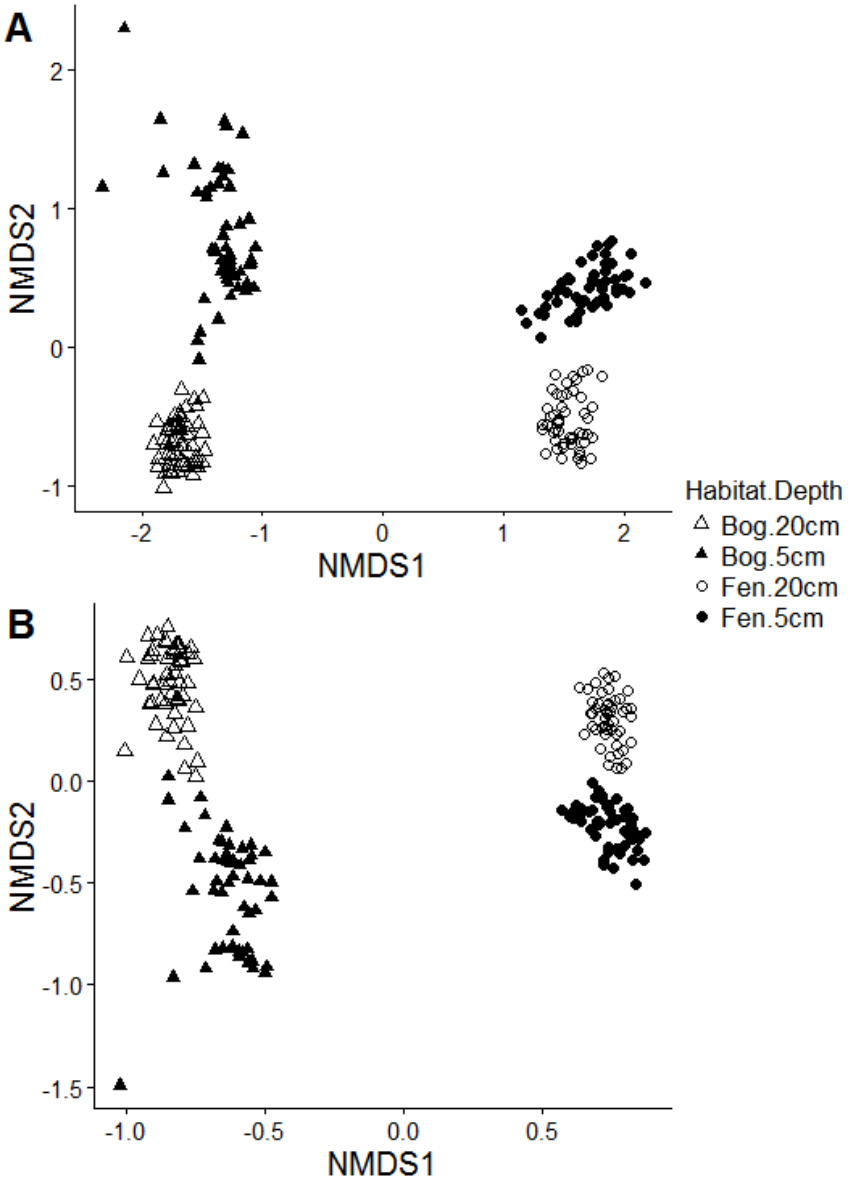

Figure 3.10: NMDS ordination of (A) prokaryotic and (B) eukaryotic communities. NMDS ordination was calculated based on Bray-Curtis dissimilarities. OTUs assigned to the following phyla were excluded from the 18S rRNA gene dataset prior to analysis: Holozoa, Metazoa, Chloroplastida and 'NA'.

3.3.4 Overall Community Composition

Large and consistent differences in eukaryotic and prokaryotic community composition were found between different habitats and depths: NMDS plots of both communities depict the two habitats separating along the first axis, with the two depths within each habitat separating along the second axis (Figure 3.10). PERMANOVA tests found that communities of both prokaryotes

and microbial eukaryotes were significantly different between habitats and depths, with a significant interaction between habitat and depth (Table 3.5). Multivariate dispersions were significantly different between habitats and depths (Table 3.6), meaning that the results of PERMANOVA tests should be viewed with caution, but the strong significance of the results and the fact that PERMANOVA is fairly robust to heterogeneous dispersions (Anderson and Walsh 2013) suggest that the effect of habitat and depth is real.

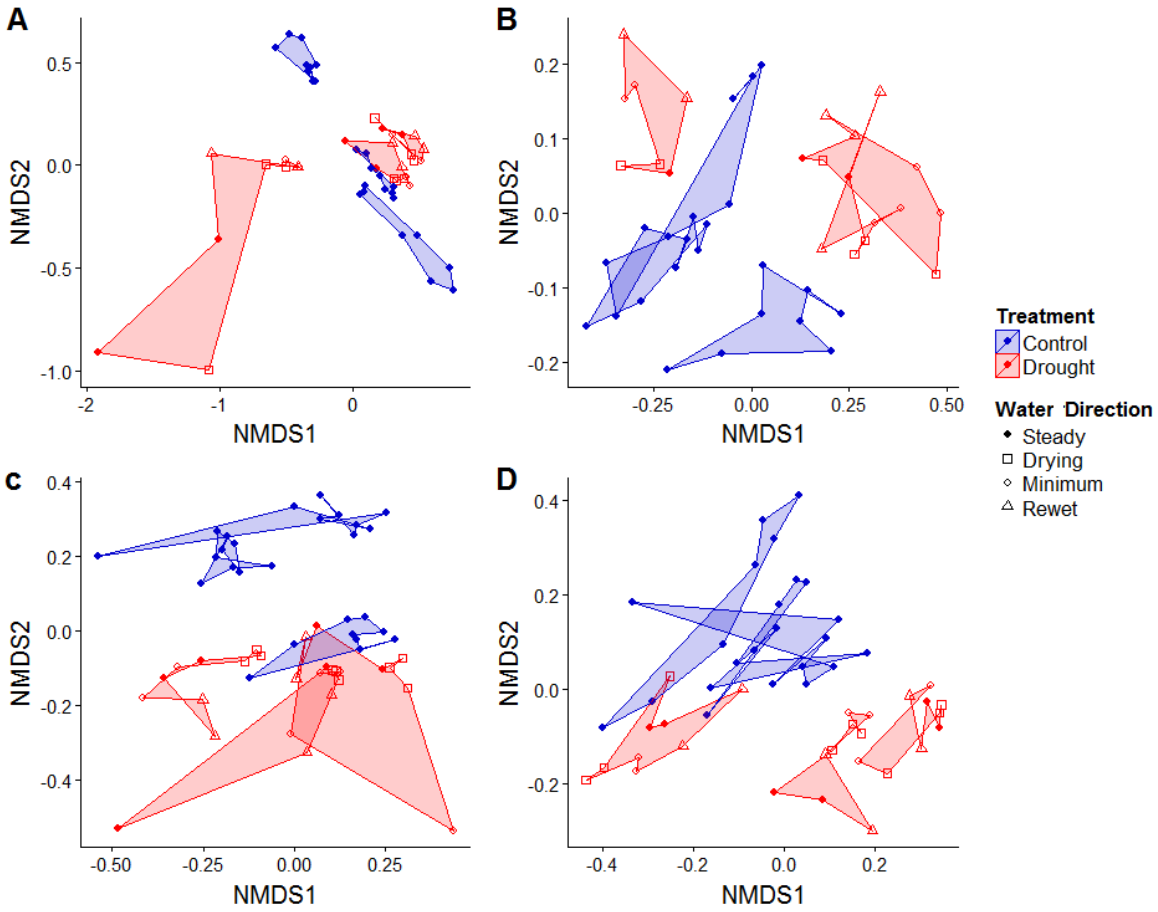

Figure 3.11: NMDS ordination of prokaryotic communities within each of the four habitat-depth combinations sampled: (A) Bog-5cm, (B) Fen-5cm, (C) Bog-20cm, and (D) Fen-20cm. All samples taken from a given core are connected to form polygons.

There was no significant effect of the interaction between time point and treatment on community composition (Table 3.7). While significant differences existed between the two treatments within every combination of habitat, depth and marker gene (Table 3.7), Figures 3.11 and 3.12 show that control and drought cores differed at all time points rather than only

differing during the drought period. For eukaryotic OTUs, multivariate dispersion was significantly different between treatment and control samples in the fen at 20 cm (Table 3.8).

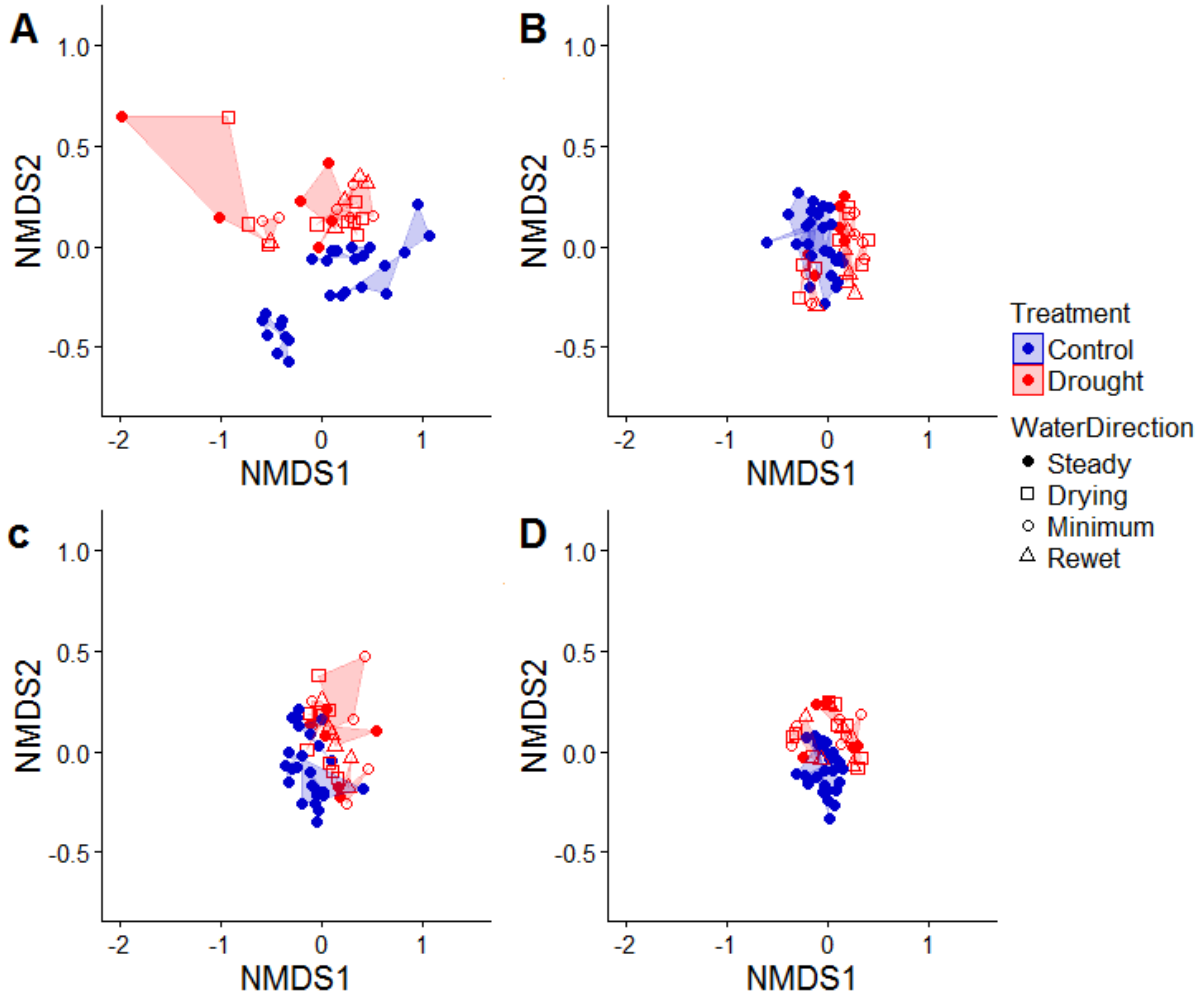

Figure 3.12: NMDS ordination of eukaryotic communities within each of the four habitat-depth combinations sampled: (A) Bog-5cm, (B) Fen-5cm, (C) Bog-20cm, and (D) Fen-20cm. All samples taken from a given core are connected to form polygons. OTUs assigned to the following phyla were excluded from the dataset prior to analysis: Holozoa, Metazoa, Chloroplastida and 'NA'.

3.3.5 Effect of Water Table

Partial constrained correspondence analysis (pCCA) was calculated with water direction as the constraining variable and core as the conditioning variable, in order to test for an effect of water direction with the effect of core removed. Constrained correspondence analysis is a method for

displaying variation in multivariate datasets which can be accounted for by a chosen set of variables (constraints), and thus is useful when a particular hypothesis about the effect of a certain variable is to be tested. Application of significance tests to pCCA found significant variation in community composition of prokaryotes with water table direction in both habitats at 5 cm depth, and in the fen at 20 cm depth (Table 3.9). Significant variation with water table direction was also found for the community composition of eukaryotes in both the bog and the fen at 5 cm depth (Table 3.9).

Table 3.7: PERMANOVA tests for the effect of time point, treatment and the time point: treatment interaction term on the community composition of prokaryotes (16S rRNA genes) and eukaryotes (18S rRNA genes). Significant p-values are denoted by * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). Marginal significance ($p < 0.1$) is denoted by '**.**'. PERMANOVA was carried out separately for each combination of habitat and depth and for both marker genes. OTUs assigned to the following phyla were excluded from the 18S rRNA gene dataset prior to analysis: Holozoa, Metazoa, Chloroplastida and 'NA'.

Marker Gene	Habitat-Depth	Factor	d.f.	F	R^2	p
16S rRNA	**Bog-5cm**	**Time Point**	8	0.6	0.58	1
		Treatment	1	3.2	0.07	0.002**
		Time Point: Treatment	8	0.4	0.07	1
	Fen- 5cm	**Time Point**	8	0.8	0.13	0.9
		Treatment	1	5.3	5.35	0.001**
		Time Point: Treatment	8	0.7	0.68	1
	Bog- 20cm	**Time Point**	8	0.4	0.07	1
		Treatment	1	8.3	0.17	0.001**
		Time Point: Treatment	8	0.3	0.04	1
	Fen- 20cm	**Time Point**	8	0.6	0.11	1
		Treatment	1	5.6	0.12	0.001**
		Time Point: Treatment	8	0.5	0.09	1
18S rRNA	**Bog-5cm**	**Time Point**	8	0.1	0.13	1
		Treatment	1	3.6	0.07	0.001**
		Time Point: Treatment	8	0.8	0.12	1
	Fen- 5cm	**Time Point**	8	0.8	0.13	1
		Treatment	1	2.4	0.47	0.001**
		Time Point: Treatment	8	0.8	0.13	1
	Bog- 20cm	**Time Point**	8	1.0	0.15	1
		Treatment	1	1.5	0.03	0.001
		Time Point: Treatment	8	0.9	0.15	1
	Fen- 20cm	**Time Point**	8	0.9	0.15	1
		Treatment	1	2.3	0.05	0.001**
		Time Point: Treatment	8	0.8	0.14	1

The OTUs exhibited a much smaller range of scores than the sites, with 'tails' of many points following the arrows which represent each water direction (Figure 3.13B; Figure 3.13D; Figure 3.13F; Figure 3.13H); this indicates that the effect of water table direction was not primarily driven by 'outlier' OTUs. However, visualisation of the CCA ordinations suggests that the effect of water direction in the bog at 5 cm depth was primarily driven by a single core for both prokaryotes and eukaryotes (Figure 3.13A; Figure 3.13G), with the remaining cores responding weakly (eukaryotic communities; Figure 3.13B) or not at all (prokaryotic communities; Figure 3.13A). Similarly, the pattern seen in the bog at 20 cm suggests that the effect of water direction is primarily observed as a result of outlier effects and differences between cores (Figure 3.13E). Only in the case of communities in the fen at 5 cm does visualisation of CCA suggest a consistent effect of drought across all treated cores (Figure 3.13C; Figure 3.13G).

Table 3.8: PERMDISP tests for the effect of time point and treatment on the multivariate homogeneity of group dispersions, implemented using the 'betadisper' function from R package 'VEGAN'. Significant p-values are denoted by * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). Marginal significance ($p < 0.1$) is denoted by '**.**'. PERMDISP was carried out separately for each combination of habitat and depth and for both marker genes. OTUs assigned to the following phyla were excluded from the 18S rRNA gene dataset prior to analysis: Holozoa, Metazoa, Chloroplastida and 'NA'.

Marker Gene	Habitat-Depth	Factor	d.f.	F	p
16S rRNA	**Bog-5cm**	**Time Point**	8	0.2	1
		Treatment	1	0.1	0.8
	Fen-5cm	**Time Point**	8	1	0.4
		Treatment	1	0.9	0.3
	Bog-20cm	**Time Point**	8	0.2	1
		Treatment	1	1.3	0.3
	Fen-20cm	**Time Point**	8	0.2	1
		Treatment	1	2.2	0.1
18S rRNA	**Bog-5cm**	**Time Point**	8	0.9	0.5
		Treatment	1	0.1	0.8
	Fen-5cm	**Time Point**	8	1.0	0.4
		Treatment	1	1.6	0.2
	Bog-20cm	**Time Point**	8	1.2	0.3
		Treatment	1	0.9	0.4
	Fen-20cm	**Time Point**	8	0.5	0.8
		Treatment	1	12.1	0.001**

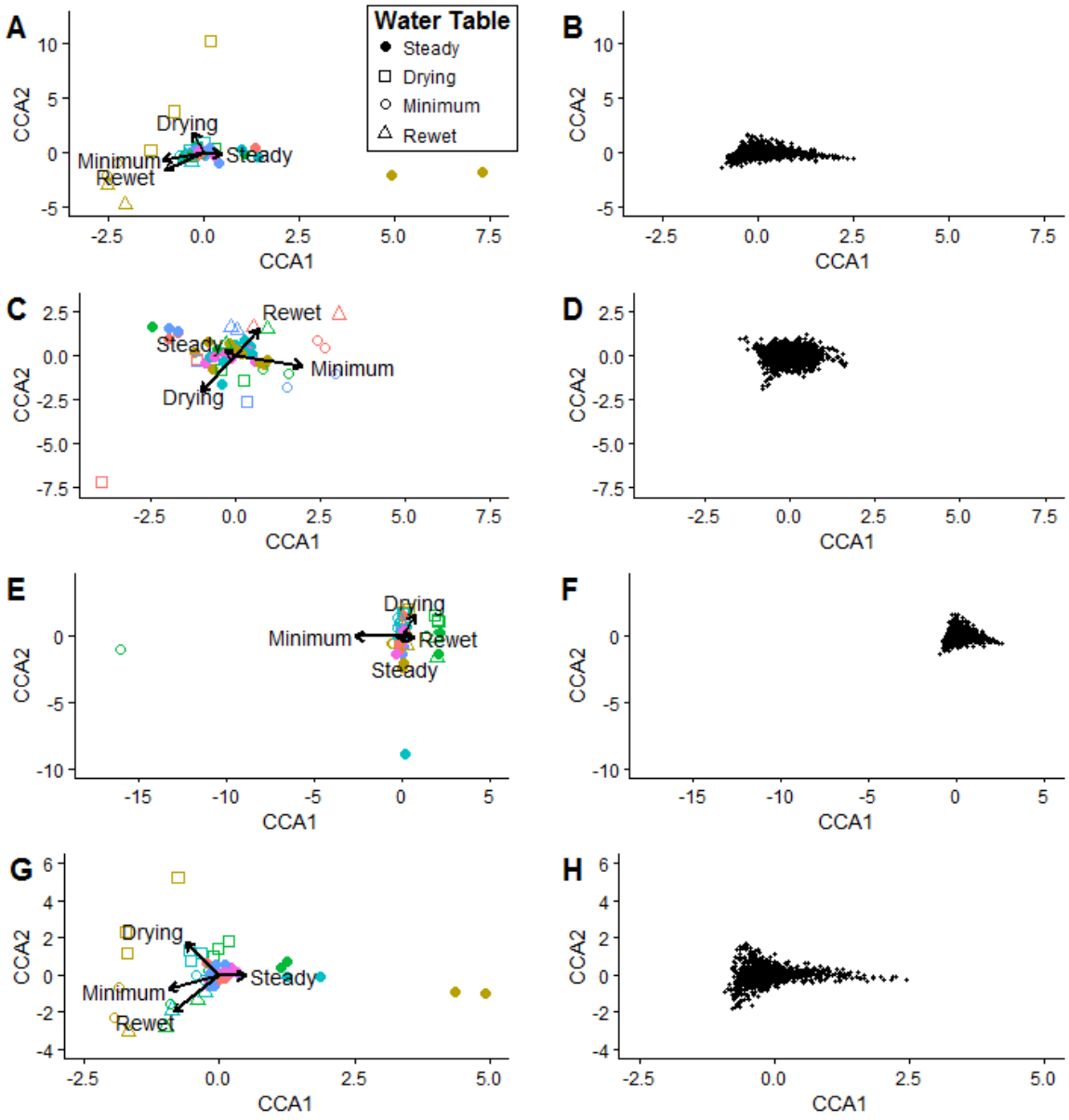

Figure 3.13: Partial constrained correspondence analysis of prokaryotic (A-F) and eukaryotic (G & H) communities, with water table direction as the only constraint. Plots on the left-hand side (A, C, E, G) show samples, with the shape of each point representing water table direction, while plots on the right-hand side (B, D, F, H) show OTUs. On plots A, C, E & G different cores are represented by different colours. Each row represents a different habitat-depth combination: A & B = bog-5cm, C & D = fen- 5cm, E & F = bog- 20cm, G & H = bog- 5cm (eukaryotes), I & J- fen 5cm (eukaryotes). OTUs assigned to the following phyla were excluded from the 18S rRNA gene dataset prior to analysis: Holozoa, Metazoa, Chloroplastida and 'NA'.

Next, linear-mixed effects models were applied to extract the subset of the community which responded significantly to drought, in a more robust manner than extracting OTUs based on

the scores in the CCA ordination. A significant interaction between time point and treatment was found for a number of OTUs, which were manually filtered to remove OTUs which were significant due to outlier effects. These steps yielded a final subset of OTUs showing a significant response to drought, hereafter referred to as 'drought-affected OTUs' (Tables 3.10-3.13). When NMDS ordination was carried out with an input matrix containing the abundances of drought-affected OTUs only, a strong effect of core was observed in the bog samples at 5 cm (Figure 3.14C). However, NMDS ordination of drought-affected prokaryotes in the fen at both 5 cm and 20 cm depth yielded a clear separation between samples taken from droughted cores during drought treatment and all other samples (i.e. samples taken from control cores at all time points, and samples from drought cores at pre-drought time points), although the effect was greater at 5 cm depth (Figure 3.14A; Figure 3.14D). NMDS plots of drought-affected eukaryotes in the fen at 5 cm did not show a strong separation of drought and control samples (Figure 3.14B).

Following Benjamini-Hochberg corrections for multiple comparisons, the only OTUs for which the interaction between time point and treatment had a significant effect were two Bacteria in the fen at 5 cm (Table 3.10): one of these belonged to the β-Proteobacteria and one to the γ-Proteobacteria. However, prior to the application of multiple corrections, many more OTUs were significantly affected by the interaction between time point and treatment: a total of 37 prokaryotes and seven eukaryotes in the fen at 5 cm; four prokaryotes and three eukaryotes in the bog at 5 cm; five prokaryotes and one eukaryote in the fen at 20 cm; and two prokaryotes in the bog at 20 cm (Tables 3.10-3.13).

Taxonomic assignment of drought-affected OTUs in the fen at 5 cm found that the majority of prokaryotes belonged to domain Bacteria, with only one OTU assigned to the Archaea (Table 3.10). Drought-affected OTUs in this habitat were dominated by Proteobacteria (15 OTUs) and Bacteroidetes (14 OTUs). A single eukaryotic OTU was assigned to each of Rhizaria, Alveolata and Metazoa by utax, with the remaining four OTUs unable to be assigned at Phylum level with a utax confidence value of 0.85; nonetheless the most probable domain for three of these was Rhizaria. There appeared to be a taxonomic pattern to the responses of different OTUs to drought, with 12 of the 14 Bacteroidetes OTUs decreasing in response to drought while 11 of the 15 OTUs assigned to Proteobacteria increased in proportional abundance (Figure 3.15; Table 3.10).

Table 3.9: ANOVA-like permutation tests for the effect of a single constraint (water direction) within partial constrained correspondence analysis (pCCA). The core from which each sample was taken was included as a conditioning variable, meaning that core effects were removed prior to fitting of constraints. CCA was carried out separately for each combination of habitat and depth, for both marker genes. Significant p-values are denoted by * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). Marginal significance ($p < 0.1$) is denoted by '**.**'. OTUs assigned to the following phyla were excluded from the 18S rRNA gene dataset prior to analysis: Holozoa, Metazoa, Chloroplastida and 'NA'.

Marker	Water	F	d.f.	p
16S rRNA Gene	**Bog-5cm**	1.8	3	0.006**
	Fen-5cm	1.4	3	0.014*
	Bog-20cm	2.2	3	0.03*
	Fen-20cm	0.9	3	0.7
18S rRNA Gene	**Bog-5cm**	1.4	3	0.001**
	Fen-5cm	1.2	3	0.025*
	Bog-20cm	1.1	3	0.4
	Fen-20cm	1.0	3	0.6

Drought-affected OTUs in the bog at 5 cm included two which were assigned to Acidobacteria, one assigned to Proteobacteria and one assigned to Bacteroidetes (Table 3.11). Of the eukaryotic drought-affected OTUs, two were assigned to the Rhizaria and one could not be assigned at phylum level. All of the drought-affected OTUs detected in the bog at 5 cm appeared to show a positive response to drought (Figure 3.16). In the fen at 20 cm, two OTUs showed a positive response to drought (Figure 3.17): OTU_75 from the 16S rRNA genes dataset (assigned to Acidobacteria) and OTU_40802 from the 18S rRNA genes (assigned to Stramenopiles). The remaining OTUs appeared to show a negative response to drought (Figure 3.17), and could not be assigned to a phylum with a confidence value of 0.85 (Table 3.12). Only two OTUs in the bog at 20 cm showed a significant response to the interaction between time point and treatment, both of which were assigned to the Acidobacteria (Table 3.13) and appeared to show a positive response to drought (Figure 3.18).

Table 3.10: Linear mixed-effects models on logit-transformed abundances of prokaryotes (16S rRNA gene) and eukaryotes (18S rRNA gene) in the fen at 5 cm. Adjusted p-values (Benjami-Hochberg correction) are shown in column 'p.adj'. Only OTUs with an (unadjusted) p-value of <0.05 and for which the interaction between time point and treatment was not due to outliers are shown. Significant adjusted p-values are denoted by * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). Marginal significance ($p < 0.1$) is denoted by '**.**'. Taxonomy was assigned using the RDP Classifier web server, as this was found to classify OTUs with more confidence than utax. Column 'graph' refers to the graph on Figure 3.15 which corresponds to each OTU.

	OTU	F	d.f.	p	p.adj	Graph	Taxonomy
16S	OTU_503	8.2	8,28	<0.0001	0.008**	A	Bacteria; Proteobacteria; Beta-Proteobacteria
	OTU_469	6.7	8,28	<0.0001	0.046*	B	Bacteria; Proteobacteria; Gamma-Proteobacteria
	OTU_871	6.0	8,28	0.0002	0.1	C	Bacteria; Proteobacteria; Beta-Proteobacteria; Burkholderiales; Oxalobacteraceae; *Massalia*
	OTU_699	5.4	8,28	0.004	0.3	D	Bacteria; Bacteroidetes
	OTU_226	5.2	8,28	0.0005	0.4	E	Bacteria; Bacteroidetes
	OTU_744	5.1	8,28	0.0005	0.4	F	Bacteria; Proteobacteria; Alpha-Proteobactera; Rhizobiales
	OTU_473	5.0	8,28	0.0007	0.5	G	Bacteria; Bacteroidetes
	OTU_1031	4.9	8,28	0.0007	0.5	H	Bacteria; Bacteroidetes
	OTU_1076	4.9	8,28	0.0007	0.5	I	Bacteria; Bacteroidetes
	OTU_845	4.2	8,28	0.002	1	J	Bacteria
	OTU_943	4.1	8,28	0.002	1	K	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromandaceae; *Paludibacter*
	OTU_20783	4.0	8,28	0.002	1	L	Bacteria; Bacteroidetes
	OTU_13911	3.9	8,28	0.004	1	M	Bacteria; Proteobacteria
	OTU_290	3.8	8,28	0.004	1	N	Bacteria
	OTU_695	3.7	8,28	0.004	1	O	Bacteria; Bacteroidetes; Spingobacteriia; Sphingobacteriales
	OTU_381	3.7	8,28	0.005	1	P	Bacteria; Proteobacteria; Beta-Proteobacteria; Burkholderiales; Oxalobacteraceae; *Duganella*
	OTU_204	3.6	8,28	0.005	1	Q	Bacteria
	OTU_2224	3.4	8,28	0.007	1	R	Bacteria
	OTU_932	3.2	8,28	0.010	1	S	Bacteria; Proteobacteria; Delta-Proteobacteria
	OTU_836	3.2	8,28	0.011	1	T	Bacteria; Bacteroidetes
	OTU_1515	3.1	8,28	0.012	1	U	Bacteria; Proteobacteria

	OTU_720	3.0	8,28	0.014	1	V	Bacteria; Proteobacteria; Alpha-Proteobacteria; Caulobacterales; Caulobacteraceae; *Caulobacter*
	OTU_1109	3.0	8,28	0.015	1	W	Bacteria; Proteobacteria
	OTU_515	3.0	8,28	0.015	1	X	Archaea; Pacearchaeota
	OTU_405	3.0	8,28	0.016	1	Y	Bacteria; Bacteroidetes
	OTU_793	2.9	8,28	0.017	1	Z	Bacteria; Bacteroidetes
	OTU_15950	2.8	8,28	0.022	1	AA	Bacteria; Proteobacteria; Delta-Proteobacteria; Desulfuromonadales; Geobacteraceae; *Geobacter*
	OTU_3414	2.7	8,28	0.024	1	AB	Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae
	OTU_545	2.7	8,28	0.026	1	AC	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales
	OTU_2189	2.6	8,28	0.027	1	AD	Bacteria; Bacteroidetes
	OTU_2114	2.6	8,28	0.028	1	AE	Bacteria
	OTU_601	2.6	8,28	0.032	1	AF	Bacteria; Proteobacteria; Delta-Proteobacteria
	OTU_1143	2.4	8,28	0.040	1	AG	Bacteria; Bacteroidetes
	OTU_14319	2.3	8,28	0.045	1	AH	Bacteria; Acidobacteria; Group 6
	OTU_26696	2.3	8,28	0.046	1	AI	Bacteria; Proteobacteria; Alpha-Proteobacteria; Sphingomonadales; Sphingomonadaceae; Sphingomonas
	OTU_683	2.3	8,28	0.048	1	AJ	Bacteria; Proteobacteria; Alpha-Proteobacteria; Rhizobiales
	OTU_37782	2.3	8,28	0.048	1	AK	Bacteria; Proteobacteria; Beta-Proteobacteria
18S	OTU_96	3.5	8,30	0.005	0.8	AL	Eukaryota; Rhizaria; Cercozoa
	OTU_389	2.8	8,30	0.02	1	AM	Eukaryota
	OTU_206	2.7	8,30	0.02	1	AN	Eukaryota
	OTU_62	2.5	8,30	0.03	1	AO	Eukaryota; Alveolata
	OTU_262	2.4	8,30	0.04	1	AP	Eukaryota
	OTU_102	2.3	8,30	0.04	1	AQ	Eukaryota
	OTU_51	2.3	8,30	0.05	1	AR	Eukaryota; Metazoa; Nematoda

Table 3.11: Linear mixed-effects models on logit-transformed abundances of prokaryotes (16S rRNA gene) and eukaryotes (18S rRNA gene) in the bog at 5 cm. Adjusted p-values (Benjami-Hochberg correction) are shown in column 'p.adj'. Only OTUs with an (unadjusted) p-value of <0.05 and for which the interaction between time point and treatment was not due to outliers are shown. Significant adjusted p-values are denoted by * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). Marginal significance ($p < 0.1$) is denoted by '**.**'. Taxonomy was assigned using the RDP Classifier web server, as this was found to classify OTUs with more confidence than utax. Column 'graph' refers to the graph on Figure 3.16 which corresponds to each OTU.

	OTU	F	d.f.	p	p.adj	Graph	Taxonomy
16S	OTU_748	3.7	8,30	<0.0001	1	A	Bacteria; Proteobacteria; Alpha-Proteobacteria; Rhodospirillales; Acetobacteraceae
	OTU_156	3.4	8,30	0.01	1	B	Bacteria; Acidobacteria; Group 6
	OTU_46137	3.1	8,30	0.01	1	C	Bacteria; Acidobacteria; Group 1
	OTU_695	2.7	8,30	0.02	1	D	Bacteria; Bacteroidetes; Spingobacteriia, Spingobacteriales
18S	OTU_189	4.9	8,30	0.00	0.06.	E	Eukaryota
	OTU_74	3.2	8,30	0.01	0.96	F	Eukaryota; Rhizaria
	OTU_23322	3.1	8,30	0.01	1	G	Eukaryota; Rhizaria; Cercozoa

Table 3.12: Linear mixed-effects models on logit-transformed abundances of prokaryotes (16S rRNA gene) and eukaryotes (18S rRNA gene) in the fen at 20 cm. Adjusted p-values (Benjami-Hochberg correction) are shown in column 'p.adj'. Only OTUs with an (unadjusted) p-value of <0.05 and for which the interaction between time point and treatment was not due to outliers are shown. Significant adjusted p-values are denoted by * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). Marginal significance ($p < 0.1$) is denoted by '**.**' Taxonomy was assigned using the RDP Classifier web server, as this was found to classify OTUs with more confidence than utax. Column 'graph' refers to the graph on Figure 3.17 which corresponds to each OTU.

	OTU	F	d.f.	p	p.adj	Graph	Taxonomy
16S	OTU_606	3.1	8, 28	0.01	1	A	Bacteria
	OTU_364	2.6	8, 28	0.03	1	B	Bacteria
	OTU_524	2.4	8, 28	0.04	1	C	Bacteria
	OTU_75	2.4	8, 28	0.04	1	D	Bacteria; Acidobacteria; Group 1
	OTU_62	2.3	8, 28	0.045	1	E	Bacteria
18S	OTU_40802	3.0	8, 28	0.01	1	F	Eukaryota; Stramenopiles

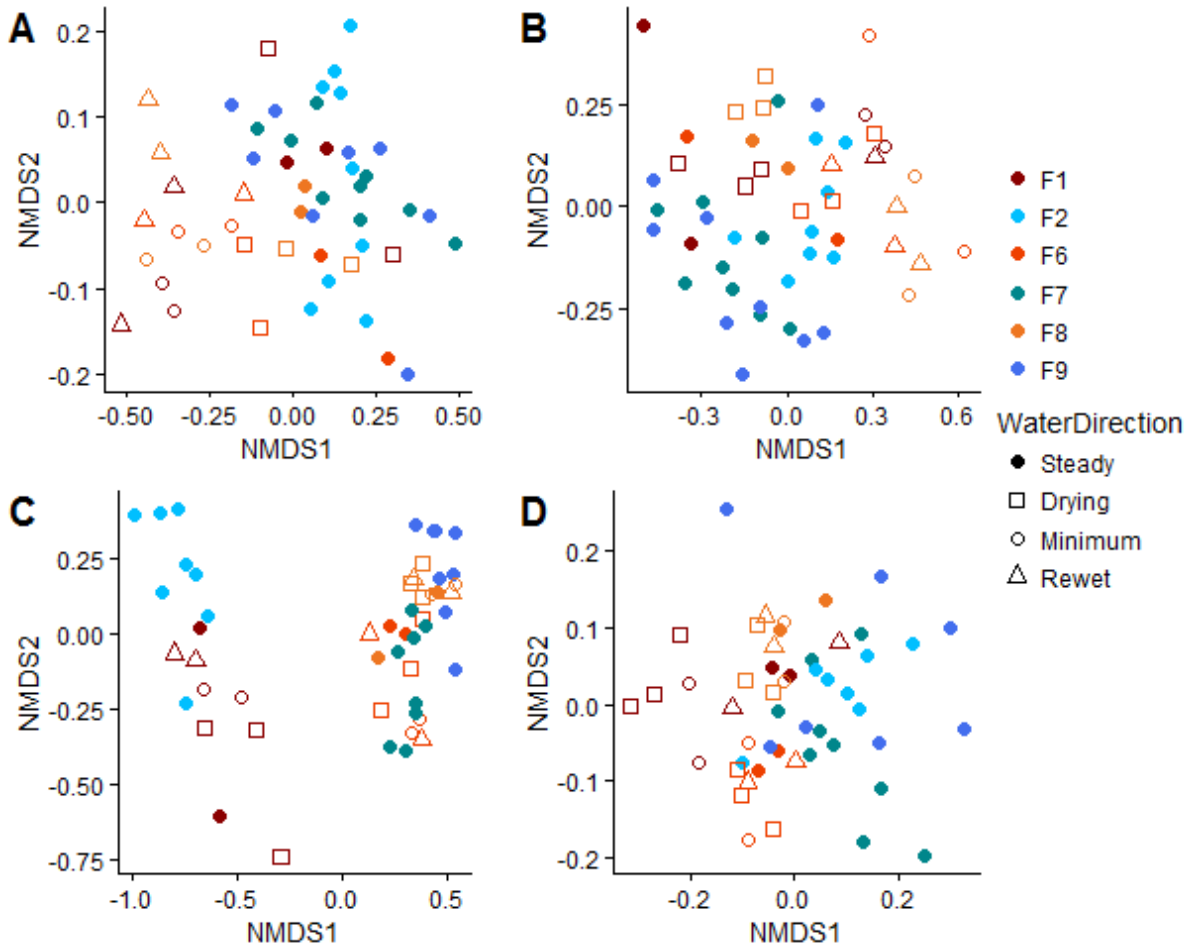

Figure 3.14: NMDS ordination of OTUs which were found to be significantly affected by time point and treatment. Plots A & B show ordination of drought-affected OTUs from the fen at 5 cm, divided into prokaryotes (A) and eukaryotes (B). Plots C & D show drought-affected prokaryotic OTUs in the bog at 5 cm (C) and the fen at 20 cm (D).

Table 3.13: Linear mixed-effects models on logit-transformed abundances of eukaryotes (16S rRNA gene) and prokaryotes (18S rRNA gene) in the bog at 20 cm. Adjusted p-values (Benjami-Hochberg correction) are shown in column 'p.adj'. Only OTUs with an (unadjusted) p-value of <0.05 and for which the interaction between time point and treatment was not due to outliers are shown. Significant adjusted p-values are denoted by * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). Marginal significance ($p < 0.1$) is denoted by '.'. Taxonomy was assigned using the RDP Classifier web server, as this was found to classify OTUs with more confidence than utax. Column 'graph' refers to the graph on Figure 3.18 which corresponds to each OTU.

	OTU	F	d.f.	p	p.adj	Graph	Taxonomy
16S	OTU_4221	2.9	8, 32	0.02	1	A	Bacteria; Acidobacteria; Group 1
	OTU_1152	2.8	8, 32	0.02	1	B	Bacteria; Acidobacteria; Group 1

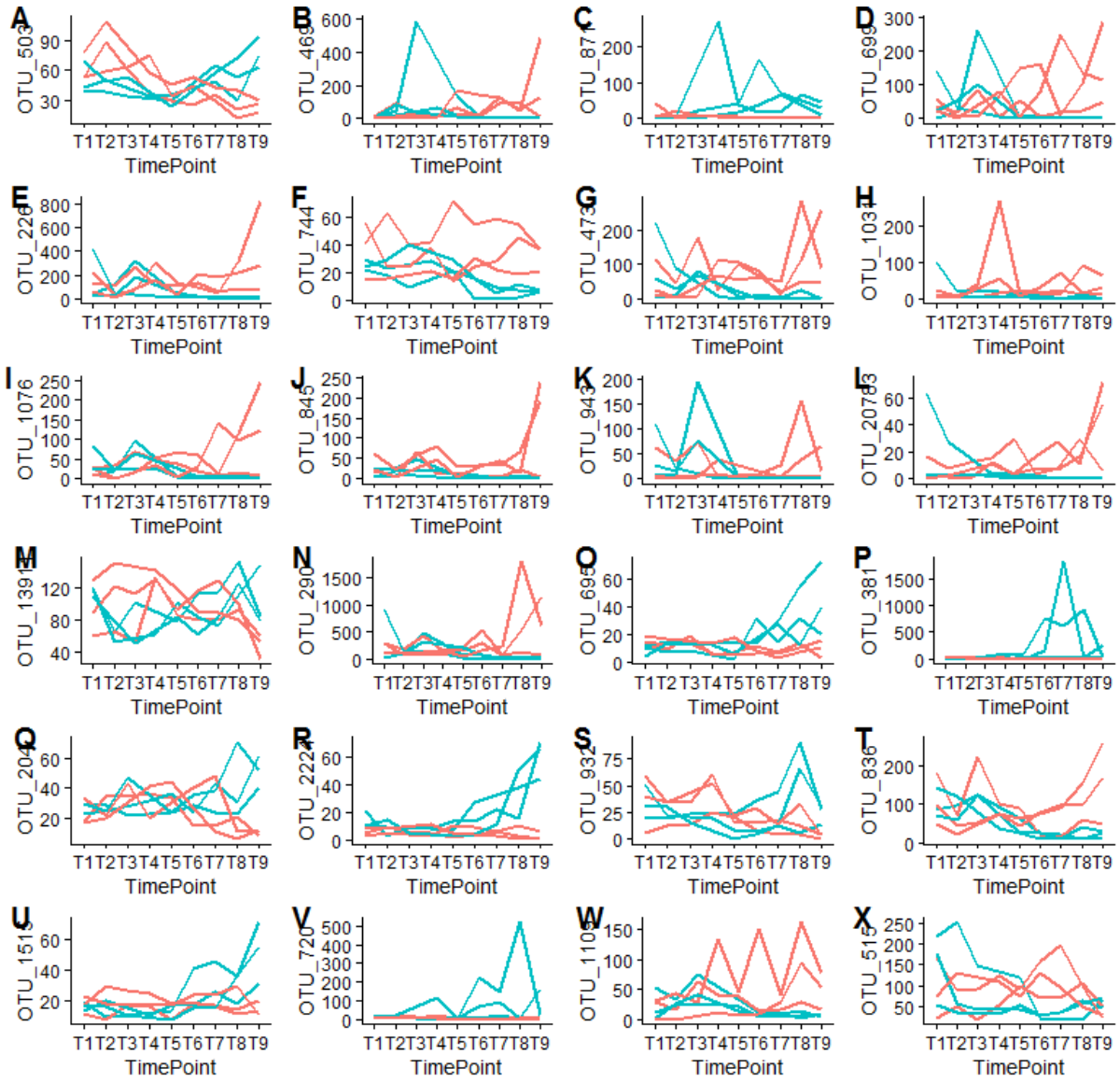

Figure 3.15.1 (continued overleaf)

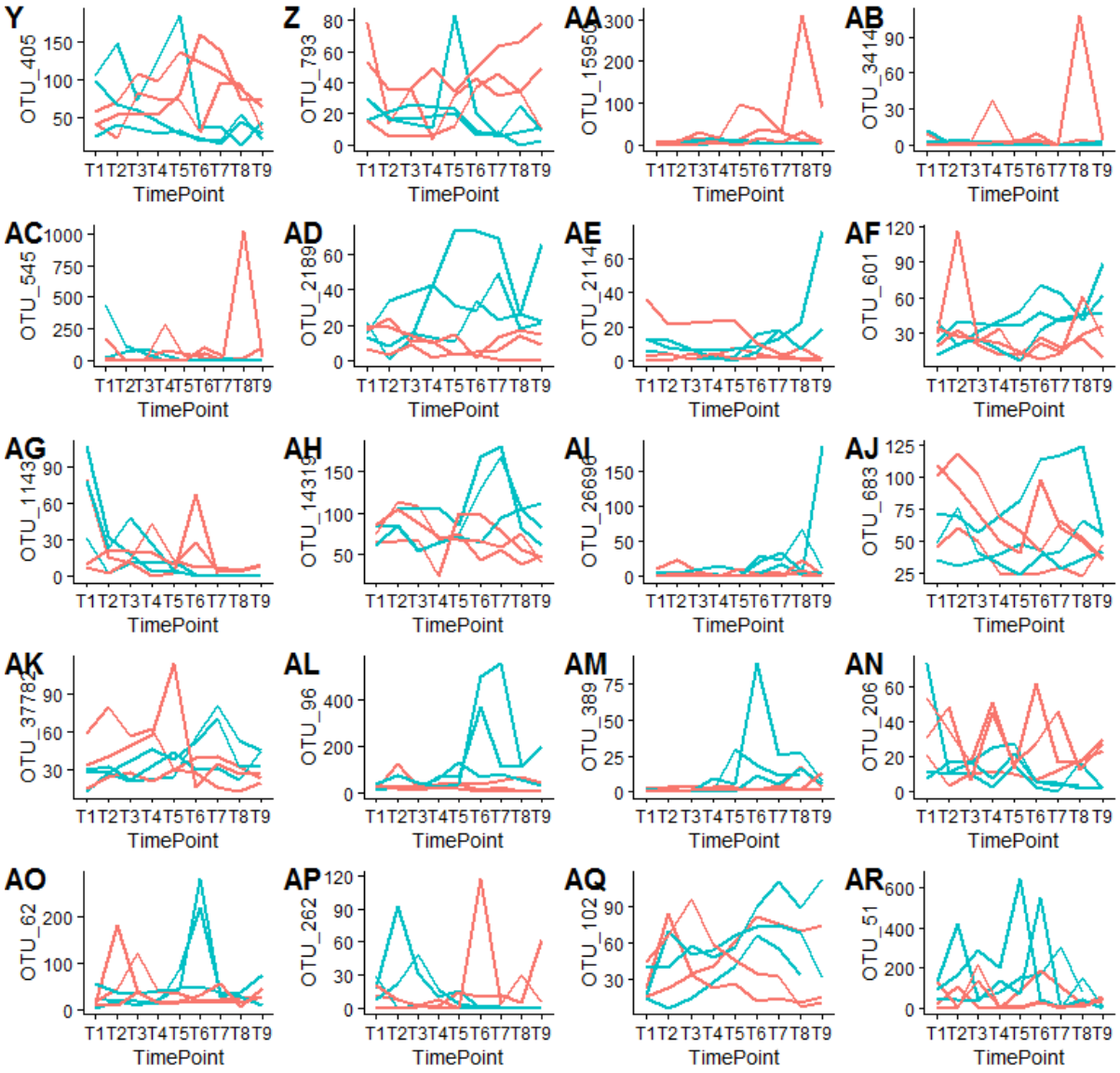

Figure 3.15.2: Line plots showing the abundance of OTUs which were found to be significantly affected by time point and treatment in the fen at 5 cm. Each fen mesocosm core is plotted individually to show the degree of individual variation in abundance between mesocosm cores: blue lines represent droughted cores, and red lines represent control cores. Further information on each OTU is displayed in Table 3.10.

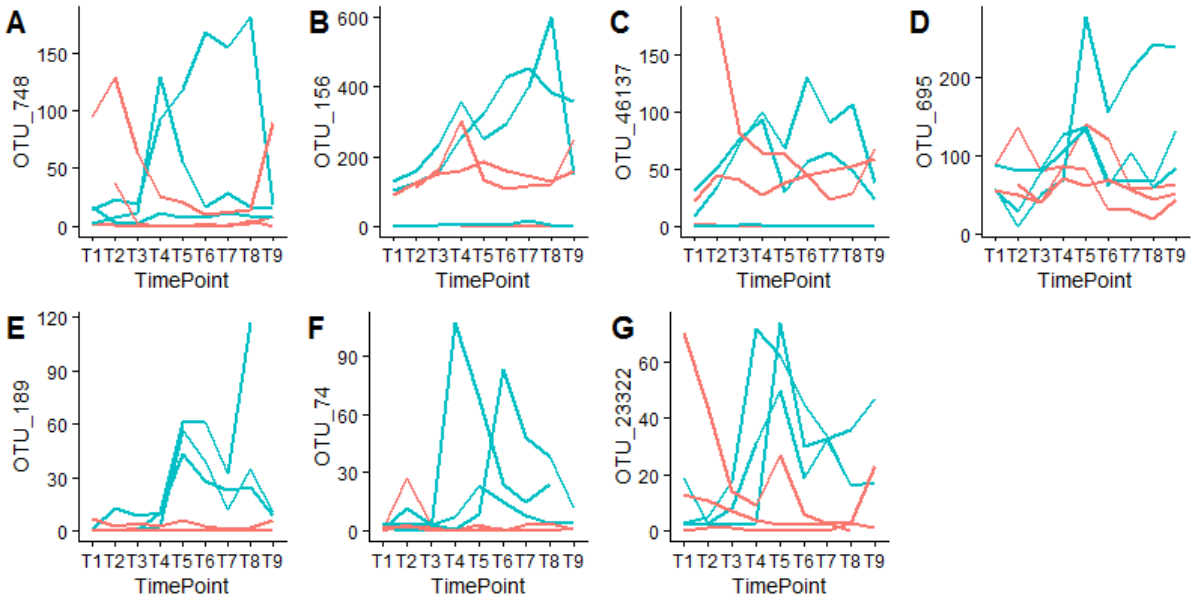

Figure 3.16: Line plots showing the abundance of OTUs which were found to be significantly affected by time point and treatment in the bog at 5 cm. Each bog core is plotted individually to show the degree of individual variation in abundances between mesocosm cores: blue lines represent droughted cores, and red lines represent control cores. Further information on each OTU is displayed in Table 3.11.

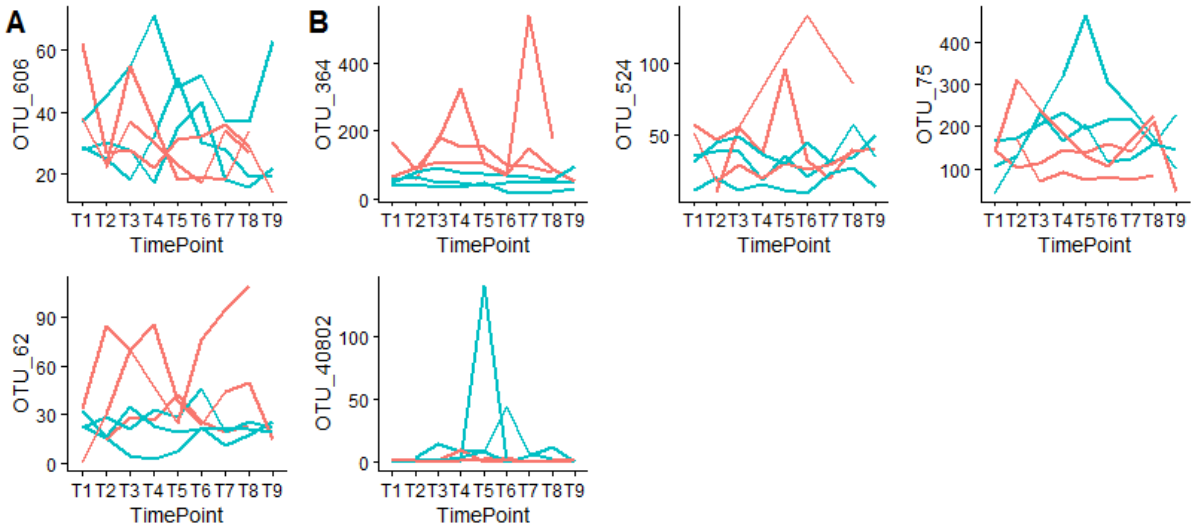

Figure 3.17: Line plots showing the abundance of OTUs which were found to be significantly affected by time point and treatment in the fen at 20 cm. Each fen core is plotted individually to show the degree of individual variation in abundances between mesocosm cores: blue lines represent droughted cores, and red lines represent control cores. Further information on each OTU is displayed in Table 3.12.

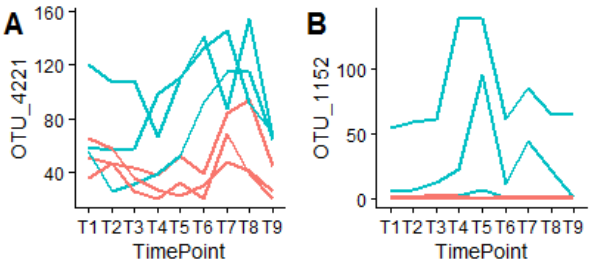

Figure 3.18: Line plots showing the abundance of OTUs which were found to be significantly affected by time point and treatment in the bog at 20 cm. Each bog core is plotted individually to show the degree of individual variation in abundances between mesocosm cores: blue lines represent droughted cores, and red lines represent control cores. Further information on each OTU is displayed in Table 3.13.

3.3.6 Effect of Core

A significant effect of mesocosm core on overall community composition was observed for both prokaryotes (Figure 3.11) and eukaryotes (Figure 3.12), and PERMANOVA found the effect of core to be strongly significant in both habitats and at both depths (Table 3.14). Differences between cores accounted for a large proportion of the variation in community composition, with R^2 values of up to 0.69 (Table 3.14). In general, R^2 values were higher for prokaryotes than for eukaryotes (Table 3.14).

Table 3.14: PERMANOVA tests for the effect of Core on the community composition of prokaryotes (16S rRNA genes) and eukaryotes (18S rRNA genes). Tests were carried out separately for each combination of habitat and depth, for both marker genes. Significant adjusted p-values are denoted by * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). OTUs assigned to the following phyla were excluded from the 18S rRNA gene dataset prior to analysis: Holozoa, Metazoa, Chloroplastida and 'NA'.

Marker Gene	Habitat-Depth	d.f.	F	R^2	p
16S rRNA	**Bog- 5cm**	5	12.6	0.58	0.001**
	Fen- 5cm	5	6.2	0.41	0.001**
	Bog- 20cm	5	21.3	0.69	0.001**
	Fen- 20cm	5	8.1	0.48	0.001**
18S rRNA	**Bog- 5cm**	5	4.5	0.33	0.001**
	Fen- 5cm	5	2.7	0.23	0.001**
	Bog- 20cm	5	1.6	0.15	0.001**
	Fen- 20cm	5	2.2	0.20	0.001**

Table 3.15: ANOVA-like permutation tests for the effect of a multiple constrains within constrained correspondence analysis (CCA). CCA was carried out separately for each combination of habitat and depth, for both marker genes. Significant p-values are denoted by * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). Marginal significance ($p < 0.1$) is denoted by '**.**'. OTUs assigned to the following phyla were excluded from the 18S rRNA gene dataset prior to analysis: Holozoa, Metazoa, Chloroplastida and 'NA'.

Marker Gene	Habitat	Effect	F	d.f.	p
16S rRNA	**Bog-5cm**	**Mosses**	1.8	1	0.04*
		Graminoids	1.4	1	0.119
		Shrubs	5.5	1	0.001**
		pH	2.1	1	0.045*
	Fen-5cm	**Mosses**	3.3	1	0.001**
		Graminoids	4.4	1	0.001**
		pH	3.4	1	0.001**
	Bog-20cm	**Mosses**	2.6	1	0.007**
		Graminoids	1.4	1	0.3
		Shrubs	4.9	1	0.001**
		pH	1.6	1	0.1
	Fen-20cm	**Mosses**	7.1	1	0.001**
		Graminoids	8.8	1	0.001**
		pH	2.9	1	0.002**
18S rRNA	**Bog-5cm**	**Mosses**	1.7	1	0.006**
		Graminoids	1.5	1	0.023*
		Shrubs	3.0	1	0.001**
		pH	1.5	1	0.046*
	Fen-5cm	**Mosses**	2.0	1	0.001**
		Graminoids	1.9	1	0.001**
		pH	1.4	1	0.008**
	Bog-20cm	**Mosses**	1.0	1	0.9
		Graminoids	0.9	1	1
		Shrubs	1.2	1	0.001**
		pH	1.2	1	0.026*
	Fen-20cm	**Mosses**	1.5	1	0.001**
		Graminoids	1.6	1	0.001**
		pH	1.2	1	0.046*

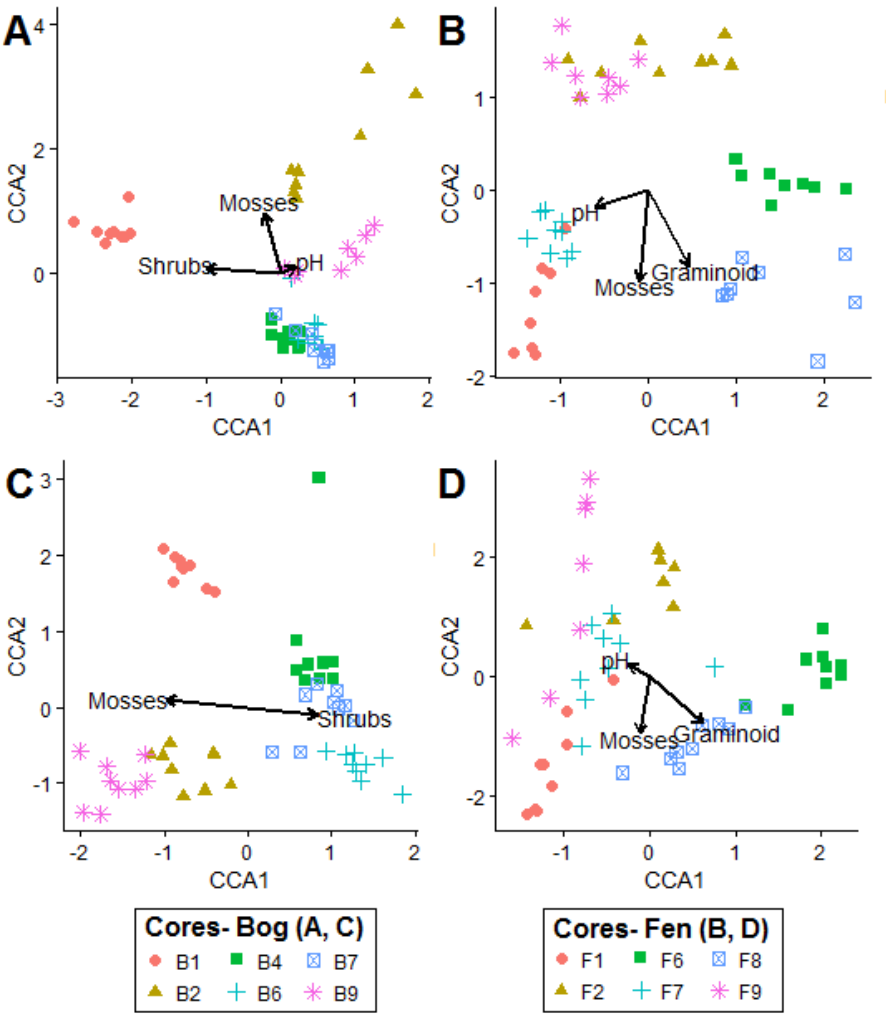

Figure 3.19: Constrained correspondence analysis of prokaryotic communities in all four habitat-depth combinations: (A) bog- 5cm, (B) fen- 5cm, (C) bog- 20cm, (D) fen- 20cm. Fitted constraints were pH, redox potential and the percentage cover of dwarf shrubs (bog only), mosses and graminoids. Different shapes and colours represent different mesocosm cores. Only statistically significant constraints are shown (see Table 3.17).

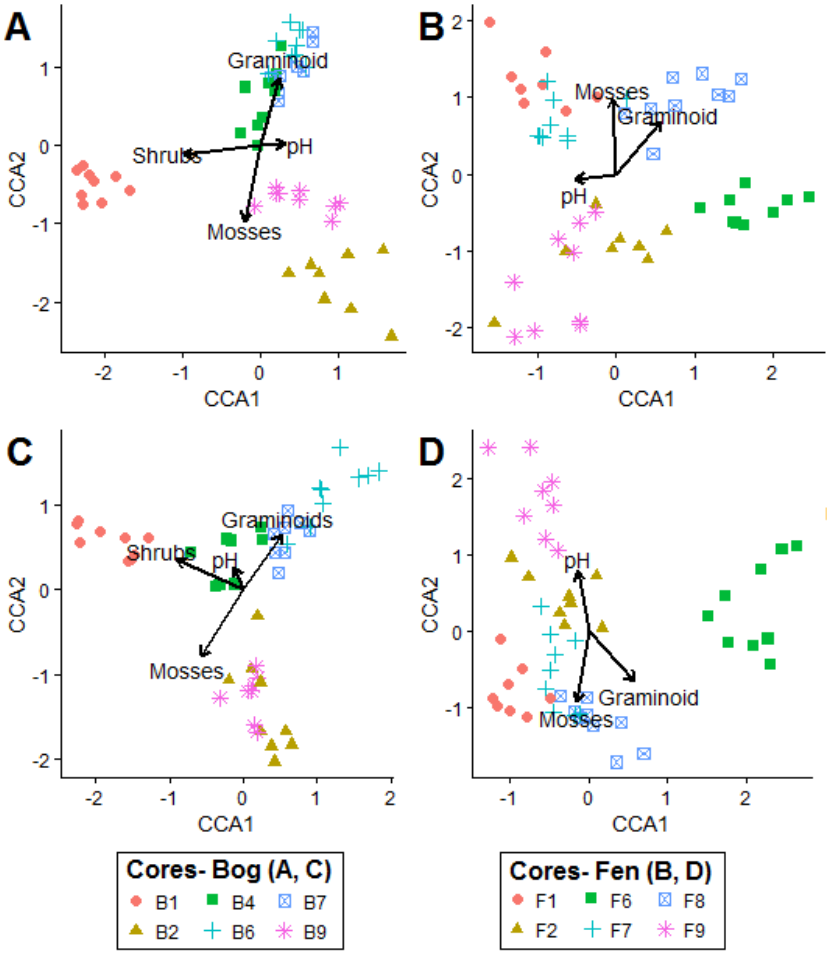

Figure 3.20: Constrained correspondence analysis of eukaryotic communities in all four habitat-depth combinations: (A) bog- 5cm, (B) fen- 5cm, (C) bog- 20cm, (D) fen- 20cm. Fitted constraints were pH, redox potential and the percentage cover of dwarf shrubs (bog only), mosses and graminoids. Different shapes and colours represent different mesocosm cores. Only statistically significant constraints are shown (see Table 3.17). OTUs assigned to the following phyla were excluded from the dataset prior to analysis: Holozoa, Metazoa, Chloroplastida and 'NA'.

To determine which variables could account for the differences in community between different cores, constrained correspondence analysis (CCA) was carried out with pH and the percentage coverage of three important plant functional groups (mosses, graminoids and shrubs) as constraining (independent) variables. On the resulting ordination diagrams, samples clearly cluster by the core from which they were collected for both prokaryotic (Figure 3.19) and eukaryotic (Figure 3.20) communities. The results of ANOVA-like permutation tests to determine the significance of each constraint are shown in Table 3.15. Vegetation was a strong driver of differences between cores. In the bog at 5 cm, percentage

cover of all three plant functional groups had a significant relationship with the community composition of both prokaryotes and microbial eukaryotes, as did pH (Figure 3.19A; Figure 3.19D; Figure 3.20A; Figure 3.20D; Table 3.15). Similarly, in the fen at both depths both pH and the percentage cover of graminoids and mosses had a significant effect on community composition (shrubs were not present in any fen mesocosm core; Figure 3.19B; Figure 3.20B; Table 3.15). In the bog at 20 cm, shrubs were the only plant functional group to significantly affect community composition, and pH also had a significant effect (Figure 3.19C; Figure 3.20C; Table 3.15). In general, CCAs of prokaryotic and eukaryotic communities within each habitat and depth were affected by similar suites of variables (Figure 3.19; Figure 3.20; Table 3.15).

3.4 Discussion

Prokaryotic and eukaryotic communities as a whole were strongly affected by habitat and depth, and also differed between mesocosm cores within each habitat. However, there was no effect of drought on overall community composition: this may either indicate that microbial communities within peat are unaffected by drought, or may be a result of dormant species and 'relic DNA' (Carini *et al.* 2016) obscuring genuine community change. A small subset of OTUs did appear to respond to drought, especially in the fen at 5 cm depth, and the proportion of the eukaryotic community made up of Rhizaria also rose during drought; nonetheless, the majority of drought-affected OTUs were not significantly affected by drought once corrections for multiple comparisons were applied.

3.4.1 Sequencing and Quality Control

Singletons made up a significant proportion of the obtained sequences: 14% of reads from the 16S rRNA gene, and over half of reads from the 18S rRNA gene. Singletons were excluded from the OTU clustering step, but after clusters had been formed singletons which belonged to existing OTUs were included in final abundance counts. Given the large number of sequences obtained in the current study, singletons were assumed to be either erroneous reads or to belong to transient or extremely rare species, neither of which would be likely to play a large ecological role. Including singletons in abundance counts of OTUs generated by clustering non-singletons meant that mildly erroneous reads or rare variants of more abundant species could be included. However, while singleton removal is common in MGA studies, it does reduce the ability of studies to recognise rare species. In particular, some singleton sequences may belong to

'conditionally rare' taxa: taxa which are at the limit of detection under 'normal' conditions, but become extremely abundant under particular environmental conditions (Caporaso *et al.* 2011b; Shade *et al.* 2014). The existence of these conditionally rare taxa suggests that rare organisms can play important ecological roles under the right conditions, and additionally shows that taxa at the limits of detection frequently represent genuine diversity rather than sequencing errors. Comparison of singletons with sequence databases or with raw data taken from other studies in similar habitats could shed further light on which of these sequences represent genuine taxa and which are erroneous reads.

3.4.2 α-Diversity

While rarefaction curves drawn for each sample suggested that coverage was adequate in both habitats (Figure 3.2), it should be noted that rarefaction curves are known to be insensitive to changes in the number of 'rare' species, where rare species are defined relative to sample size i.e. as species occurring less than once per sample of a given size (Haegeman *et al.* 2013). Therefore, it is not possible to draw conclusions about the overall species richness in either habitat and it is possible that a great number of rare species were missing.

The OTU richness of both prokaryotic and eukaryotic communities was significantly higher in the fen than the bog (Figure 3.3A; Figure 3.3C; Table 3.1), corresponding to the results of an earlier study (Lin *et al.* 2012). The lower diversity of microbial communities in bog peat is likely linked to lower pH in this habitat (Bridgham *et al.* 2000): pH is one of the most important drivers of soil microbial communities, and is negatively correlated to both prokaryotic (Fierer and Jackson 2006; Griffiths *et al.* 2011) and eukaryotic (Dupont *et al.* 2016) species richness in soils. Unlike the results of MGA, ARISA fingerprinting found that bacterial fragment richness did not differ between habitats (Chapter 2). However, measures of species richness from MGA are likely more accurate: when ARISA fingerprinting is carried out on diverse communities, multiple species may produce fragments of the same length, leading to underestimates of diversity (Kovacs *et al.* 2010).

The effect of depth on OTU richness was weaker than the effect of habitat, but was nevertheless significant. Bacterial communities had lower OTU richness at 20 cm depth than at 5 cm in both habitats (Figure 3.3A), while OTU richness of microbial eukaryotic communities was higher at 5 cm than 20 cm in the bog but did not differ between depths in the fen (Figure 3.3C). Similar to differences in diversity between habitats, differences in OTU richness between depths may

be caused by pH differences: pH was slightly higher at 5 cm than 20 cm in both habitats (Chapter 2). However, the effect of depth on pH was relatively weak, suggesting other factors may play a role: for example, the species richness of both fungi and bacteria is strongly affected by soil organic matter and nitrogen (Siciliano *et al.* 2014). While organic matter content remains high throughout the peat profile, the lignocellulose index increases with depth (Hill *et al.* 2014), potentially making organic matter more difficult for microbial communities to access.

The effect of habitat and depth on Simpson's Index was different for prokaryotic and eukaryotic communities (Figure 3.3B; Figure 3.3D). For prokaryotic communities, Simpson's Index was lowest for communities in the bog at 20 cm. All samples taken from the bog at 20 cm contained a particular OTU at very high abundances (making up on average 19% of the community), while no single OTU made up such a large proportion of the community in any other habitat-depth combination. This OTU was assigned to subgroup 1 of phylum Acidobacteria, members of which are most abundant in habitats with low pH and low rates of carbon mineralisation (Fierer *et al.* 2007; Jones *et al.* 2009; Rousk *et al.* 2010). Therefore, it is likely that the combination of low pH and low enzyme activities in the bog at 20 cm depth create conditions that are only favourable for a limited subset of the community, leading to low OTU richness and high abundances of oligotrophic taxa. The Simpson's Index of eukaryotic communities was lowest in the bog, potentially reflecting reduced diversity due to the low pH of this habitat (Dupont *et al.* 2016).

Drought did not have a significant effect on OTU richness of either prokaryotic or eukaryotic communities (Table 3.1), in agreement with a previous study based on T-RFLP fingerprinting (Kim *et al.* 2008). The lack of a drought effect on OTU richness could be due to a number of factors: for example, species may persist in a dormant state under drought conditions (Manzoni *et al.* 2014), or a pattern of some species increasing and others decreasing could allow community change without a change in α-diversity. However, in the current study the response of the overall community to drought was weak (Section 3.3.4) and only a small subset of OTUs responded to drought (Section 3.3.5). The weak overall community response means that it is likely that the overall community was not affected strongly enough for overall community properties such as α-diversity to be affected. However, the Simpson's Index of eukaryotic communities in the droughted mesocosm cores rose between time points 3 and 5, potentially indicating an increase in the diversity of microbial eukaryotes during the early stage of the

drought manipulation, although this did not result in a significant difference in Simpson's Index between the two treatments.

The number of 'core' OTUs (cOTUs) within each habitat and at each depth did not always reflect overall OTU richness. For example, in both habitats prokaryotic OTU richness was higher at 5 cm than 20 cm; however, while more prokaryotic cOTUs were found at 5 cm than 20 cm in the fen, the opposite pattern was seen in the bog (Figure 3.5A). The higher number of cOTUs at 20 cm in the bog could reflect higher spatial or temporal turnover of OTUs at 5 cm depth, possibly due to a high proportion of dormant OTUs in the bog at 20 cm: enzyme activities at this depth were much lower than at 5 cm (Chapter 2) and the community was dominated by Acidobacteria, a phylum which often contains a high proportion of inactive members (Jones and Lennon 2010; Lin *et al.* 2014b). High levels of dormancy at 20 cm could be linked to labile carbon limitation, which occurs with increasing depth as the available organic matter becomes more recalcitrant (Hill *et al.* 2014) and may be particularly severe in bogs where much of the dissolved organic matter (DOM) is highly resistant to degradation when oxygen is lacking (Tfaily *et al.* 2013). For eukaryotes, more cOTUs occurred in the fen than the bog and differences in the number of cOTUs between depths were small (Figure 3.5B), reflecting the pattern seen for OTU richness.

3.4.3 Relative Abundances of Domains & Abundant Phyla

Bacteria made up a far higher proportion of the 16S rRNA gene dataset than Archaea did (Figure 3.6A), although a surprisingly large proportion of the community (up to 30%) could not be identified to domain level with confidence (Figure 3.6A). An even higher proportion of OTUs could not be assigned to phylum level. The proportion of unassigned OTUs was higher in the fen than in the bog, especially at 20 cm depth: this likely reflects the fact that a wide variety of anaerobic prokaryotes have been poorly studied, especially those belonging to the domain Archaea or to candidate phyla within the bacteria. For example, a number of recent studies describe new members of several candidate phyla which exhibit fermentative lifestyles (Wrighton *et al.* 2012; Kantor *et al.* 2013), meaning that they may be well suited to life in the fen environment where low redox potential is combined with abundant organic matter.

Aside from 'Unassigned' OTUs, the prokaryotic phyla with the highest relative abundances were Acidobacteria (particularly in the bog) and Proteobacteria, corresponding to previous MGA studies of bacterial communities in peatlands (Lin *et al.* 2012; Serkebaeva *et al.* 2013;

Lin *et al.* 2014b). Proteobacteria and Acidobacteria are also abundant in non-wetland soils. In non-wetland soils Actinobacteria also tend to make up a large proportion of the community (e.g. Delmont *et al.* 2012; Luo *et al.* 2014; Chodak *et al.* 2015), which was not the case in the current study. The comparative rarity of Actinobacteria in peatlands (Lin *et al.* 2012; 2014b) may have important consequences for carbon cycling, as Actinobacteria contain many genes involved in degradation of phenolic compounds in peatlands (Tveit *et al.* 2013; Lin *et al.* 2014a) and are one of the few bacterial phyla to contain members with the capability to break down lignin (Bugg *et al.* 2011).

The proportion of the community made up by each of the most abundant phyla was affected by habitat and depth (Table 3.3; Figure 3.6B). Four phyla were more abundant in the bog than the fen: Acidobacteria, which flourish in soils with low pH values (Jones *et al.* 2009) such as bogs (Chapter 2); Actinobacteria, many of which are strict aerobes (Goodfellow and Williams 1983) and thus may have preferred the higher redox potential of the bog mesocosm cores (Chapter 2); Verrucomicrobia, which are important in the degradation of recalcitrant organic matter in marine ecosystems (Martinez-Garcia *et al.* 2012) and thus may make use of recalcitrant organic matter found in bogs (Tfaily *et al.* 2013); and Chloroplasts/Cyanobacteria, which were likely present as a result of chloroplasts in undecomposed *Sphagnum* moss. Bacteroidetes were most abundant in the fen and at 5 cm depth, probably because abundance of this phylum increases with pH (Fierer *et al.* 2007; Frank-Fahle *et al.* 2014) and pH was considerably higher in the fen than the bog (Chapter 2). Overall, Proteobacteria were more abundant in the fen, but the relative abundance of Proteobacteria varied more with depth in the bog than in the fen (Figure 3.6B). Proteobacteria made up a large proportion of the prokaryotic community in the bog at 5 cm, with α-Proteobacteria particularly abundant. Large numbers of α-Proteobacteria are associated with the microbiome of *Sphagnum* moss (Bragina *et al.* 2014). *Sphagnum* was abundant in many of the bog cores, suggesting that the *Sphagnum* microbiome persists in the early stages of decomposition before being replaced as decomposition proceeds with increasing depth along the peat profile. Conversely, the proportion of Proteobacteria in the bog at 20 cm was very low: many Proteobacteria respond positively to nutrient additions (Fierer *et al.* 2012) and high carbon availability (Fierer *et al.* 2007), so the decrease in Proteobacteria with depth may be caused by depth-dependent phosphorus limitation (Lin *et al.* 2014a) or by decreasing carbon availability with depth (Hill *et al.* 2014).

Cyanobacteria/Chloroplast rRNA was the only phylum within the 16S rRNA gene dataset to significantly respond to time point, showing a significant fall in abundance at time point T3 (Figure 3.7). Most members of this group were affiliated with Chloroplasts rather than Cyanobacteria, and thus likely originated from undecomposed *Sphagnum* moss. Chloroplast abundance in *Sphagnum* is known to be affected by stress (Gerdol *et al.* 1996); however, the fact that Chloroplast abundance did not fall until T3 suggests that it is unlikely to be a response to changing conditions when mesocosm cores were collected and placed in the controlled temperature room.

Fungi were the most abundant eukaryotic phylum at 5 cm depth, with Ascomycota and Basidiomycota being the most abundant subphyla of fungi, corresponding to earlier research in peatlands (Lara *et al.* 2011; Lin *et al.* 2012; 2014b) as well as other soils (Dupont *et al.* 2016). Chloroplastida (green algae and land plants) were also abundant, probably as a result of undecomposed plant material. Each of the three most abundant protist taxa (Alveolata, Rhizaria and Stramenopiles) were more abundant in the fen than the bog. The low proportion of protists in the bog is likely as a result of low pH: fungi dominate communities of microbial eukaryotes in soils with low pH values, with protists becoming more abundant as the pH approaches neutral (Dupont *et al.* 2016). Within the protists, the most abundant phyla were similar to those in non–peat soils (Dupont *et al.* 2016): these included the Apicomplexa (Alveolata), Ciliphora (Alveolata), Cercozoa (Rhizaria), and the Oomycota (Stramenopiles). Many of the eukaryotic phyla tested were significantly more abundant at 5 cm than 20 cm depth (Table 3.4), a trend which was driven by the extremely high proportion of the community made up by unassigned OTUs at 20 cm depth (Figure 3.8). The properties of peatland ecosystems are sufficiently unusual (e.g. anoxic, low pH and low nutrients) that many of the taxa in peatland ecosystems may be unknown. A limited amount of research into protozoa in peatlands has been carried out, but this is primarily focused on communities within living *Sphagnum* moss (i.e. 5 cm depth or less) and relies on morphological methods to divide protozoa into functional groups (e.g. Mieczan 2007; Jassey *et al.* 2013; Jassey *et al.* 2015). The current work highlights the need for further research into the diversity of microbial eukaryotes and for continued expansion of existing databases

Of the six eukaryotic phyla tested, only Rhizaria were significantly affected by drought (Figure 3.9; Table 3.4). Unfortunately, few Rhizarian OTUs in the current dataset could be assigned to taxonomic levels below phylum, making it difficult to infer the functional roles of these

organisms in the community. Within the Rhizaria, the most abundant class was the Cercozoa: this group, like Rhizaria as a whole, includes a wide variety of lifestyles and morphologies (Burki and Keeling 2014; Harder *et al.* 2016). Existing evidence suggests that the proportional abundance of testate amoeba within the Cercozoa may respond to drought (Harder *et al.* 2016), and that a decrease in the biomass of mixotrophic testate amoeba leads to increasing carbon emissions from warmed peatlands (Jassey *et al.* 2015). However, further research is required to fully understand the effect of drought on Rhizaria and the role they play in carbon dioxide release from peat under drought conditions.

3.4.4 Overall Community Composition

Bogs and fens contained very different microbial communities, with relatively little overlap in core OTUs (Figure 3.5) and clear separation of the two habitats along the first axes of NMDS ordination plots based on both the 16S and 18S rRNA genes (Figure 3.10). Differences between the microbial communities present in each habitat likely reflect the large environmental differences between bog and fen peat (Chapter 2). In particular, pH is hugely important in determining the community composition of both microbial eukaryotes and prokaryotes in soils (Fierer and Jackson 2006; Rousk *et al.* 2010; Griffiths *et al.* 2011; Dupont *et al.* 2016) and the pH of bog peat was much lower than that of fen peat (Chapter 2). Most species of bacteria and fungi have optimum pH ranges of much less than two pH units (Hung and Trappe 1983; Wheeler *et al.* 1991; Rosso *et al.* 1995; Fernández-Calviño and Bååth 2010), which was the approximate difference in pH between the bog and the fen, thus explaining why so few 'core' OTUs were shared between habitats. Many other environmental variables also differed between the two habitats: for example, the fen had a lower redox potential, higher water content and a different plant community to the bog. All of the aforementioned variables have been found to significantly affect microbial community composition (Peralta *et al.* 2014; Dupont *et al.* 2016), and likely contribute to the differing microbial communities found in each habitat. The two depths (5 cm and 20 cm) also contained distinct microbial communities, although differences between depths were weaker than those between habitats (Figure 3.5; Figure 3.10). Peat chemistry differed less between depths than between habitats: for example, redox potential was not significantly different between depths, while pH was only slightly higher at 5 cm than 20 cm (Chapter 2).

Community composition within each habitat-depth subset was significantly different between the two treatments for both prokaryotes and eukaryotes (Table 3.5; Table 3.6), but this effect was due to differences between mesocosm cores which were observed at all time points (including pre-drought) and drought did not affect the overall community composition (Figure 3.11; Figure 3.12). Similarly, there was no significant effect of time point on either prokaryotic or eukaryotic communities despite the fact that water content rose at the beginning of the experiment (Chapter 2). These results suggest that peatland microbial communities as a whole are relatively resistant to drought, at least in the short term. It is possible that many bacteria survive droughts by becoming dormant (Manzoni *et al.* 2014): dormancy is a widespread phenomenon in environmental bacterial communities (Jones and Lennon 2010). The abundance of 16S rRNA genes changes much more slowly than the abundance of 16S rRNA transcripts during drought in dryland ecosystems (Barnard *et al.* 2013; Barnard *et al.* 2015), suggesting that the 'active' community is responding more strongly than the 'present' community. Dormancy usually plays a weaker role in the maintenance of eukaryotic communities under stressful conditions (Jones and Lennon 2010), although fungi may form spores and protists cysts. An additional explanation for the slow rate of change in microbial communities when DNA-based (rather than RNA-based) markers are used is the persistence of 'relic DNA', i.e. DNA which persists from dead organisms. A recent study demonstrated that approximately 40% of fungal and prokaryotic DNA in soils is not contained within cells, suggesting that much of the DNA in the soil environment belongs to dead organisms (Carini *et al.* 2016). An earlier study suggested that the majority of extracellular DNA in soils was degraded within 30 days (Morrissey *et al.* 2015), but its persistence is affected by binding to clay minerals or humics. Therefore, extracellular 'relic' DNA has the potential to make the effects of short term environmental change difficult to detect using DNA-based methodologies. Future work on the effects of environmental change on soil microbial communities should therefore analyse community RNA as well as DNA, as RNA has a much more rapid turnover time in soil (Moran *et al.* 2013).

3.4.5 Effect of Water Table

While no effect of drought on the overall community composition was found by NMDS ordination or by PERMANOVA tests, there was evidence that drought significantly affected a subset of the community. Partial constrained correspondence analysis (pCCA) carried out on a filtered subset of the prokaryotic and eukaryotic community (i.e. those OTUs which were found

in at least 20% of samples) showed that there was a significant effect of water table depth on bacterial communities in the bog at both depths and in the fen at 5 cm, as well as on eukaryotic communities in the bog at 5 cm (although the effects in the bog were caused in large part by outlier effects or differences between mesocosm cores). Constrained correspondence analysis is a method which only considers the variation within a dataset which can be explained by the chosen constraints (in this case water table direction), and so the significant effect of water direction suggests that a subset of OTUs responded to drought (although many other OTUs did not). The effect of water table direction was most obvious in the fen at 5 cm, where all droughted mesocosm cores responded in a similar matter at minimum water table and during rewetting (Figure 3.13C; Figure 3.13I). Likewise, the fen at 5 cm harboured the largest number of OTUs which were significantly affected by the interaction between time point and treatment, was the only environment in which the effect of drought on some OTUs remained significant following correction for multiple comparison (Table 3.10), and was also the environment in which NMDS ordination of drought-affected OTUs showed the clearest community response to drought (Figure 3.14C). In the other habitats and depths the overall effect of drought was weak or was overshadowed by the effects of core (Figure 3.13; Figure 3.14). The low redox potential in the fen under normal conditions (Chapter 2) likely means that fen microbial communities were adapted to anoxic conditions and thus were more strongly affected by the rise in redox potential which occurs during drought than communities in the bog. Alternatively, a high proportion of relic DNA may obscure the effect of drought on microbial communities in the bog: the proportion of relic DNA is highest in acidic soils with low availability of base cations such as Ca^{2+} (Carini *et al.* 2016), and the bog had both a lower pH and a lower concentration of Ca^{2+} than the fen.

In the fen at 5 cm, two phyla were overrepresented amongst drought-affected OTUs relative to the dataset as a whole: Proteobacteria made up 27% of total OTUs and 41% of drought-affected OTUs, while Bacteroidetes made up only 7% of total OTUs but 39% of drought affected OTUs (Figure 3.15; Table 3.10). The majority of drought-affected bacteria in phylum Bacteroidetes responded negatively to drought while Proteobacteria tended to respond positively, although there were exceptions. Only two of the Bacteroidetes OTUs which responded negatively to drought could be assigned below phylum level: both were affiliated with Bacteroidales, an order which has been previously shown to be negatively correlated to redox potential in permafrost peats, with many members exhibiting fermentative lifestyles (Lipson *et al.* 2013).

One of the Bacteroidetes OTUs was additionally identified as belonging to genus *Paludibacter*: the sole described member of this genus is strict anaerobe which lacks oxidase and catalase activity (Ueki *et al.* 2006), two enzymes important for the survival of anaerobic bacteria under oxic conditions (Rolfe *et al.* 1978; Brioukhanov and Netrusov 2007). On its discovery, genus *Paludibacter* was very distinct from its closest cultured relatives both in terms of sequence similarity and metabolism (Ueki *et al.* 2006): therefore, it is possible to speculate that many novel fermentative members of the Bacteroidetes remain to be discovered, and that many of the remaining drought-responsive Bacteroidetes may fall into this category. In addition, four Proteobacteria responded negatively to drought in the fen at 5 cm: one was assigned to genus *Geobacter*, a genus consisting of Fe(III)-reducing bacteria, many of which are also able to reduce sulphate and Mn(IV) (Lovley *et al.* 2011). Given that the redox potential in the fen mesocosm cores was within the range for Fe(III) and Mn(IV)-reduction (Chapter 2), the presence of *Geobacter* is unsurprising. The higher redox potential observed during drought may put metal-reducing species at a disadvantage: while there is evidence that some *Geobacter* species tolerate oxygen at low levels and even use it as an electron acceptor (Lin *et al.* 2004), it is unclear whether this is the case for all species or whether these species are able to compete with obligate aerobes under oxic conditions. A single OTU belonging to the Firmicutes also appeared to respond negatively to drought: this OTU was assigned to family Ruminococcaceae, which consists of strict anaerobes which often occur in the mammalian gut or in the rumen of ruminants. Finally, the only drought-affected Archaea responded negatively to the drought and was assigned to phylum Pacearcheaota: this phylum has been little researched, but appears to contain at least some fermentative members (Castelle *et al.* 2015). Therefore, all negatively drought-affected OTUs in the fen at 5 cm which could be meaningfully assigned appear to be affiliated with anaerobic taxa, including obligate anaerobes. The effect of these OTUs decreasing in abundance is unclear: many of the drought-responsive OTUs were only present at relatively low abundances, and thus their effects on carbon fluxes might be expected to be weak.

Of the 17 bacterial OTUs showing a positive response to drought, 11 belonged to the Proteobacteria (Table 3.10). Interestingly, two of these OTUs are affiliated with taxa that are commonly associated with alkane degradation in petroleum-contaminated soils: genus *Caulobacter* and family Sphingomonadaceae (Yergeau *et al.* 2012; Yang *et al.* 2014; Tsuboi *et al.* 2015). Both taxa contain aerobic bacteria, suggesting that aeration during drought might

allow both OTUs to increase in abundance, but it is unclear whether the association with alkane degradation is relevant to their presence in peat. The abundance of saturated, deoxygenated carbon compounds increases with depth (D'Andrilli *et al.* 2010; Tfaily *et al.* 2013). As alkanes are saturated, deoxygenated chains, this potentially indicates that alkanes and related compounds are relatively slow to degrade in the peatland environment. Likewise, the potential effects of alkane degradation on peatland carbon fluxes is unclear: while one previous study found a negative correlation between the concentration of lipids (which have alkane-like 'tails') and anaerobic production of CO_2 and CH_4 (Reiche *et al.* 2010), this analysis was potentially complicated by the fact that lipids were most abundant in deep peat layers where microbial metabolism is suppressed by other factors. It is likely that taxa which are enriched following petroleum contamination are additionally able to degrade other forms of recalcitrant carbon such as phenolic compounds: *Caulobacter cresentus*, which belongs to the same genus as one of the enriched OTUs, possesses a cluster of genes homologous to those required for the degradation of aromatics (Nierman *et al.* 2001), and members of family Sphingomonadaceae generally contain diverse degradative enzymes (Aylward *et al.* 2013). Two of the other positively drought-affected OTUs belonged to genera *Massilia* and *Duganella* within family Oxalobacteraceae. Intriguingly, the abundance of family Oxalobacteraceae, and genus *Massilia* in particular, is sometimes significantly decreased following petroleum contamination of permafrost soils (Yang *et al.* 2014): this is the opposite response to that of *Caulobacter* and Sphingomonadaceae. Instead, these genera increase in response to enrichment with chitin (Cretoiu *et al.* 2014) or glucose (Padmanabhan *et al.* 2003), suggesting that these two OTUs could potentially represent opportunistic ('copiotrophic') bacteria taking advantage of the increased carbon and nutrient availability which occurs during drought as a result of increased hydrolase activity (Fenner and Freeman 2011). However, β-glucosidase activity did not increase during drought in the current experiment, and although other hydrolases are affected by drought (Fenner and Freeman 2011), these were not measured in the current experiment. Therefore, without further research these possibilities remain speculative.

Of the drought-affected OTUs in the fen at 5 cm from the 18S rRNA dataset, two were assigned to protist groups (one to Alveolata and one to Rhizaria), one to Nematoda and four could not be assigned at phylum level (Table 3.10). However, three of the four unassigned OTUs were assigned to Rhizaria (class Cercozoa) with low confidence values. When combined with a significant increase in the abundance of Rhizaria as a whole this suggests that Rhizaria may

respond significantly to drought. However, none of the drought-affected Rhizaria could be assigned below class level, making it difficult to ascertain the functional roles they play in peat soils. Nonetheless, it is likely that Rhizaria are relevant to carbon fluxes in peatlands, either directly or indirectly: for example, bacterial predators within the Rhizaria strongly influence bacterial communities (Glücksman *et al.* 2010) while mixotrophic testate amoebae affect peatland CO_2 emissions by switching between heterotrophic and autotrophic modes of living (Jassey *et al.* 2015).

Far fewer OTUs were significantly affected by drought in the bog at 5 cm than in the fen at 5 cm (Table 3.11), and all drought-affected OTUs in the bog at 5 cm showed a positive response to drought. Two of the four drought-affected prokaryotic OTUs in the bog at 5 cm were assigned to the Acidobacteria, reflecting the high proportion of Acidobacteria in this habitat. Another was assigned to family Acetobacteraceae within the α-Proteobacteria: this family contains obligate aerobes (Kersters *et al.* 2006), possibly explaining the positive response of this OTU to drought. Both Acetobacteraceae and Acidobacteria are tolerant of low pH, potentially demonstrating the limitations that the low pH of bog habitats imposes on the microbial community. The final drought-affected prokaryotic OTU belonged to order Sphingobacteriales within the Bacteroidetes: this order has previously been found to respond positively to drought in arid soils (Placella *et al.* 2012; Amend *et al.* 2016). Similar to the results in the fen, eukaryotic drought-affected OTUs were dominated by Rhizaria: two of the three drought-affected OTUs were assigned to this phyla, lending support to the notion that Rhizaria may be one of the microbial groups showing the strongest response to drought.

The number of drought-affected OTUs detected at 20 cm was considerably less than the number detected at 5 cm, an unsurprising finding given that 20 cm represented minimum water table. At this depth, drought did not affect the water content of the peat but did cause a significant increase in redox potential, although this was weaker than the rise in redox potential observed at 5 cm depth (Chapter 2). Across both habitats, only three of the prokaryotic OTUs affected by drought at 20 cm could be assigned to a taxonomic level more precise than Bacteria (Tables 3.12 & 3.13): each of these was assigned to Acidobacteria (subdivision 1) and each increased during drought. Acidobacteria isolated from peat are commonly aerobic (Pankratov *et al.* 2008) and so likely benefit from increased redox potential during drought. In the fen at 20 cm, four further OTUs showed a significant negative response to drought but could not be

taxonomically assigned with a confidence value that reached the threshold. However, the most likely assignment for two of these were families of obligate anaerobes: Ruminococcaceae and Anaerolineaceae, respectively. A single eukaryotic OTU responded positively to drought at 20 cm and was assigned to the Stramenopiles.

It should be noted that for the majority of 'drought-affected OTUs' described above, the effect of the interaction between time point and treatment did not remain significant following the application of corrections for multiple comparisons. Unfortunately, resources were not available to sequence all of the mesocosm cores included in the experiment, meaning that the sample size was relatively low (especially compared to the number of OTUs). In addition, different mesocosm cores not only contained heterogeneous communities (Section 3.3.6), but also showed different responses to drought: for example, OTU_156 showed a strong positive response to treatment in two of the droughted bog mesocosm cores at 5 cm depth, but in the third droughted core was only present at very low abundances throughout the experiment and did not respond to drought (Figure 3.16B). A greater number of mesocosm cores would have made it easier to distinguish between noise and genuine drought effects. Despite the low sample size, the current analysis acts as an effective hypothesis-generating exercise and suggests a number of future avenues of research which could not have been generated from previous data: in particular, the potential role played by Rhizaria (especially Cercozoa) in drought-driven changes to peat biogeochemistry and carbon fluxes. Combinations of morphological identification (to classify protists into functional groups) and targeted sequencing of the 18S rRNA gene could be used to fully understand the role Rhizaria play in drought-driven carbon release.

3.4.6 Effect of Core

Microbial community composition significantly varied between mesocosm cores, and the effect of mesocosm core accounted for a large proportion of the variation in the composition of communities of both prokaryotes and microbial eukaryotes (Table 3.14; Figure 3.11; Figure 3.12). Constrained correspondence analysis with vegetation and pH as constraining variables suggested that both factors played a role in structuring the differences between microbial communities in different cores (Table 3.15; Figure 3.19; Figure 3.20), although the exact combination of variables and the relative effect of each varied between habitats, depths and marker genes. The strong effect of vegetation on the microbial community may be mediated

by organic matter quality: shrubs are likely to contribute woody tissue, which contains high levels of lignin (a highly recalcitrant polymer). However, *Sphagnum*, the dominant genus of mosses in bogs, is also particularly slow to decompose (Lang *et al.* 2009), potentially due to the inhibitory effect of phenolic compounds or other functional groups derived from Sphagnum (Painter 1991; Børsheim *et al.* 2001; Hajek *et al.* 2011). Additionally, vascular plants (such as graminoids and shrubs) may release root exudates and thus provide an additional source of carbon for microbial communities in soil (Corbett *et al.* 2013). Finally, many of the shrubs found in peatlands are ericoid mycorrhizal, while graminoids and mosses are not (Thormann *et al.* 1999). The effect of pH on community composition was generally weaker than the effect of vegetation despite the known impact of pH on microbial communities (Rousk *et al.* 2010; Dupont *et al.* 2016). However, within-habitat pH variations were much smaller than between-habitat variations and thus it is likely that the amount of variation in pH was not enough to be a large source of between-core variation.

3.5 Conclusions

1. The prokaryotic community in all mesocosm cores was dominated by Bacteria, with few OTUs assigned to Archaea. Within the Bacteria, Acidobacteria and Proteobacteria were the most abundant phyla by far. Of the eukaryotic OTUs which could be assigned to phyla, Fungi and Chloroplastida were dominant. However, a large proportion of OTUs could not confidently be assigned at phylum level, suggesting a high level of unknown diversity occurs in peat soils.

2. The overall composition of prokaryotic and eukaryotic communities was very different in bog and fen mesocosm cores, and was also affected by depth. However, no effect of drought was observed on the community as a whole.

3. A subset of the community appeared to show a response to drought, especially in the fen at 5cm: this was potentially a result of the lower redox potential in the fen (Chapter 2). In the fen at 5cm, drought led to an increase in the proportion of the eukaryotic community made up by Rhizaria. Drought also affected abundances of a number of individual OTUs, especially in the fen at 5cm: several positively drought-affected OTUs were affiliated to taxa previously observed to respond positively to oil contamination or to the addition of labile substrates, while negatively drought-affected OTUs were affiliated with obligate anaerobes. However, the majority of drought-affected OTUs did not show a significant response to drought once corrections for multiple comparisons were applied.

3.6 References

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Chapter 4

Shotgun metagenomic sequencing of bog microbial communities under conditions of drought and of stable water table

4.1 Introduction

One of the earliest descriptions of the soil metagenome defined it as "the collective genomes of soil microflora" (Handelsman et al. 1998), and metagenomic analyses attempt to characterise the metagenome of an environment through random sequencing of DNA directly extracted from the environment. Metagenomics shares many of the advantages of marker gene analysis (MGA), in that it enables the study of microbial communities without the biases introduced by culture-based techniques, but metagenomics additionally has a number of advantages over MGA. Metagenomic methods are able to avoid many of the biases introduced by MGA workflows, especially those introduced by PCR primers (Ahn *et al.* 2012; Cai *et al.* 2013). While some of the library preparation methods commonly employed in metagenomic studies do involve PCR amplification (e.g. Nextera XT & TruSeq Nano HT DNA Library Preparation Kits), primer bias is avoided by random insertion of primer-binding sites during the DNA fragmentation step. Consequently, metagenomic methods often outperform amplicon sequencing methods when analysing microbial community composition (Shakya *et al.* 2013; Poretsky *et al.* 2014).

In addition to avoiding primer bias, shotgun metagenomic sequencing data contains a snapshot of all genetic material present in the environment (rather than only the selected marker gene(s)) and thus provides a wealth of information which cannot be obtained using MGA alone. There are several advantages to sequencing all available genetic material: firstly, functional genes are obtained in addition to taxonomic markers, which allows insights into changes in the functional potential of microbial communities and how these relate to changes in soil function. The microbial community response to environmental stresses commonly includes changes in the functional potential of the community as well the taxonomic composition, and changes in functional potential may in turn be linked to changes in carbon fluxes (e.g. Allison and Martiny 2008; Matulich and Martiny 2014). For example, warming leads to an increase in genes related to the utilisation of labile carbon sources which is linked to an overall increase in soil respiration (Luo *et al.* 2014). Work is also underway to fully integrate microbial functional genes into biogeochemical models (Reed *et al.* 2014). A second potential advantage of sequencing all genetic material is that a wider range of approaches to taxonomic analysis are possible, avoiding problems with taxonomic assignment based on small-subunit (SSU) rRNA genes such as copy-number heterogeneity (Větrovský and Baldrian 2013) and intragenomic variability (Sun *et al.* 2013). Examples of algorithms for taxonomic assignment of

metagenomic reads include Phylosift (Darling *et al.* 2014), which uses a set of universal single copy protein families as well as ribosomal genes, and MetaPhlAn (Segata *et al.* 2012), which identifies clade-specific marker genes. Unfortunately, these novel approaches come with a number of disadvantages: they may provide less intuitive outputs than SSU-based methods, are difficult to compare to existing analyses, and often require installation of dependencies or downloading of specific databases to enable usage. This means that many recent studies rely on the SSU region to assign taxonomy within shotgun sequencing datasets (e.g. Bragina *et al.* 2014; Luo *et al.* 2014).

Recently, shotgun metagenomic sequencing has been applied to North American bog and fen habitats (Lin *et al.* 2014b), as well as to related habitats such as permafrost (Tveit *et al.* 2014; Hultman *et al.* 2015) and *Sphagnum* moss (Bragina *et al.* 2014). The aforementioned studies have revealed that the functional potential of temperate bogs and fens is distinct from other soils, including permafrost peat, and demonstrate that peat represents a unique set of challenges for microbial communities. For example, the surface communities of both bogs and fens contain abundant genes for phosphorus transport (Lin *et al*. 2014a), probably as a result of the phosphorus limitation which is common to peatland ecosystems (Hill *et al*. 2014; Lin *et al*. 2014b). Deep bog peat is enriched in genes for anaerobic respiration (sulfate reduction and methanogenesis) and glycoside hydrolases (Lin *et al*. 2014a), potentially because slow rates of decomposition in upper layers of bog peat allows carbohydrates to build up in deeper layers (Tfaily *et al*. 2013). Metagenomes from both bog and fen peat contain fewer copies of copper oxidase genes than those from other soils (Lin *et al*. 2014a): this of particular interest as this family includes laccases, an important group of phenol oxidase enzymes (Sinsabaugh 2010). Copper oxidase genes are therefore of potential relevance to peat carbon sequestration since low phenol oxidase activity may be one of the main factors allowing the build-up of carbon in peatlands (Freeman *et al*. 2001; Fenner and Freeman 2011).

The microbial mechanisms underlying drought-driven carbon loss from peatlands are at present not well-understood, despite growing evidence that the microbial community in peatlands responds to water level (Kim *et al*. 2008; Nunes *et al*. 2015; Peltoniemi *et al*. 2015). Previous studies have shown that environment-driven taxonomic changes are accompanied by changes in functional potential (Luo *et al*. 2014; Tas *et al*. 2014), which could not be accurately detected using MGA alone. However, while a number of recent studies have used MGA to examine the effect of drought on the taxonomic composition of microbial communities (Barnard *et al*. 2013;

2015), at the time of writing shotgun metagenomic sequencing had not been used to analyse changes in the microbial community during drought in any habitat and the effect of drought in the peat acrotelm had not been fully established using DNA-based techniques. Therefore, our understanding of the linkage between microbial community changes and functional changes remains incomplete.

Aims and Objectives of Chapter

Biogeochemical analyses suggested that enzyme activities (phenol oxidase and β-glucosidase) were higher at 5cm below the peat surface than 20cm below it, and this remained true during drought (Chapter 2). This indicates that microbial communities in the upper layer of peat are potentially of great interest, as they drive the highest potential rates of decomposition. In addition, ARISA fingerprinting indicated that microbial communities at this depth were significantly affected by a drought-rewetting treatment (Chapter 2), leading to questions about the ways in which the community at this depth changes (both taxonomically and functionally).

The aim of this chapter was to use full metagenome shotgun sequencing to investigate changes in both the taxonomic composition and functional potential of microbial communities during drought and rewetting in a peat bog at 5cm depth. The key objectives were as follows:

1. To determine the taxonomic profile and composition of functional genes of microbial communities in a Welsh bog at 5cm depth
2. To investigate changes in the taxonomic composition and α-diversity of bacterial and fungal communities in this environment over the course of drought and rewetting
3. To identify SEED subsystems which are differentially abundant during drought or rewetting compared to permanently waterlogged controls
4. To compare the relative abundance of phenol oxidase genes and genes involved with anaerobic metabolism in peat undergoing drought and rewetting with that of permanently waterlogged peat

To this end, a subset of DNA extracts from the experiment described in Chapter 2 were selected for full metagenomic shotgun sequencing. The selected extracts came from the 10 bog mesocosm cores at a depth of 5cm and represented 5 time points (T2, T4, T6, T7 and T9), yielding a total of 50 samples for sequencing.

4.2 Methods

4.2.1 Library Preparation, Sequencing and Quality Control

The design of the experiment, as well as methods for DNA extraction and purification, are described fully in Chapter 2. Based on the results presented in Chapter 2, a subset of samples were chosen for sequencing: these samples represented all ten bog mesocosm cores at 5cm depth and at five selected time points (T2, T4, T6, T7 and T9; see Chapter 2). The pre-drought period was represented by T2, while the other four samples represent different stages of drought and rewetting. In addition to DNA extracts from the mesocosm cores, a negative control was prepared by carrying out extraction and purification protocols on sterile PCR-grade water. Negative extraction and purification controls were then pooled into a single negative control.

Library preparation was carried out using a Nextera XT Kit (Illumina) according to manufacturer's instructions, in combination with the Nextera XT Index Kit V2 Set A (Illumina). The prepared libraries were pooled in equal quantities before initial sequencing on an Illumina MiSeq to test quality and concentration of DNA within each library. Following this initial MiSeq run, a final pooled library was created by adjusting the concentration of each library to reflect DNA concentration (as indicated by the number of reads per sample in the initial MiSeq data). The final pool was sequenced on an Illumina HiSeq 2500 in Rapid Run mode. Sequencing was carried out by the Institute for Microbiology and Infection at the University of Birmingham.

Reads were trimmed at the sequencing facility using Trimmomatic (Bolger *et al.* 2014) to remove adapter sequences and poor quality reads (i.e. those containing Phred scores below 28). Prior to taxonomic and functional assignment, reads which matched sequences in the negative control were removed using the script 'bbsplit.sh' from BBMap v35.43 (http://sourceforge.net/projects/bbmap). Within the MG-RAST workflow, artificial replicates and reads matching to *Homo sapiens* were also removed. Metagenome coverage was calculated using Nonpareil (Rodriguez-R and Konstantinidis 2013), with a minimum overlap of 25% and all other parameters set to defaults. Nonpareil calculates coverage based on sequence redundancy (i.e. how many reads are present multiple times in the dataset).

4.2.2 Taxonomic Assignment

To examine the taxonomic composition of the peat bog under normal and water-saturated conditions, taxonomic assignments were carried out during the first sequenced time point (T2), prior to the application of the drought treatment. Three methods of taxonomic assignment were compared: Phylosift, MG-RAST annotation of SSU sequences (MGRAST-SSU), and MG-RAST annotation of all reads (MGRAST-M5NR). Phylosift (Darling *et al.* 2014) was run with default parameters, using unmerged paired reads as inputs. The MG- RAST webserver carries out read annotations using BLAT (Glass *et al.* 2010). Due to the low proportion of read pairs with a significant overlap, reads were left unpaired prior to uploading to MG-RAST and only the first read of each pair was uploaded to avoid biasing the results. Taxonomic annotations were downloaded in biom format using the MG-RAST API with a minimum percentage identity of 97%, alignment length of 30 and only the best hit per read considered. To obtain MGRAST-SSU annotations, reads identified as SSU rRNA were annotated against the SILVA database (Pruesse *et al.* 2007), and to obtain MGRAST-M5NR annotations, all reads were searched against the M5NR database for whole-metagenome annotation (Wilke *et al.* 2013).

4.2.3 Effect of Drought on Taxonomic Composition (SILVA Annotations)

The effect of drought on the taxonomic composition of microbial communities was analysed based on MGRAST-SSU annotations. MGRAST-SSU annotations were chosen because the databases for SSU sequences are more complete than the M5NR database, thereby reducing the bias towards well-characterised taxa, and because Phylosift output takes the form of summed probabilities which are difficult to interpret and unsuitable for the majority of existing statistical tools. Prior to analysis, the number of annotations was rarefied using the 'rarefy_even_depth' command in the Phyloseq package (McMurdie and Holmes 2013). Fungi and bacteria were selected as the focus of the analyses in this chapter, as the methodology was inappropriate for analyses of macrofauna and protozoa were poorly represented in the annotations from MG-RAST.

To test for a significant effect of time and treatment on OTU richness of bacteria and fungi, the number of OTUs was calculated using function 'specnumber' in package 'vegan' (Dixon and Palmer 2003). Generalised linear models were then fitted using function 'glm'. Models were initially fitted using Poisson errors, but in cases where evidence of overdispersion was found

(i.e. the presence of greater variability than would be expected within the Poisson distribution), standard errors were corrected using a quasi-GLM method in which the variance was the mean multiplied by a dispersion parameter (in the Poisson distribution the variance is assumed to be equal to the mean; 'quasipoisson' errors). The significance of each interaction term was then analysed by analysis of deviance (function 'drop1.glm') using a Chi-squared test. Where significant differences were found, post-hoc tests were carried out using package 'lsmeans' (Lenth 2016).

Abundant bacterial and eukaryotic phyla (defined as those with more than 1000 reads in total) were tested for significant effects of time point, treatment, or the interaction of time point and treatment. The proportion of the rarefied dataset made up by each phyla was logit transformed (Warton and Hui 2011) before application of linear mixed-effects models with '~1|Core' as the random effects.

Next, OTUs occurring in fewer than five samples were removed and NMDS plots were generated using the 'metaMDS' function from R package 'VEGAN' (Dixon and Palmer 2003), based on quantitative Jaccard distances and with all other parameters as defaults. Within the package 'VEGAN', the quantitative Jaccard distance (hereafter simply Jaccard distance, D_J) is calculated as follows:

$D_J = \frac{2D_B}{1+D_B}$, where D_B = the Bray-Curtis dissimilarity.

The Bray-Curtis dissimilarity between samples *j* and *k*, B_{jk}, is defined as:

$$B_{jk} = \sum \frac{abs(x_{ij} - x_{ik})}{\sum x_{ij} + x_{ik}}$$

In the above equation, *i* stands for a given species. Separate NMDS plots were generated based on both family- and species-level annotations. In order to study the within-phylum community composition for the most abundant phyla, NMDS plots were generated using only OTUs which belonged to each of the most abundant phyla. PERMANOVA tests were run to test for differences between time points and treatments, and for the effects of the interaction between time point and treatment, using function 'adonis' from package 'VEGAN'. The relationship between environmental variables and community composition was investigated by applying the 'envfit' procedure from package 'VEGAN'. Envfit first calculates the direction of the effect

of a given variable: for ‘vectors’ (continuous variables) this is done by calculating the direction of maximum correlation between the variable and the ordination scores, while for ‘factors’ (discrete variables) envfit calculates the average ordination score for each factor level. Next, significance values are calculated for each variable using a permutation test.

Additionally, partial canonical correspondence analysis (pCCA) ordination was carried out to test the hypothesis that water table affected community composition. Canonical correspondence analysis is a constrained method: i.e. rather than attempting to display all variation present in the data (e.g. as NMDS ordination does), CCA only displays the component of the variation which is explained by the constraints of interest. Therefore, CCA is of value when testing clear hypotheses about the effect of a particular variable on the community (Legendre and Legendre 2012; Oksanen *et al.* 2015). In this case a factor encoding the water table direction was used as the constraining variable, with three different levels: steady (all control cores, and drought cores at T2), falling (drought cores at T4 and T6) and rising (drought cores at T7 and T9). To remove confounding effects caused by inter-core differences, the core from which a sample was taken was included as a conditioning variable. Ordinations were fitted using command ‘cca’ from package ‘VEGAN’ (Oksanen *et al.* 2015), and tested for an effect of water table using the ‘anova.cca’ command.

Next, a generalised estimation equation model (GEE) was fitted to counts of each OTU in order to test for significant interaction effects between time point and treatment, following Zuur *et al.* (2009). GEEs were chosen over alternative models because they allow for a dependence structure to be fitted (in this case, samples taken from the same core are correlated), rather than assuming all data points are independent, and because they can be fitted to non-normal data such as counts. First, OTUs were filtered to select only abundant organisms: those making up at least 0.1% of the community in at least one sample, and occurring in at least 40% of samples. GEEs were fitted with the Poisson distribution and with an ‘exchangeable’ correlation structure (all observations within a core are equally correlated to one another). After model fitting, the significance of interaction between time point and treatment was calculated using a Wald test and the obtained *p*-values were adjusted for multiple comparisons using a Benjami-Hochberg correction (function ‘p.adjust’). All GEE models were fitted with package ‘geepack’ (Yan *et al.* 2012).

4.2.4 Effect of Mesocosm Core on Taxonomic Composition (SILVA Annotations)

To further investigate the large differences in community composition which were observed between different mesocosm cores, tests of differential abundance were carried out at phylum and domain level using package 'DESeq2' (Love *et al.* 2014). DESeq2 fits generalised linear models with negative binomial distributions, with dispersion estimated across multiple genes with similar expression. Although DESeq2 was initially developed for use with RNA-seq data, it is also applicable to other data types where the input matrix describes raw counts of sequences assigned to different genes, including metagenomic datasets.

4.2.5 Assembly of novel 16S rRNA regions (EMIRGE Assemblies)

In order to examine the effect of drought on bacterial OTUs not present in the SILVA database, full-length 16S sequences were assembled using EMIRGE (Miller *et al* 2011). Briefly, the EMIRGE algorithm begins with a set of reference sequences, maps sample reads to the reference sequences, and then 'corrects' the reference based on the probability that it gave rise to the sample reads. Repeated correction of sequences is carried out over forty iterations, with each iteration increasing the probability that the set of assembled sequences gave rise to the sample data. EMIRGE was run on paired end reads using SILVA v111 (Pruesse *et al.* 2007) as the initial reference database, with an average insert size of 300 and standard deviation of the input size of 20. The SILVA database was chosen as a reference because it was the database used for testing of EMIRGE by the initial authors (Miller *et al.* 2011).

Due to computational constraints, EMIRGE was run individually on each sample and the results merged by combining the outputs from each sample and clustering at 97% similarity using usearch v7.0 (Edgar 2010). Next, the representative assembled 16S rRNA gene sequences were used as a reference database for assigning reads to OTUs using the 'pick_closed_reference_otus.py' script in QIIME (Caporaso *et al.* 2010). These abundances were combined into a single data frame and rarefied to give an even number of OTUs per sample. OTUs occurring in fewer than five samples were removed prior to analysis. Community composition was analysed using the 'VEGAN' package in R (Dixon and Palmer 2003): NMDS plots were generated based on Jaccard distances, and the effect of environmental variables was fitted to ordinations using function 'envfit'. Taxonomy was assigned using the

'assign_taxonomy.py' script in QIIME, with the SILVA database v119 as a reference (Pruesse *et al* 2007).

4.2.6 Functional Assignment

As with taxonomic assignment, functional assignments were carried out by BLAT within the MG-RAST webserver (Glass *et al.* 2010). Annotations against the M5NR database were downloaded in biom format through the MG-RAST API with a minimum percentage identity of 90% and a minimum alignment length of 30. Only the best hit for each read was considered. Pathways and gene functions were obtained from the SEED database, and annotations belonging to eukaryotes and bacteria were downloaded separately.

Table 4.1: Properties of HMMs representing genes of interest. These represent either genes for oxidase enzymes or genes which represent key steps in pathways of anaerobic metabolism.

	Gene/Family	Source	I.D.	Pathway
lcc-A	Laccase- Ascomycete	FunGene	lcc-A	Oxidase
lcc-B	Laccase-Basidiomycete	FunGene	lcc-B	Oxidase
ppo	Polyphenol Oxidase	FunGene	ppo	Oxidase
mnp	Manganese peroxidase	FunGene	mnp	Oxidase
lip	Lignin peroxidase	FunGene	lip	Oxidase
vp1	Versatile peroxidase	FunGene	vp1	Oxidase
cuo3	Multi-copper oxidase Type III	PFAM	PF07732	Oxidase
cuo4	Multi-copper oxidase Type IV	PFAM	PF00394	Oxidase
diox	Dioxygenase	PFAM	PF00775	Oxidase
perox	Peroxidase	PFAM	PF00141	Oxidase
tyros	Tyrosinase	PFAM	PF00264	Oxidase
pm	Phenol mono-oxygenase	PFAM	PF04663	Oxidase
dsrA	Dissimilatory sulfate reductase	FunGene	dsrA	Sulfate reduction
hydA	Hydrogenase	FunGene	hydA	Fermentation
nirK	Nitrite reductase	FunGene	nirK	Denitrification
nosZ	Nitrous oxide reductase	FunGene	nosZ	Denitrification
nirS	Nitrite reductase	FunGene	nirS	Denitrification
pmoA	Particulate methane mono-oxygenase	FunGene	pmoA	Methane oxidation
alkB	Alkane hydroxylase	FunGene	alkB	Alkane degradation

Annotations were rarefied using the 'rarefy_even_depth' command in the Phyloseq package (McMurdie and Holmes 2013) and combined at the level of module (the third level of the hierarchy). Modules with less than one annotation were removed. Next, NMDS plots were generated using the 'metaMDS' function in package 'VEGAN' (Dixon and Palmer 2003), based on Jaccard distances. The impact of environmental variables on functional composition was tested by applying the 'envfit' procedure from package 'VEGAN' to NMDS ordinations.

To test for a significant effect of the time point: treatment interaction on the abundances of any SEED module, GEEs were fitted on the count abundances of the most abundant genes. First, OTUs were filtered to select only abundant modules: those making up at least 1% of the community in at least one sample, and occurring in at least 40% of samples. GEEs were fitted with the Poisson distribution and with an 'exchangeable' correlation structure (all observations within a core are equally correlated). After model fitting, the significance of the interaction term between sampling time point and treatment was calculated using a Wald test and the obtained *p*-values were adjusted for multiple comparisons using a Benjami-Hochberg correction (with function 'p.adjust'). All GEE models were fitted with package 'geepack' (Yan *et al.* 2012).

4.2.7 Abundance of genes involved in phenol degradation and anaerobic metabolism

In order to examine the effect of drought on abundances of phenol oxidase genes and on marker genes for anaerobic respiration and alkane hydroxylase genes, nine hidden Markov models (HMMs) were taken from the FunGene (Fish *et al* 2013) and PFAM (Finn *et al* 2013) databases. All selected HMMs are described in Table 4.1. Additionally, an HMM of the conserved single-copy gene *rpoB* was downloaded from FunGene to use in normalising counts of each of the other genes.

The command 'hmmsearch' from HMMER (Eddy 2011) was used to find matches for each HMM, using default parameters. The taxonomic affiliations of oxidase genes was determined by using blastp (Camacho *et al.* 2009) to search all matches to the HMM against the protein sequences in the family in question (downloaded from PFAM). The LCA algorithm in MEGAN (Huson *et al.* 2007) was then used to find the most likely phylum assignment for each of the blastp hits.

Gene numbers per cell were calculated by dividing the number of hits to each HMM of interest by the number of hits to the *rpoB* HMM. This gave a proportion which was arcsine transformed prior to fitting of a general linear model. Random effects were chosen by fitting the maximal model using two random effects terms (~1|core or ~core|time) as well as with fixed effects only, and comparing likelihood ratios of each.

4.3 Results

4.3.1 Read Counts and Coverage

Following trimming and removal of the negative control a total of 349,042,939 read pairs remained, representing an average of 6,980,859 read pairs per sample (982,587,443 nucleotides, or just under 1 gigabase). However, there was a large degree of variation in the number of reads per sample, with a minimum of 2,787,753 pairs and a maximum of 17,492,126 pairs. A mean of 6,576,636 reads per sample passed quality control in MG-RAST. Samples contained a mean of 5,527,859 reads predicted to originate from proteins and 536,927 reads predicted to originate from rRNA sequences, of which on average 1,618,285 proteins and 38,024 rRNA sequences could be identified (i.e. 29% of predicted proteins and 7% of predicted rRNAs could be annotated). However, the majority of BLAT hits were of poor quality, with a mean percentage identity of 48%.

Table 4.2: Results of generalised linear models (glm) applied to test for the effect of drought on species and family richness of both bacteria and fungi in metagenomic shotgun sequencing dataset.

Taxa	Variable	Factor		d.f.	p (> Chi)
Bacteria	**Species Richness**	Time Point		4	0.002**
		Treatment		1	0.001**
		Time	Point:	4	0.1
	Family Richness	Time Point		4	0.3
		Treatment		1	0.04*
		Time	Point:	4	0.8
Fungi	**Species Richness**	Time Point		4	0.1
		Treatment		1	0.01*
		Time	Point:	4	0.8
	Family Richness	Time Point		4	0.3
		Treatment		1	0.02*
		Time	Point:	4	0.8

Estimated coverage ranged from 30% to 78%, with a mean of 53%. Nonpareil estimated that to reach 95% coverage in this habitat an average of 6,672,189,963 base pairs would be required per sample, requiring three orders of magnitude more sequencing effort than in the current study.

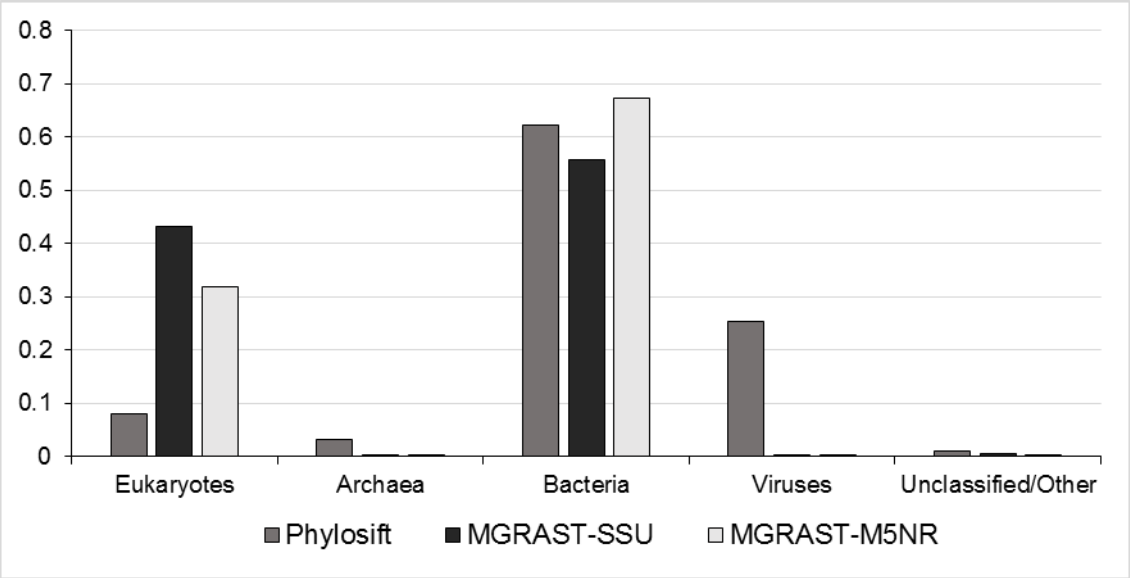

Figure 4.1: Mean proportion of reads in all cores at time point two (pre-drought) assigned to each domain of cellular life or to viruses by three methodologies: Phylosift (Darling et al. 2014), BLAT annotation of SSU reads in MGRAST (MGRAST-SSU) and BLAT annotation of all predicted genes in MGRAST (MGRAST-M5NR). Mean values were calculated across all mesocosm cores at time point 2 (pre-drought).

4.3.2 Undisturbed Community Composition

Bacteria were the most abundant domain in the metagenomes regardless of the methodology used to assign taxonomy, and comprised a mean of 61% of SSU reads (Figure 4.1). However, the three methodologies gave results which differed considerably in the relative importance of other high-level classifications: viruses formed a quarter of the community when taxonomy was assigned by Phylosift but made up a much smaller proportion of MG-RAST annotations. Conversely, eukaryotes made up a larger proportion of annotations from both MG-RAST workflows (43% of MGRAST-SSU annotations, and 32% of MGRAST-M5NR annotations) than from PhyloSift annotations. Archaea made up a very small proportion of the community, contributing less than 1% of MG-RAST annotations and 3% of annotations according to Phylosift. Fungi were the most abundant group of eukaryotes, although Streptophyta also made

up a large proportion of the community (Figure 4.2). Protists made up only a small proportion of the community: the most abundant protist group was the Apicomplexa, with 0.3% of total reads.

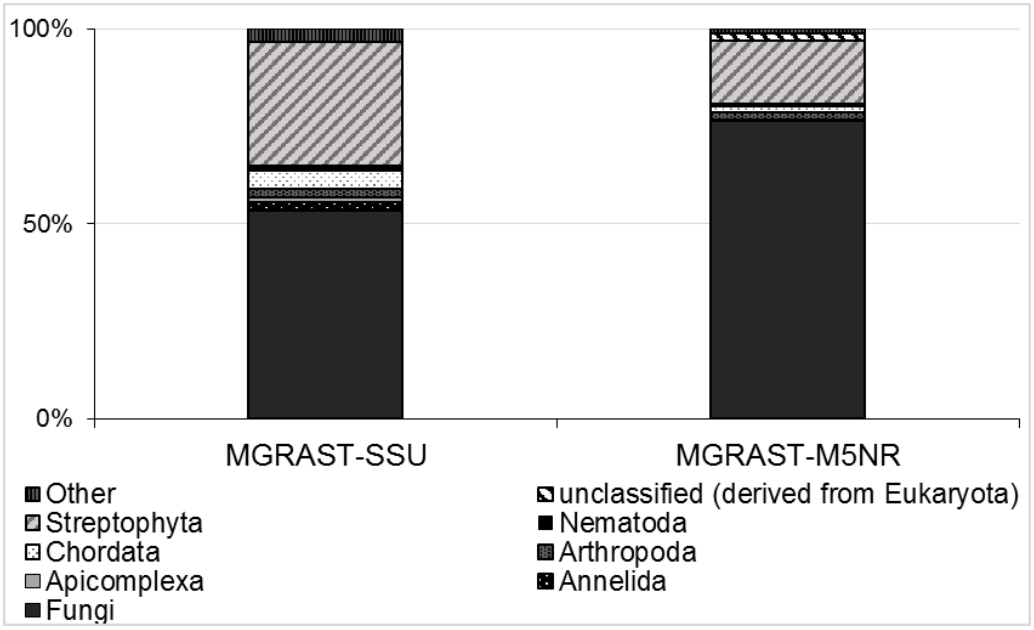

Figure 4.2: Proportion of eukaryotic reads belonging to each of the most abundant eukaryotic groups. See figure 4.4 for fungal phyla. Only phyla which made up >1% of the community are shown, with the remainder summed as 'Other'. Mean values were calculated across all mesocosm cores at time point 2 (pre-drought).

Within the domain Bacteria, the three methodologies gave very different community compositions at phylum level (Figure 4.3). Proteobacteria were highly abundant regardless of methodology, but the proportion of the community composed of Proteobacteria ranged from 69% (MGRAST-M5NR) to 22% (MGRAST-SSU). The proportion of Acidobacteria likewise varied considerably with methodology, ranging from 25% of Phylosift annotations to only 1% of MGRAST-M5NR annotations. According to MGRAST-SSU annotations, α-Proteobacteria dominated within the Proteobacteria (36% of total Proteobacteria), followed by β-Proteobacteria (23%), γ-Proteobacteria (23%) and δ-Proteobacteria (14%). Both ε- and ζ-Proteobacteria were rare, making up less than 1% of annotated Proteobacteria. Abundant orders within the Proteobacteria included Rhizobiales (α-Proteobacteria), Burkholderiales (β-Proteobacteria), Neisseriales (β-Proteobacteria) and Pseudomonadales (γ-Proteobacteria). Within the Acidobacteria, Acidobacteriales and Solibacterales each made up approximately a third of annotations, while the remaining third could not be assigned to a class. Abundant orders

(> 1000 annotations) within other phyla included Actinomycetales (Actinobacteria), Bacteroidales (Bacteroidetes), Sphingobacteriales (Bacteroidetes), Bacillales (Firmicutes), Clostridiales (Firmicutes), Planctomycetales (Planctomycetes) and Verrucomicrobiales (Verrucomicrobia).

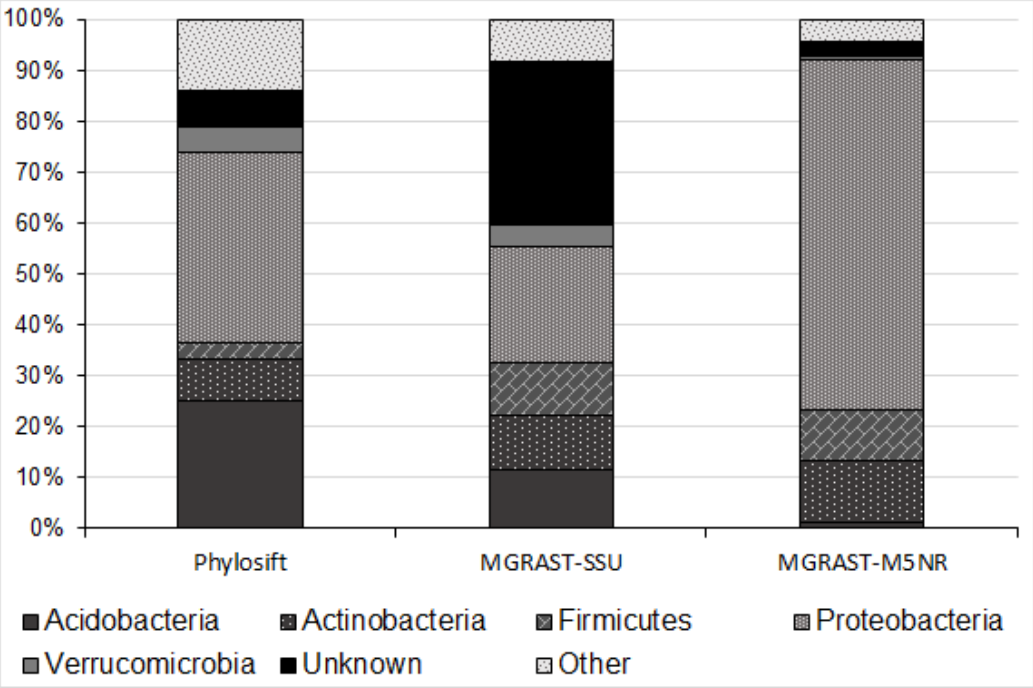

Figure 4.3: Proportion of bacterial reads which belonged to each of the most abundant phyla within the domain Bacteria. Only phyla which made up >1% of the community are shown, with the remainder summed as 'Other'. Mean values calculated across all mesocosm cores at time point 2 (pre-drought).

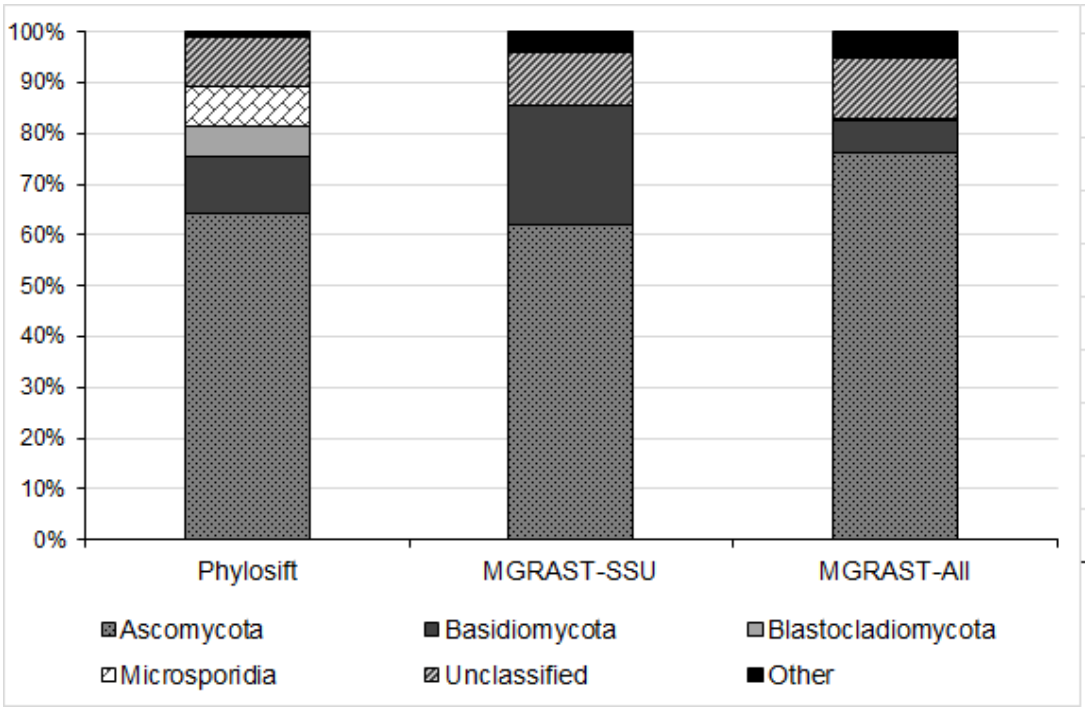

Figure 4.4: Proportion of fungal reads which belonged to each of the most abundant fungal phyla. Only phyla which made up >1% of the total are shown, with the remainder summed as 'Other'. Mean values were calculated across all mesocosm cores at time point 2 (pre-drought).

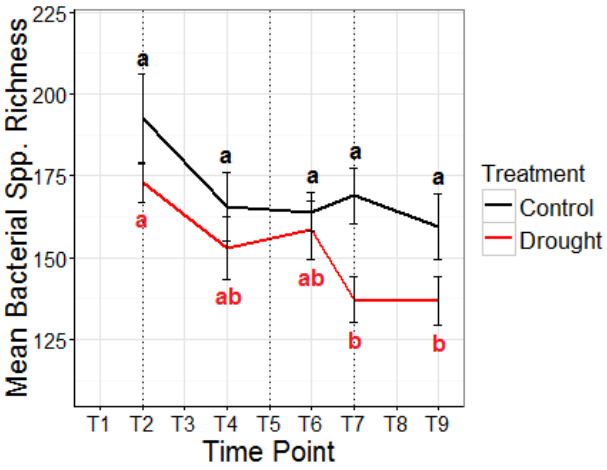

Figure 4.5: Mean bacterial species richness in droughted and control mesocosm cores by time point. Error bars represent standard errors.

Table 4.3: Linear mixed-effects models to test for the effect of drought on the proportion of the dataset made up by the most abundant bacterial and fungal phyla. The random effects term in each case was "~1|Core". Proportions were logit transformed prior to model fitting.

Phylum	Factor		F	d.f.	p
Acidobacteria	**Time Point**		0.3	4, 32	0.9
	Treatment		0.3	1, 8	0.6
	Time	**Point:**	0.7	4, 32	0.6
Actinobacteria	**Time Point**		0.8	4, 32	0.5
	Treatment		1.7	1, 8	0.2
	Time	**Point:**	1.7	4, 32	0.2
Firmicutes	**Time Point**		0.8	4, 32	0.5
	Treatment		<0.1	1, 8	0.9
	Time	**Point:**	2.4	4, 32	0.07.
Proteobacteria	**Time Point**		<0.1	4, 32	1
	Treatment		3.8	1, 8	0.09.
	Time	**Point:**	1.7	4, 32	0.2
Verrucomicrobia	**Time Point**		1.7	4, 32	0.2
	Treatment		0.2	1, 8	0.7
	Time	**Point:**	0.3	4, 32	0.9
Bacteroidetes	**Time Point**		0.8	4, 32	0.5
	Treatment		<0.1	1, 8	0.9
	Time	**Point:**	2.2	4, 32	0.1
Ascomycota	**Time Point**		0.7	4, 32	0.6
	Treatment		1.5	1, 8	0.2
	Time	**Point:**	0.5	4, 32	0.7
Basidiomycota	**Time Point**		0.9	4, 32	0.5
	Treatment		0.1	1, 8	0.8
	Time	**Point:**	0.5	4, 32	0.7

Regardless of the methodology used for taxonomic assignment, Ascomycota were the dominant fungal phylum (Figure 4.4), making up between 59% (Phylosift) and 76% (MGRAST-M5NR) of fungi. Among the less abundant phyla there were large differences between methodologies: annotation of MGRAST-SSU sequences indicated a higher proportion of Basidiomycota (25%) than was suggested by MGRAST-M5NR (6%) or Phylosift (11%) annotations. Microsporidia and Blastocladiomycota made up a significant proportion of the community when Phylosift was used for taxonomic assignment, but were barely detected by MG-RAST (Figure 4.4). Within the MGRAST-SSU rRNA annotations, abundant classes (>1000 hits) within phylum Ascomycota were Leotiomycetes, Sordariomycetes,

Dothideomycetes and Eurotiomycetes and abundant families within phylum Basidomycota were Agaricomycetes and Tremellomcyetes.

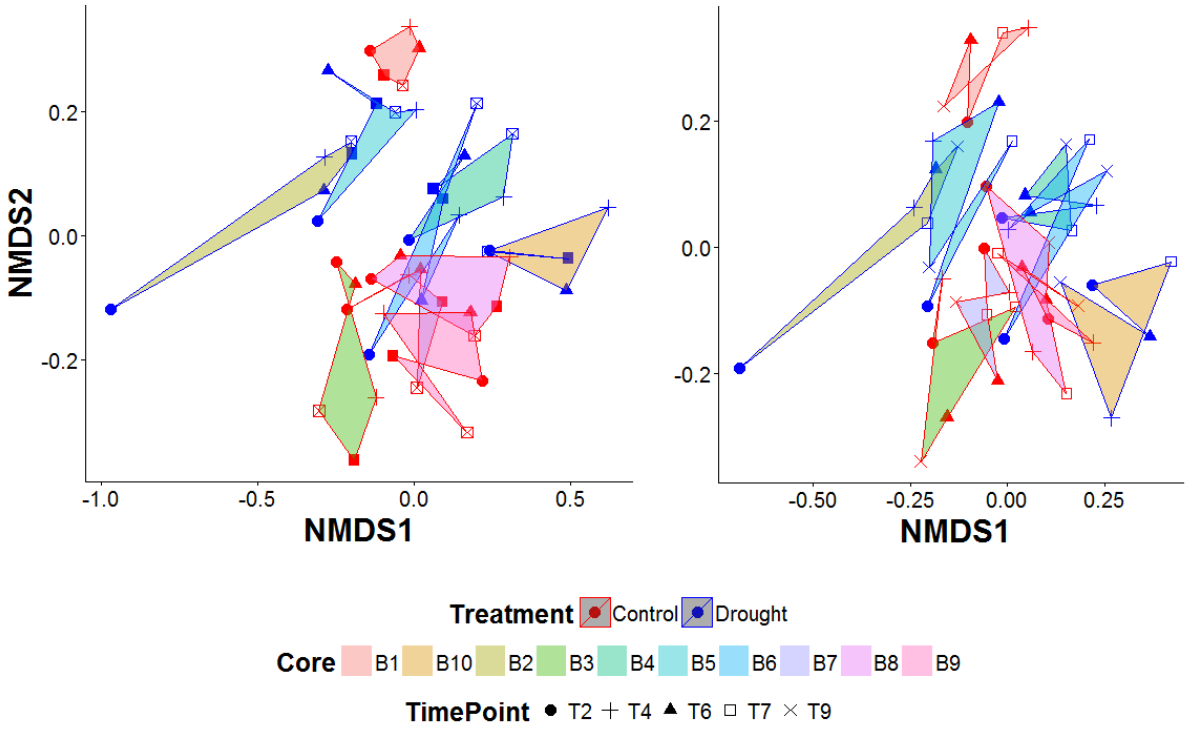

Figure 4.6: NMDS ordination of bacterial communities based on (A) species-level annotations and (B) family-level annotations. Ordination was based on Jaccard distances, and all annotations were carried out in MG-RAST against the SILVA SSU rRNA database.

4.3.3 Effect of Drought on Taxonomic Composition (SILVA Annotations)

Treatment had a significant effect on OTU and family richness of both bacteria and fungi (Table 4.2). Additionally, bacterial OTU richness varied significantly between sampling time points: bacterial OTU richness fell over time, and this fall was greatest in droughted cores (Figure 4.5). However, the interaction between time point and treatment was not significant for bacteria or fungi (Table 4.2). Neither time point nor treatment had a significant effect on the abundance of any of the most abundant bacterial phyla (Table 4.3), although the interaction between time point and treatment had a marginally significant effect on the abundance of Firmicutes (which decreased with time in all cores, but less so in droughted cores; p=0.07) and

treatment had a marginally significant effect on the abundance of Proteobacteria (more abundant in droughted cores; p=0.09).

Table 4.4: PERMANOVA to test for effects of treatment, time point, and the interaction term on bacterial and fungal community composition.

Taxa	Factor	F	d.f.	R^2	p
Bacteria	**Treatment**	1.2	1	0.02	0.1
	Time Point	0.9	4	0.08	0.8
	Treatment: Time Point	0.9	4	0.07	1.0
Fungi	**Treatment**	1.4	1	0.03	0.007**
	Time Point	1.0	4	0.08	0.8
	Treatment: Time Point	0.9	4	0.08	0.9

There was no significant effect of time point, treatment, or the interaction between time point and treatment on bacterial community composition (Table 4.4). Bacterial communities clustered by mesocosm core rather than treatment in NMDS ordinations, with a similar pattern shown regardless whether the ordination was based on OTU-level or family-level annotations (Figure 4.6). However, application of 'envfit' to the NMDS ordination of bacterial communities revealed that bacterial community composition was significantly correlated to treatment, phenol oxidase activity, β-glucosidase activity, redox potential and percentage cover of dwarf shrubs and bare ground (Figure 4.7; Table 4.5). There was also a significant relationship between the mesocosm core from which samples were collected and bacterial community composition (Figure 4.7; Table 4.5). OTUs showed a degree of clustering according to phyla: for example, the majority of Bacteroidetes had values on the first axis which are between 0 and -0.5, while Actinobacteria almost exclusively exhibited positive scores on at least one of the two axes shown.

Next, NMDS ordinations were calculated based on rarefied abundances of OTUs within the five most abundant bacterial phyla (Proteobacteria, Acidobacteria, Actinobacteria, Firmicutes and Verrucomicrobia) and 'envfit' correlations calculated (Table 4.5). Community composition within phylum Proteobacteria showed a significant relationship with the concentration of phenolic compounds, redox potential, percentage cover of graminoids and core; community composition within phylum Actinobacteria showed a significant relationship with redox potential, the percentage cover of mosses, dwarf shrubs and bare ground, and core;

community composition within phylum Firmicutes showed a significant relationship with phenol oxidase, redox potential and core; and community composition within phylum Verrucomicrobia showed a significant relationship with β-glucosidase activity, core, and percentage cover of bare ground and dwarf shrubs. The direction of these relationships is shown in Figure 4.8. No significant relationships were found between Acidobacteria community composition and any environmental variable tested.

Table 4.5: Results from application of 'envfit' to NMDS ordinations of communities, based on annotation of SSU rRNA genes against the SILVA database. NMDS ordinations were based on Jaccard distances of OTUs which were assigned by annotating SSU reads to species level. POX= phenol oxidase activity, b-gluc = β-glucosidase activity; redox = redox potential; shrubs = percentage cover of dwarf shrubs; bare/litter = percentage cover of bare ground and/or litter; mosses = percentage cover of all mosses; graminoids = percentage cover of all graminoids; core = the mesocosm core from which samples were taken. Only significant values are shown. A single outlying sample was removed from the Actinobacterial data prior to running 'envfit' model.

Taxa	Variable	R^2	p
All Bacteria	**POX**	0.3214	0.002**
	B-gluc	0.2848	0.003**
	Redox	0.5674	0.001***
	Shrubs	0.4185	0.001***
	Bare/Litter	0.2154	0.014*
	Core	0.835	0.003**
	Treatment	0.1029	0.022*
Proteobacteria	**Phenol**	0.1915	0.017*
	Redox	0.1595	0.046*
	Graminoids	0.1978	0.02*
	Core	0.4361	0.001***
Actinobacteria	**Redox**	0.1514	0.030*
	Mosses	0.1516	0.048*
	Shrubs	0.189	0.021*
	Bare/Litter	0.2285	0.009**
	Core	0.5936	0.001***
Firmicutes	**POX**	0.1582	0.038*
	Redox	0.1683	0.034*
	Core	0.5004	0.001***
Verrucomicrobia	**B-gluc**	0.3868	0.00***
	Shrubs	0.3651	0.001***
	Bare/Litter	0.1911	0.022*
	Core	0.5143	0.001***
All Fungi	**Redox**	0.1704	0.020*
Ascomycetes	**Phenol**	0.1984	0.023*
	Core	0.3703	0.03*

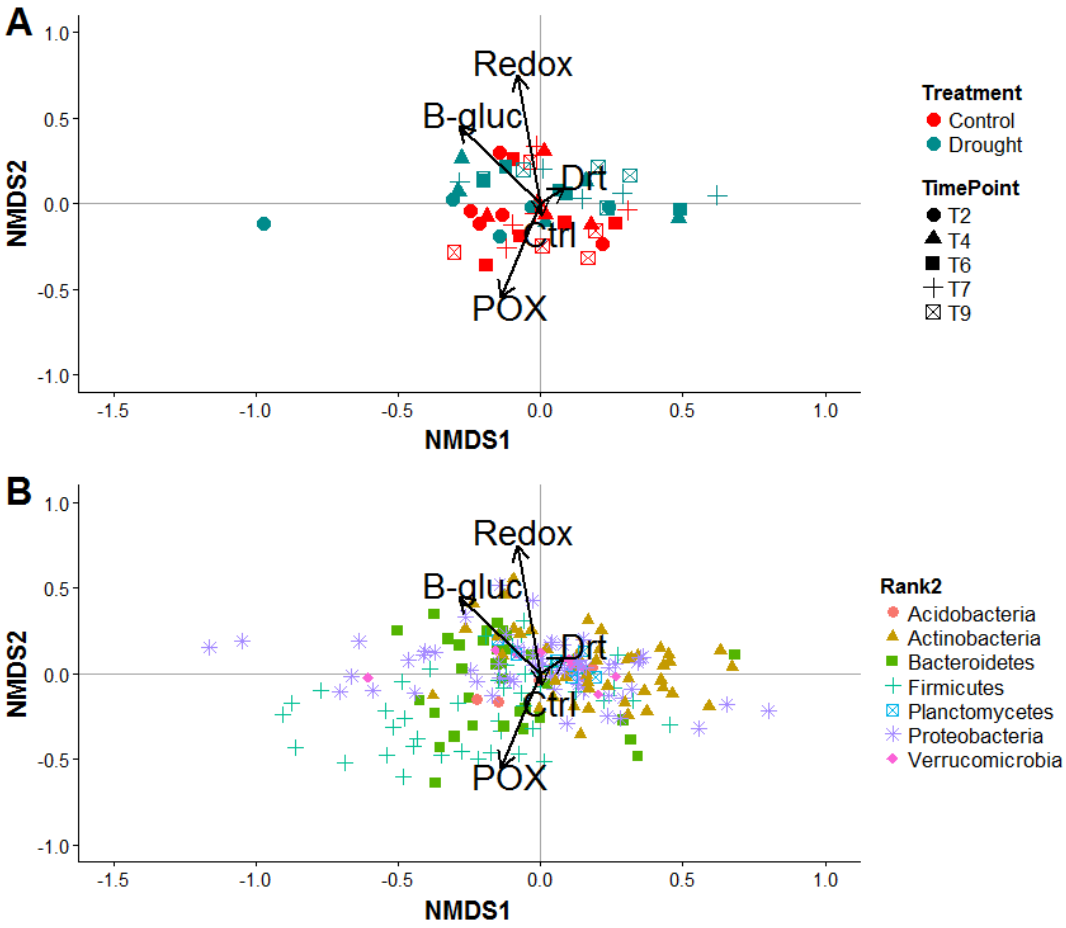

Figure 4.7: NMDS ordination of bacterial communities, depicting (A) samples and (B) OTUs. Arrows depict the result of 'envfit' (i.e. correlations between community composition and environmental variables). Ordination was based on Jaccard distances, and all annotations were carried out in MG-RAST against the SILVA SSU rRNA gene database. B-gluc=β-glucosidase activity; POX = phenol oxidase activity; Redox = redox potential; Drt = drought-treated mesocosm cores; Ctrl= control mesocosm cores.

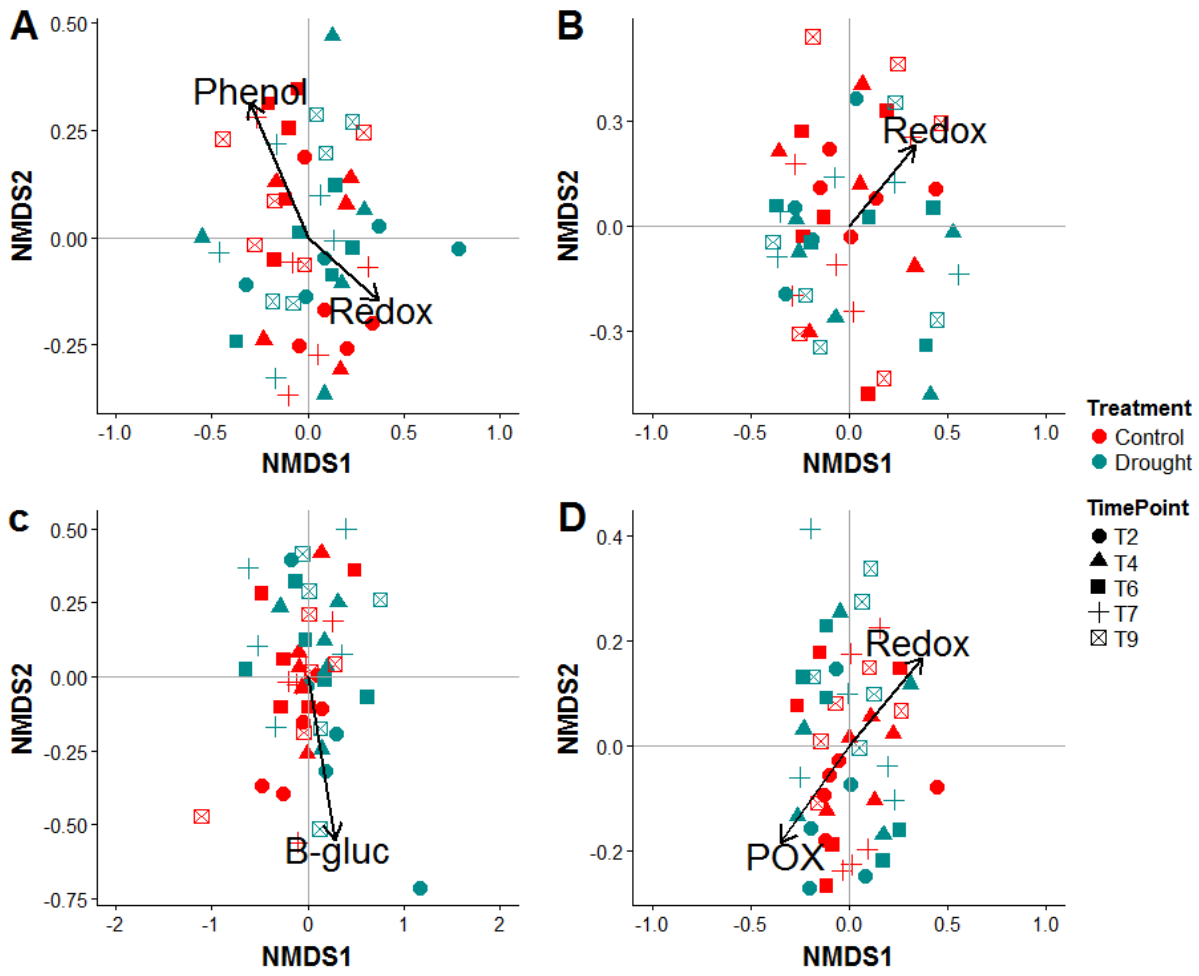

Figure 4.8: NMDS ordination of within-phylum community composition of samples, based on OTUs within four of the most abundant bacterial phyla: (A) Proteobacteria, (B) Actinobacteria, (C) Verrucomicrobia and (D) Firmicutes. Arrows depict the significant results of 'envfit' (i.e. significant correlations between community composition and environmental variables). Ordination was based on Jaccard distances, and all annotations were carried out in MG-RAST against the SILVA SSU rRNA gene database. B-gluc=β-glucosidase activity; POX = phenol oxidase activity; Redox = redox potential; Phenol =concentration of soluble phenolic compounds.

PERMANOVA detected a significant effect of treatment on fungal community composition, although this was not accompanied by any significant effect of time point or of the interaction between time point and treatment (Table 4.4). Mesocosm cores clustered more weakly in NMDS ordinations of fungal communities than bacterial communities, although the majority of variation was dominated by two samples belonging to a single core (Figure 4.9). Application of 'envfit' to fungal NMDS found that fungal community composition was significantly related

to redox potential (Table 4.5), and treatments showed weak separation along the second axis (the direction of strongest correlation between community composition and both redox potential and percentage water; Figure 4.10). On an NMDS plot of OTUs, the two main phyla showed patterns along the second axis: the majority of Basidiomycota occupied the lower part of this axis, while Ascomycota dominated the centre of the plot. Ascomycota community composition was significantly related to concentration of soluble phenolic compounds and core.

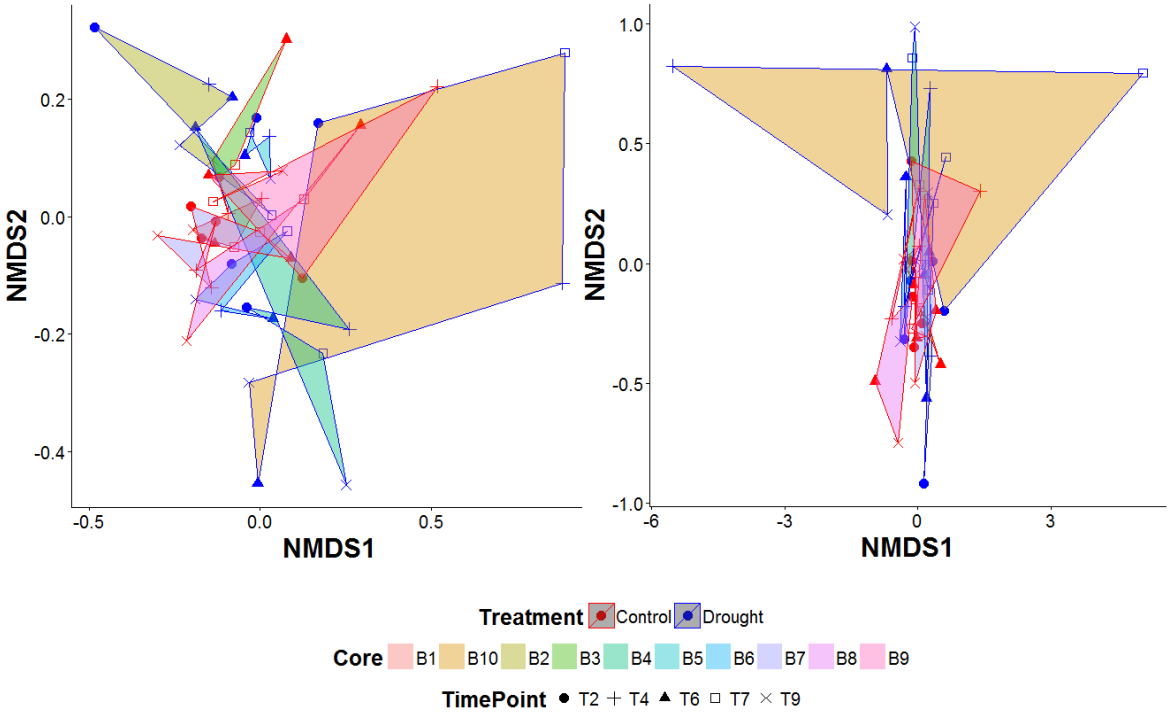

Figure 4.9: NMDS ordination of fungal communities based on (A) species-level annotations and (B) family-level annotations. Ordination was based on Jaccard distances, and all annotations were carried out in MG-RAST against the SILVA SSU rRNA gene database.

Canonical correspondence analysis followed by permutation tests (function 'cca.anova') found that water table did not have a significant effect on either bacterial (χ^2=0.05, d.f. = 2, p = 0.3) or fungal (χ^2=0.15, d.f. = 2, p = 0.5) community composition. Wald tests performed on generalised estimation equation (GEE) models of rarefied OTU abundances found that

abundances were not significantly affected by the interaction between time point and treatment for any of the individual OTUs tested.

Table 4.6: Results of tests for differential abundance between cores carried out in DESeq2. Phyla and domains were analysed separately. Adjusted *p*-values represent the result of likelihood ratio tests, adjusted using the Benjamini-Hochberg procedure.

Level	Taxon	Mean	Chi-sq.	p.adj
Domain	Archaea	2814.6	71.0	<0.001***
Domain	Eukaryota	42.8	55.5	<0.001***
Domain	Bacteria	4497.7	40.6	<0.001***
Phylum	Acidobacteria	523.8	63.8	<0.001***
Phylum	Actinobacteria	419.0	54.4	<0.001***
Phylum	Annelida	56.5	42.7	<0.001***
Phylum	Arthropoda	85.0	66.6	<0.001***
Phylum	Ascomycota	768.1	39.5	<0.001***
Phylum	Bacillariophyta	5.6	21.8	0.03*
Phylum	Bacteroidetes	190.6	53.0	<0.001***
Phylum	Basidiomycota	258.0	21.9	0.03*
Phylum	Blastocladiomycota	4.9	30.1	0.002**
Phylum	Brachiopoda	2.5	28.8	0.003**
Phylum	Chlorobi	2.6	37.6	<0.001***
Phylum	Chordata	264.5	49.8	<0.001***
Phylum	Crenarchaeota	4.3	55.4	<0.001***
Phylum	Euryarchaeota	15.7	40.1	<0.001***
Phylum	Firmicutes	325.3	38.2	<0.001***
Phylum	Mollusca	4.4	28.8	0.003**
Phylum	Nitrospirae	11.0	65.7	<0.001***
Phylum	Platyhelminthes	51.0	22.9	0.02*
Phylum	Proteobacteria	853.1	32.2	0.001**
Phylum	Streptophyta	700.4	43.9	<0.001***
Phylum	Thaumarchaeota	3.8	55.5	<0.001***
Phylum	Verrucomicrobia	204.6	47.6	<0.001***
Phylum	Xanthophyceae	2.7	24.7	0.01*
Phylum	unclassified (derived from Archaea)	2.1	36.8	<0.001***
Phylum	unclassified (derived from Bacteria)	1342.1	45.9	<0.001***
Phylum	unclassified (derived from Eukaryota)	215.6	63.0	<0.001***

4.3.4 Effect of Mesocosm Core on Taxonomic Composition (SILVA Annotations)

The abundances of all three domains (Bacteria, Archaea and Eukaryota) were found to show significant differences between mesocosm cores (Table 4.6; Figure 4.11; Figure 4.12). In

addition, six phyla were significantly differentially abundant between cores: Arthropoda, Acidobacteria, Actinobacteria, Annelida, Ascomycota and Bacillariophyta (Table 4.6). In particular, samples taken from core B10 contained a very low proportion of reads belonging to eukaryotes and a high proportion of archaea (Figure 4.11A).

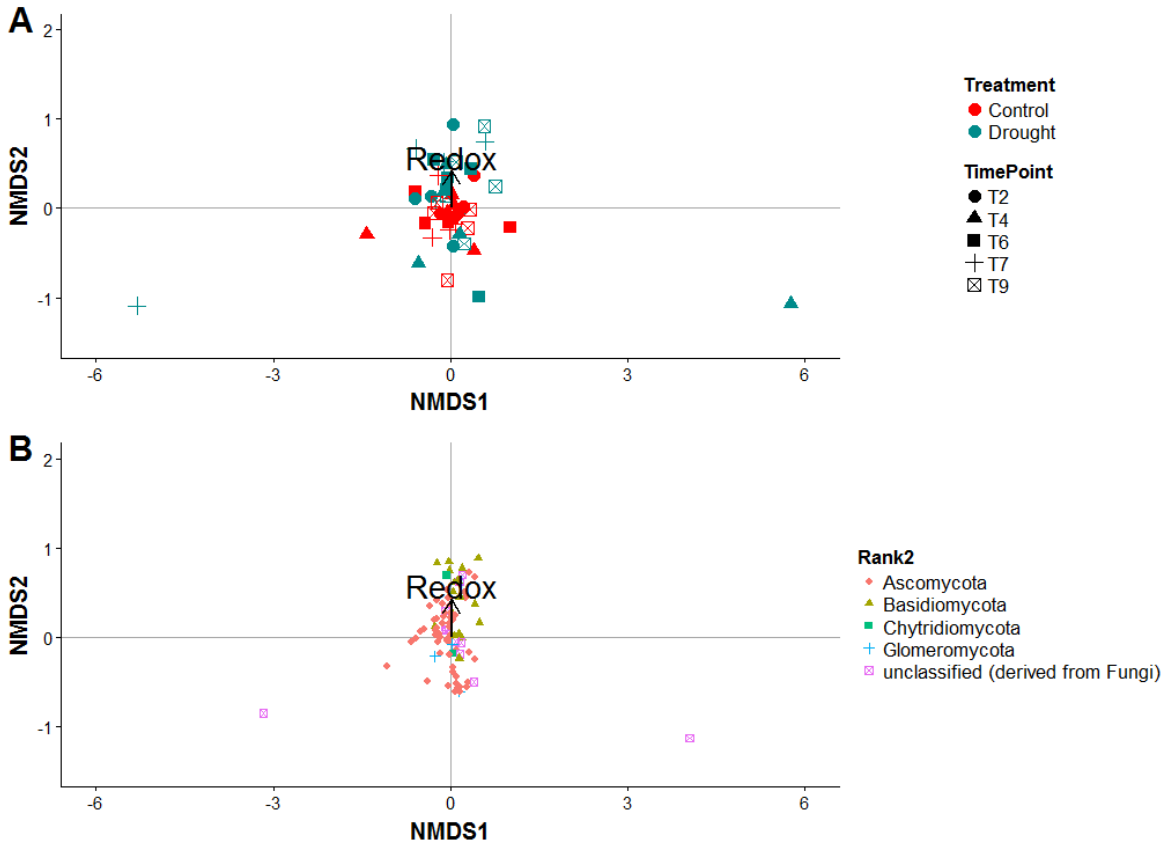

Figure 4.10: NMDS ordination of fungal communities, depicting (A) samples and (B) OTUs. Arrows depict the result of 'envfit' (i.e. correlations between community composition and environmental variables). Ordination was based on Jaccard distances, and all annotations were carried out in MG-RAST against the SILVA SSU rRNA gene database. B-gluc=β-glucosidase activity; POX = phenol oxidase activity; Redox = redox potential; S=steady water table; F = falling water table; R = rising water table.

4.3.5 Effect of Drought on Taxonomic Composition (EMIRGE Assemblies)

In total, EMIRGE assembly followed by clustering of the obtained sequences at 97% similarity yielded a total of 2,652 sequences, each representing a full-length 16S rRNA gene ("EMIRGE-assemblies"). By far the most abundant phyla amongst EMIRGE-assemblies was Proteobacteria, making up 40% of total sequences, followed by Acidobacteria (16%) (Figure

4.13). Considerable numbers of EMIRGE-assemblies were also assigned to Actinobacteria (7% of EMIRGE-assemblies), Bacteroidetes (5%), Firmicutes (4%), Planctomycetes (8%) and Verrucomicrobia (7%). In addition, several of the EMIRGE-assemblies belonged to rare phyla which were not detected by MG-RAST annotation of SSU reads: for example, candidate divisions OD1, OP11, OP3 and TM7. Candidate divisions are deeply-branching lineages with no cultured representatives, although in the case of TM7 isolates have been obtained since the initial identification of this lineage (Soro *et al.* 2014; He *et al.* 2015).

Table 4.7: Results from application of 'envfit' to NMDS ordinations of "EMIRGE-communities", i.e. communities based on closed-reference OTU-picking against a database made up of full-length 16S rRNA genes assembled using EMIRGE. NMDS ordinations were based on Jaccard distances. %water= water content. Only significant values are shown.

Taxa	Variable	R^2	p
All Bacteria	**%Water**	0.1629	0.045*
Proteobacteria	**pH**	0.2189	0.013*
	Treatment	0.1041	0.012*

Non-metric multidimensional scaling (NMDS) ordination of EMIRGE-communities showed no strong clustering by treatment, although 'envfit' detected a significant correlation between community composition and water content (Table 4.7; Figure 4.14). However, this correlation ceased to be significant if a single outlier was removed (data not shown). Similar to the NMDS plot of MG-RAST SSU annotations, certain bacterial phyla clustered together on the NMDS plot of the EMIRGE-communities: in particular, most Actinobacteria exhibited negative values on the second axis. When the most abundant phyla were analysed individually, community composition of Proteobacteria was significantly related to both pH and treatment (Table 4.7).

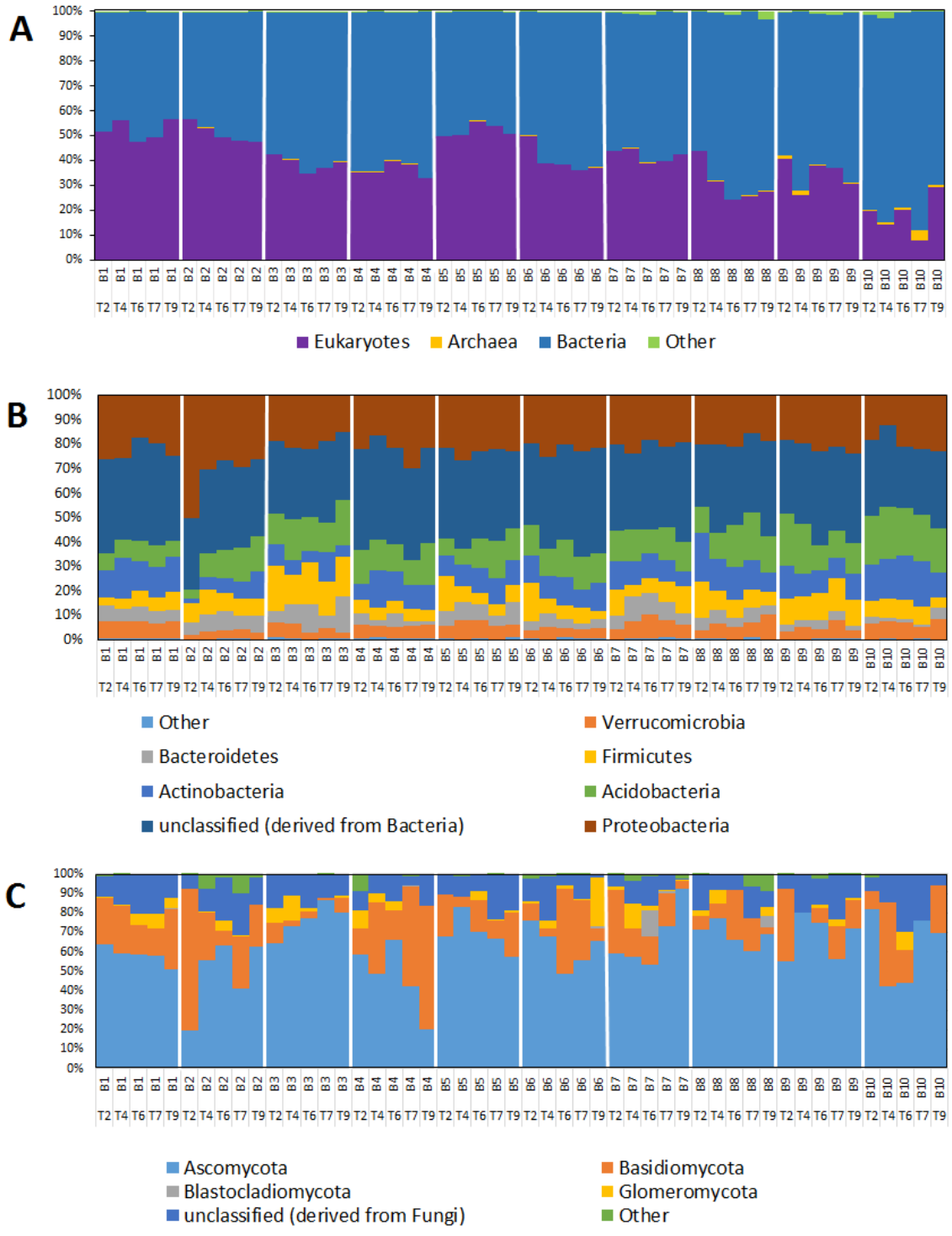

Figure 4.11: Proportion of the community made up by (A) each domain, (B) bacterial phyla which comprised at least 1% of bacterial reads in a single sample, and (C) fungal phyla which made up at least 10% of fungal reads in at least one sample. Samples taken from each core are separated by white lines and are ordered according to time point (earliest to latest).

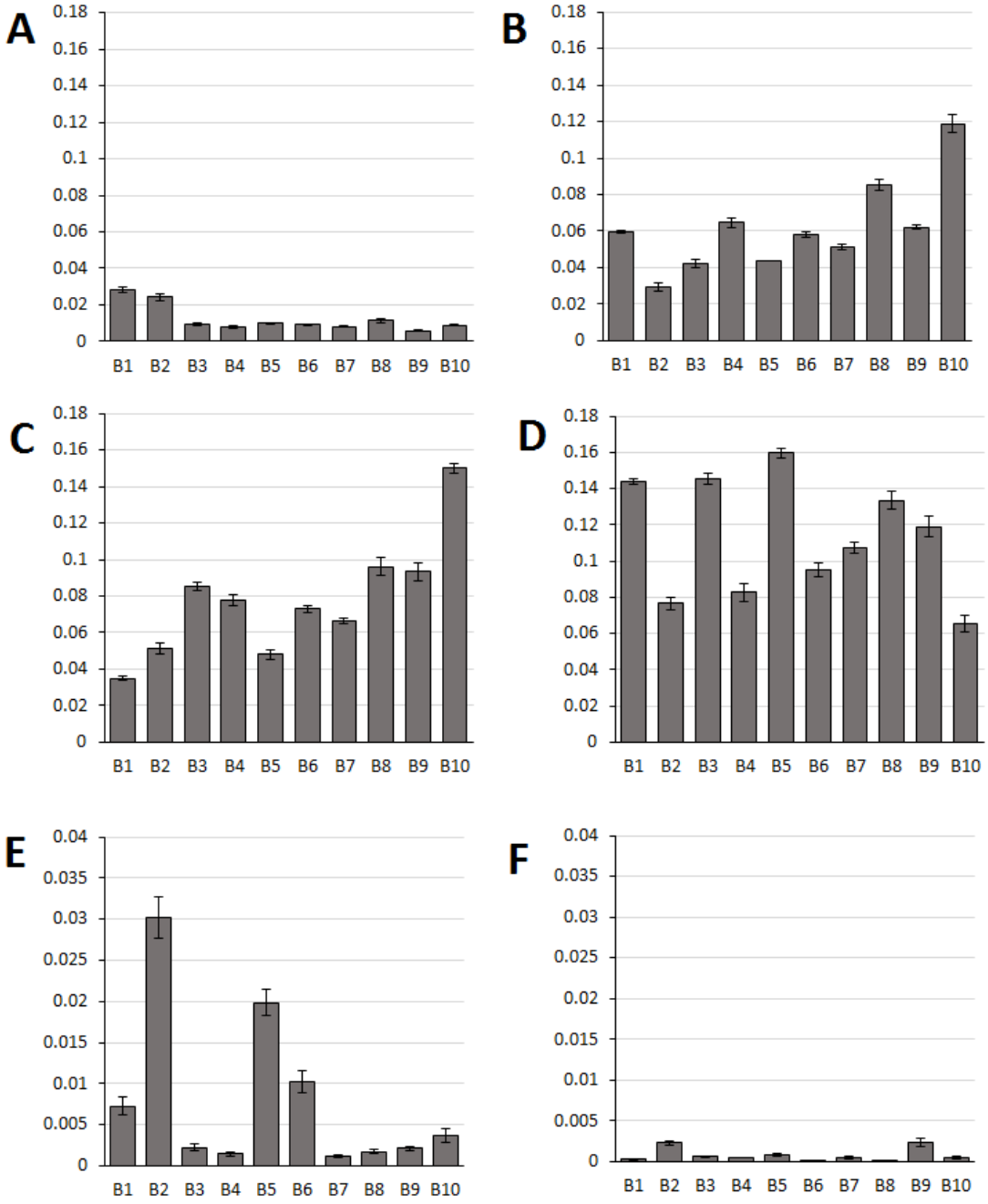

Figure 4.12: Proportion of the community made up by the six phyla which differed significantly between cores: (A) Arthropoda, (B) Actinobacteria, (C) Acidobacteria, (D) Annelida, (E) Ascomycota, and (F) Bacillariophyta. Error bars represent standard errors. Note the difference in scale along the y-axes of graphs E&F and A-D.Closed-reference OTU picking using EMIRGE-assembled 16S rRNA genes as a reference yielded 18,207 hits.

The taxonomic composition of the obtained community ("EMIRGE-community") was different to the taxonomic composition of the EMIRGE-assembled reads used as a reference:

Proteobacteria, Planctomycetes and 'Other' comprised a larger proportion of the EMIRGE-assemblies than of the EMIRGE-community, while Acidobacteria showed the opposite pattern (Figure 4.13). In addition, the EMIRGE-community differed from the community determined by BLAT annotation against the SILVA database (Figure 4.13): for example, both Proteobacteria and Acidobacteria were more abundant in the EMIRGE-community than in the MGRAST-SSU annotations.

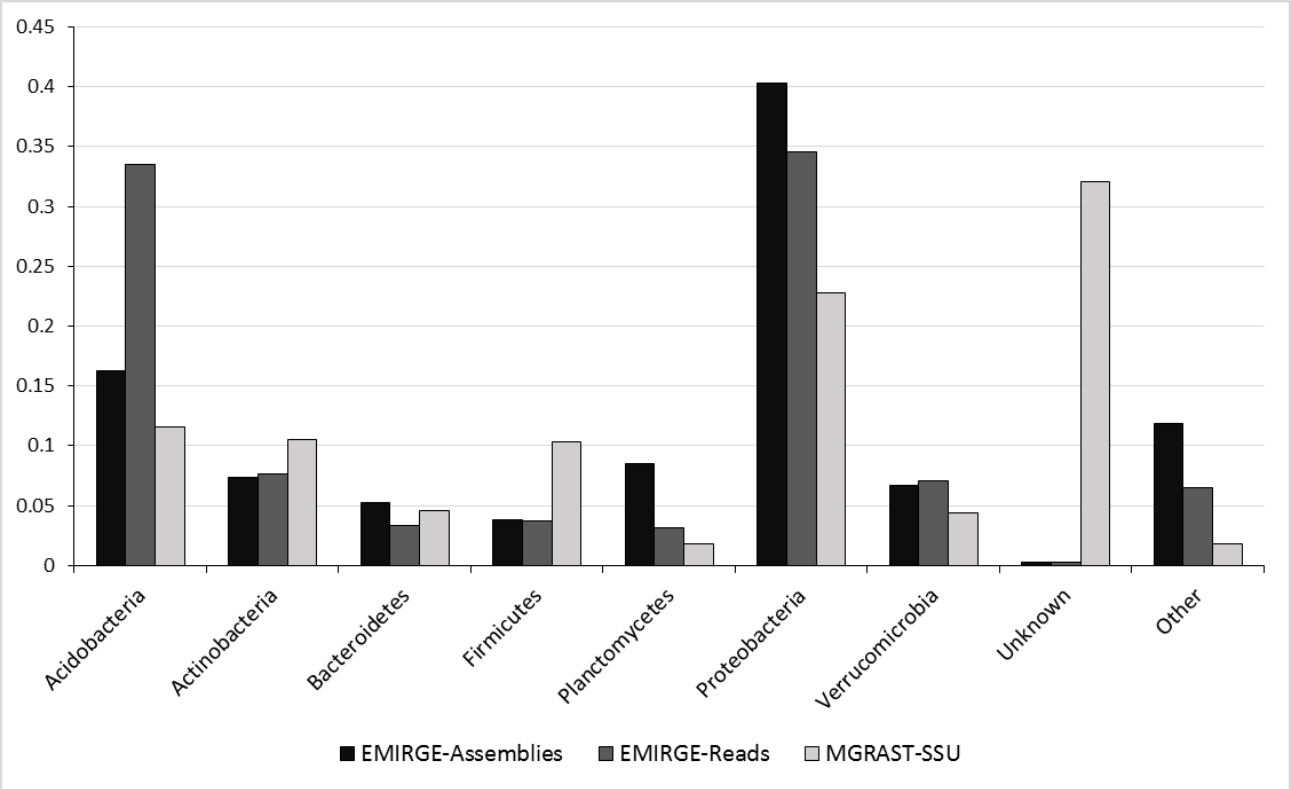

Figure 4.13: Taxonomic distribution of full-length 16S SSU sequences assembled by EMIRGE ('EMIRGE-Assemblies') and of total reads within 'EMIRGE OTUs' ('EMIRGE-Reads'), i.e. OTUs resulting from closed-reference clustering with EMIRGE-assembled 16S sequences used as the reference. Only phyla which made up at least 5% of the community according to at least one method are shown.

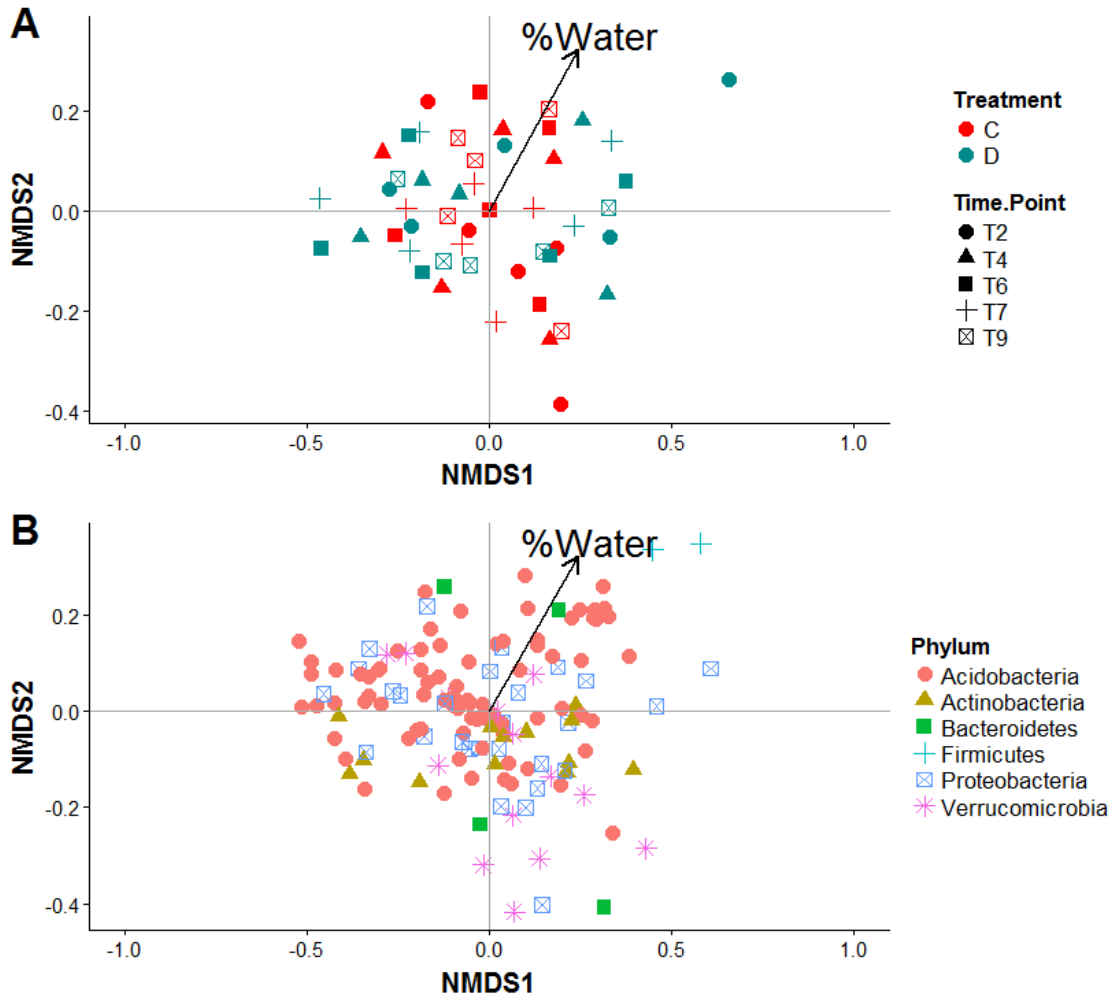

Figure 4.14: NMDS ordination of bacterial communities based on EMIRGE OTUs, depicting ordination of (A) samples and (B) OTUs. Arrows depict the result of 'envfit' (i.e. correlations between community composition and environmental variables). OTUs were assigned by closed-reference clustering with full-length 16S assembled generated by EMIRGE. %Water = water content of peat.

4.3.6 Functional Composition

Protein metabolism was the most abundant SEED subsystem, and this was consistent across each of the most abundant phyla (Figure 4.15; Table 4.8). Protein metabolism accounted for 59% of bacterial and 54% of eukaryotic functional assignments. Within both eukaryotic and prokaryotic SEED annotations the most important category within the protein metabolism module was protein biosynthesis (particularly SSU ribosomal DNA). Aside from protein metabolism the relative abundances of SEED subsystems varied between bacteria and eukaryotes. Carbohydrate metabolism made up 14% of functional annotations within the

domain Bacteria, with the remaining SEED subsystems each making up less than 3% of annotated bacterial genes. Within carbohydrate metabolism, the most abundant subsystems at level 2 of the SEED hierarchy were CO_2 fixation (45%) and organic acid metabolism (27%). Abundant subsystems within the eukaryotic annotations were photosynthesis (19%), RNA metabolism (13%) and respiration (8%). Genes for nitrogen metabolism made up a relatively small proportion of total annotations and were not detected for eukaryotes, but within the bacteria the majority of genes in the nitrogen metabolism subsystem were in the pathways of allantoin utilisation and ammonium assimilation.

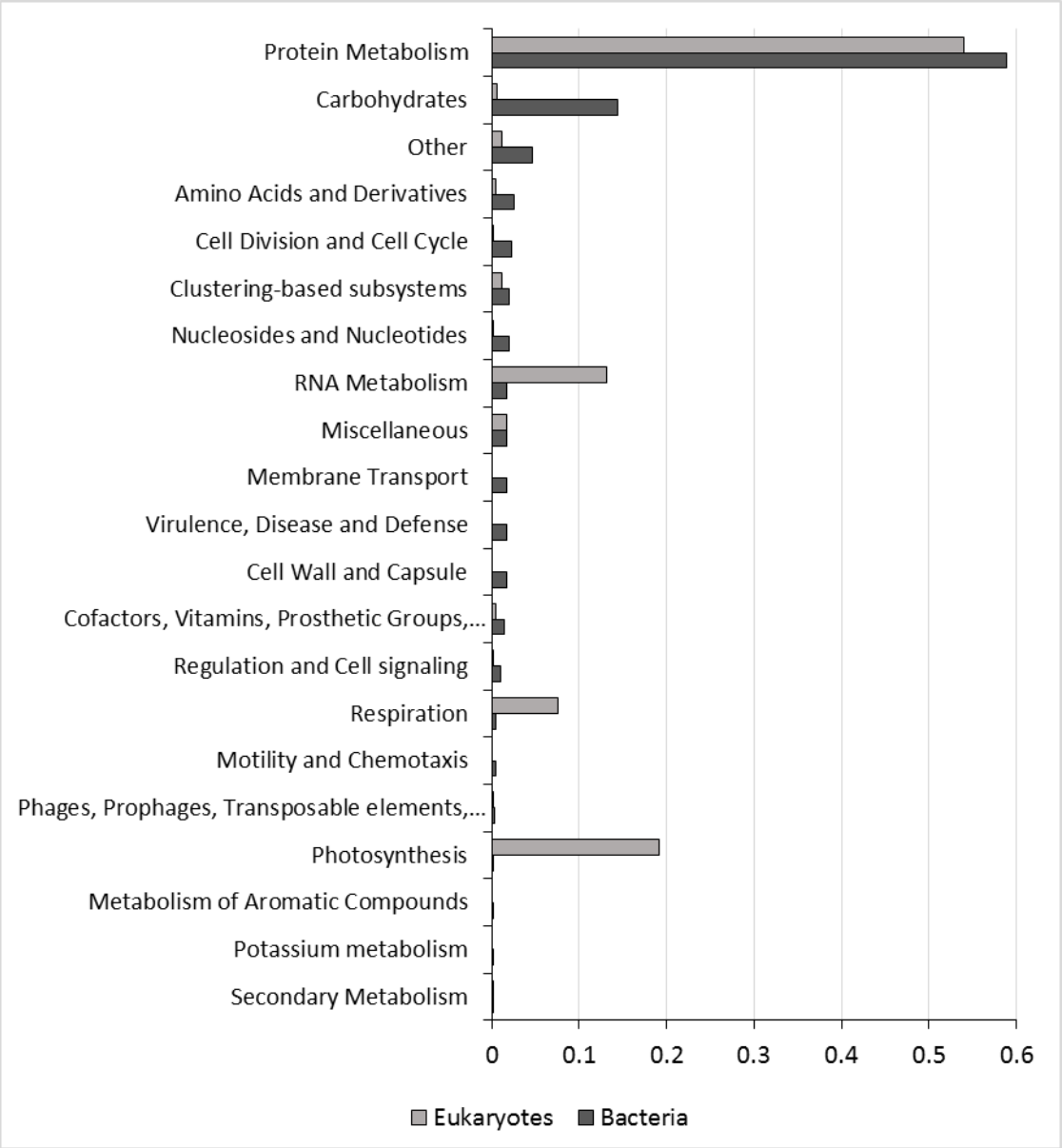

Figure 4.15: Percentage abundance of Level 1 SEED subsystems (i.e. the highest level in the SEED hierarchy). Only subsystems which made up more than 1% of annotated reads are shown.

Table 4.8: Percentage of annotated reads within the five most abundant bacterial phyla which belong to SEED Level 1 subsystems. Proteo=Proteobacteria, Acido=Acidobacteira, Actino=Actinobacteria, Verru=Verrucomicrobia, Firmi=Firmicutes.

Module	Proteo	Acido	Actino	Verru	Firmi
Amino Acids and Derivatives	2.9%	0.2%	3.2%	0.1%	2.2%
Carbohydrates	22.3%	0.0%	7.8%	6.7%	3.4%
Cell Division and Cell Cycle	2.2%	4.8%	0.7%	3.0%	2.1%
Cell Wall and Capsule	1.7%	0.0%	1.2%	0.1%	3.7%
Clustering-based subsystems	2.0%	0.8%	3.4%	0.8%	4.7%
Cofactors, Vitamins, Prosthetic Groups, Pigments	2.6%	0.0%	0.4%	0.0%	0.4%
DNA Metabolism	1.0%	0.0%	0.0%	0.1%	0.4%
Dormancy and Sporulation	0.0%	0.0%	0.0%	0.0%	1.0%
Fatty Acids, Lipids, and Isoprenoids	0.2%	0.0%	0.2%	0.0%	1.0%
Iron acquisition and metabolism	1.3%	0.0%	0.2%	0.0%	4.2%
Membrane Transport	3.2%	0.0%	0.4%	0.0%	2.8%
Metabolism of Aromatic Compounds	0.5%	0.0%	0.0%	0.0%	0.0%
Miscellaneous	2.0%	0.3%	0.1%	0.2%	2.8%
Motility and Chemotaxis	0.4%	0.9%	0.1%	1.4%	0.5%
Nitrogen Metabolism	1.6%	0.0%	1.2%	0.0%	0.3%
Nucleosides and Nucleotides	2.8%	0.0%	4.5%	0.1%	1.1%
Phages, Prophages, Transposable elements, Plasmids	0.1%	0.1%	0.0%	0.0%	2.4%
Phosphorus Metabolism	0.2%	0.0%	0.2%	0.0%	0.1%
Photosynthesis	0.0%	0.0%	0.0%	0.0%	0.0%
Potassium metabolism	0.1%	0.0%	0.1%	0.0%	0.1%
Protein Metabolism	46.6%	90.5%	72.5%	84.8%	50.2%
Regulation and Cell signalling	1.2%	0.0%	1.4%	0.0%	2.1%
Respiration	0.4%	0.2%	0.9%	0.5%	0.9%
RNA Metabolism	1.9%	2.0%	0.8%	2.1%	1.2%
Secondary Metabolism	0.1%	0.0%	0.2%	0.0%	0.0%
Stress Response	1.6%	0.1%	0.1%	0.0%	0.9%
Sulfur Metabolism	0.2%	0.0%	0.0%	0.0%	0.1%
Virulence, Disease and Defense	0.9%	0.0%	0.5%	0.0%	11.6%

Relationships between functional community composition and environmental variables were weak: there were no significant relationships between the overall functional composition and any environmental variable for either bacteria or eukaryotes (Table 4.9). NMDS ordination plots of the functional composition of both bacterial and eukaryote annotations show a large degree of overlap between cores and treatments (Figure 4.16; Figure 4.17). However, when the two most abundant bacterial SEED categories (‘Protein Metabolism’ and ‘Carbohydrates’) were analysed separately a weakly significant relationship was found between functional

composition within the carbohydrates module and two environmental variables: redox potential and phenol oxidase activity (Figure 4.18; Table 4.9).

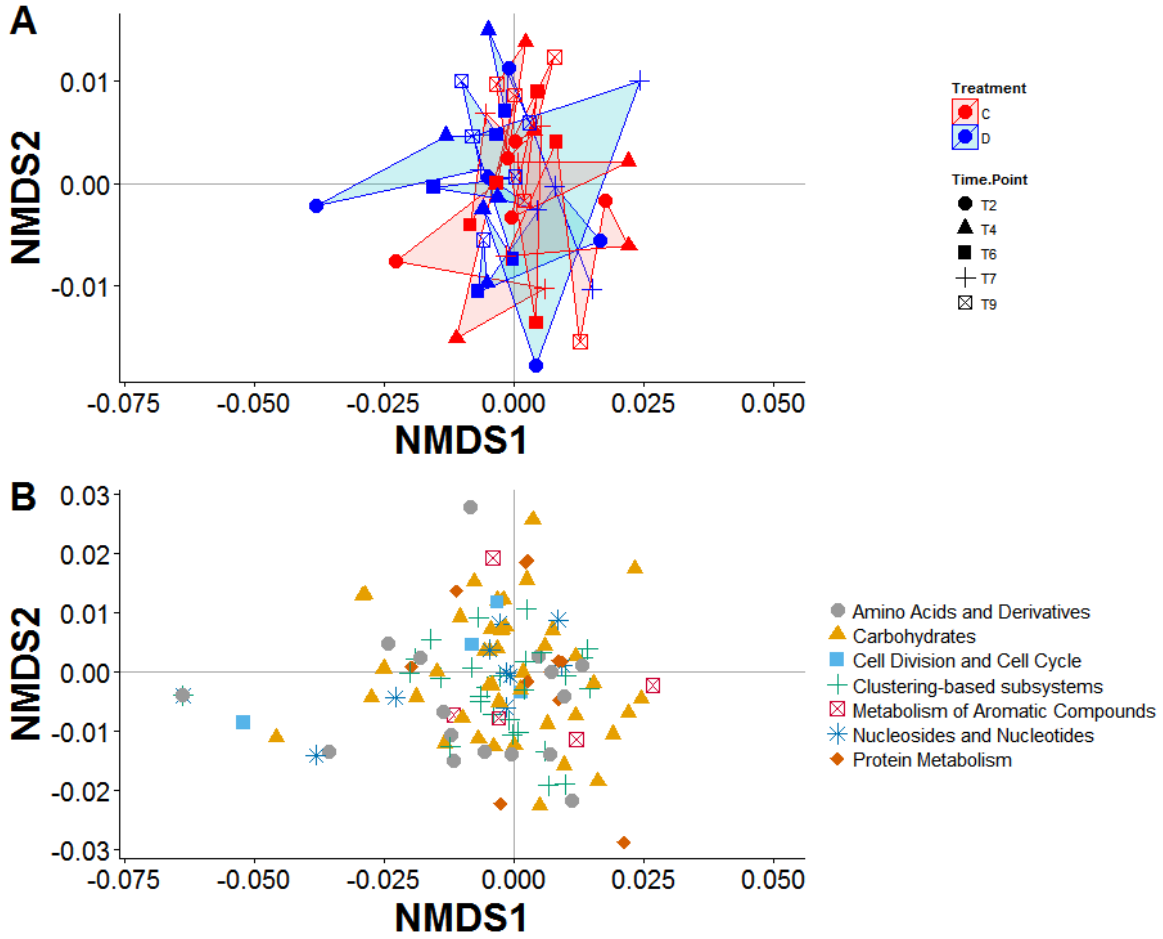

Figure 4.16: NMDS ordination of the functional potential of bacterial communities, depicting ordination of (A) samples and (B) OTUs. Arrows depict the result of 'envfit' (i.e. correlations between community composition and environmental variables). Ordinations are based on Level 3 SEED subsystems.

Table 4.9: Results from application of 'envfit' to NMDS ordinations of SEED pathway abundances. Ordination within module 'metabolism' did not significantly correlate to any environmental variable. POX= phenol oxidase activity, b-gluc = β-glucosidase activity; redox = redox potential. Only significant values are shown.

Taxa	Subsystem	Variable	R^2	p
Bacteria	**All**	**None**	n.s.	n.s.
	Protein	**None**	n.s.	n.s.
	Carbohydrates	**POX**	0.1485	0.048*
		Redox	0.1646	0.035*
Eukaryotes	**All**	**None**	n.s.	n.s.

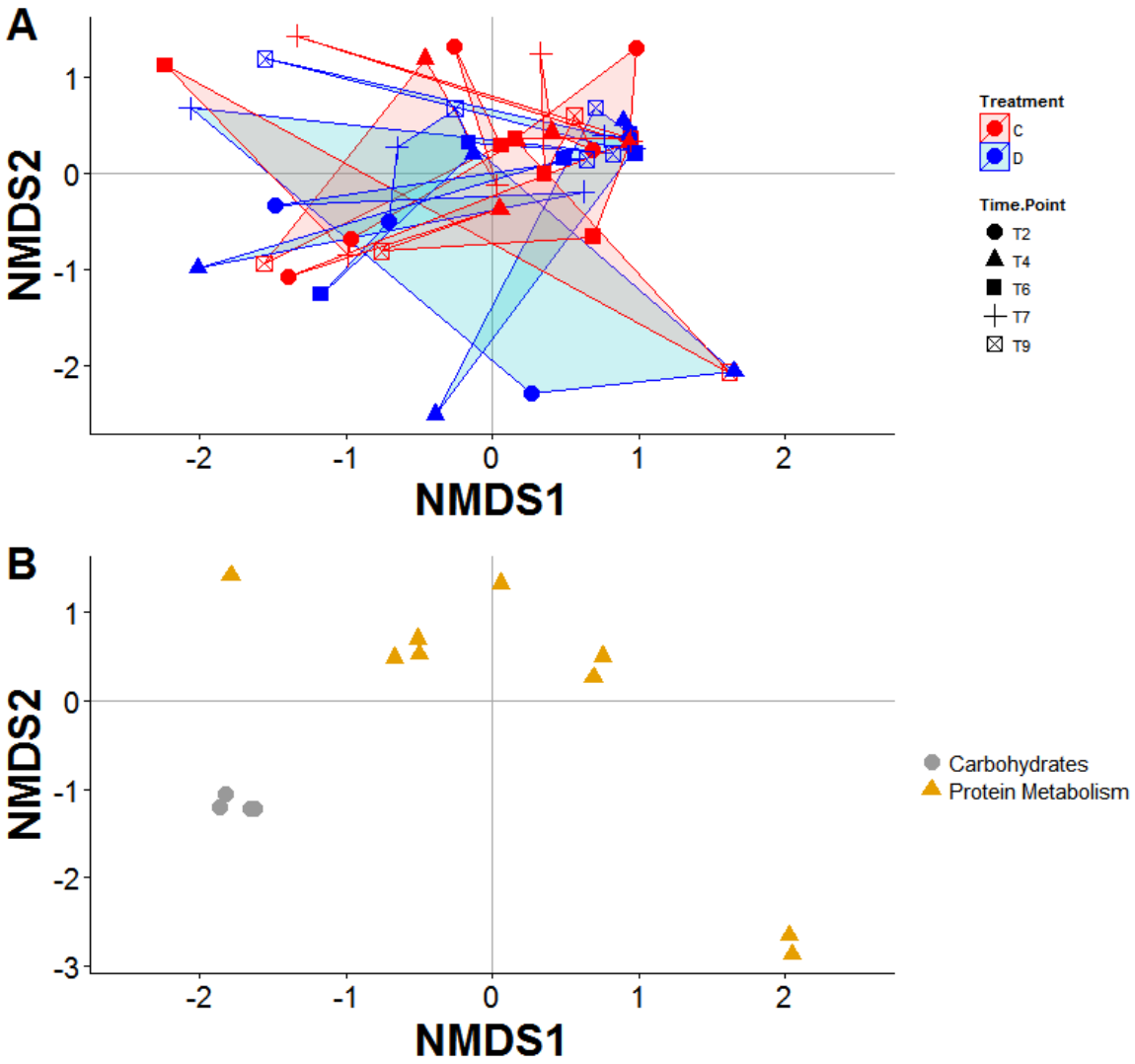

Figure 4.17: NMDS ordination of the functional potential of eukaryotic communities, depicting ordination of (A) samples and (B) OTUs. Arrows depict the result of 'envfit' (i.e. correlations between community composition and environmental variables). Ordinations are based on Level 3 SEED subsystems.

Very few SEED modules passed the abundance filtering step applied prior to fitting of GEE models: 6 modules for bacteria and 2 modules for eukaryotes. Of these, only bacterial subsystem SS11428 (ribosomal protein gene *S12p*) was significantly affected by the interaction effect between time point and treatment (χ^2_4=18.9, adjusted *p*-value = 0.005): this subsystem was significantly more abundant in the control than drought mesocosm cores at T4 (z = 4.0,

p=0.0001) (Figure 4.19). There was no significant main effect of time point or treatment on the abundance of SS11428.

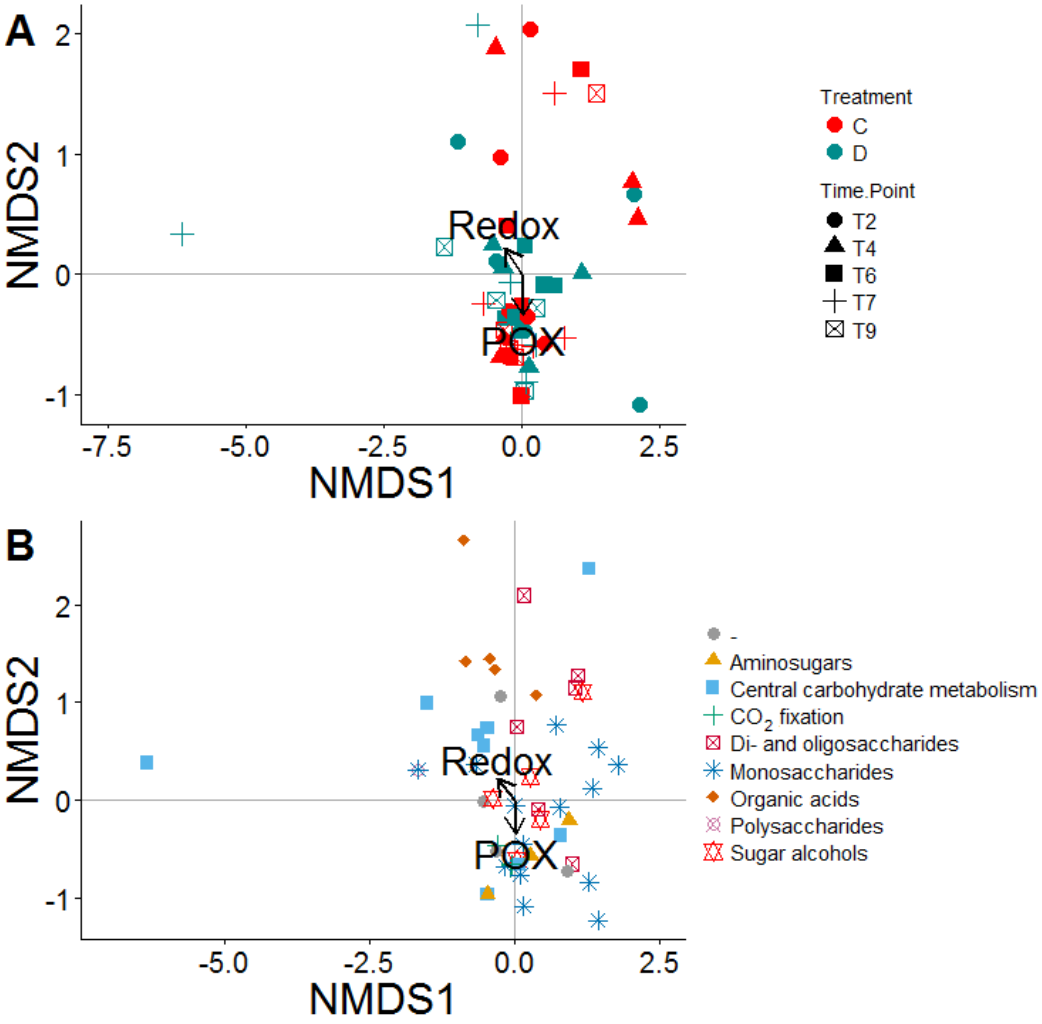

Figure 4.18: NMDS ordination of functional potential within the carbohydrate metabolism subsystem of bacterial communities, depicting ordination of (A) samples and (B) OTUs. Arrows depict the result of 'envfit' (i.e. correlations between community composition and environmental variables). Ordinations are based on Level 3 SEED subsystems.

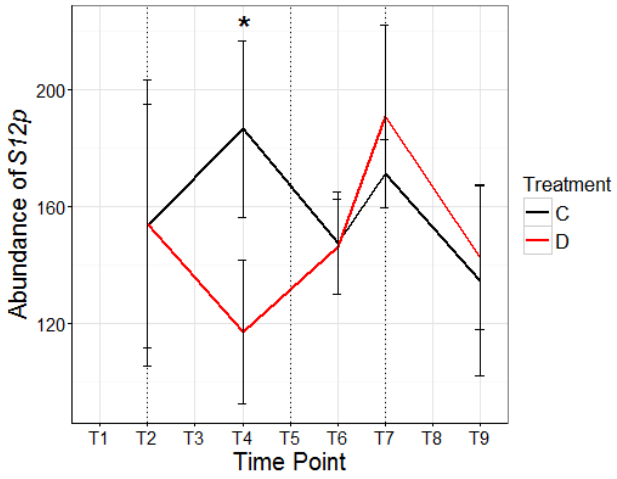

Figure 4.19: Number of *S12p* genes in rarefied dataset (containing a total of 482 annotations per sample). Error bars represent standard errors.

Table 4.10: Proportion of oxidase domains belonging to both prokaryotic and eukaryotic phyla. Phyla possessing <1% of every oxidase gene are summed in 'other'.

Group	Phylum	Cu-ox 3	Cu-ox 4	Dioxygenase	Peroxidase	Tyrosinase
Bacteria	**Acidobacteria**	44%	0%	0%	3%	1%
Bacteria	**Actinobacteria**	2%	0%	12%	5%	1%
Bacteria	**Bacteroidetes**	2%	0%	0%	0%	3%
Bacteria	**Cyanobacteria**	0%	0%	0%	0%	5%
Bacteria	**Planctomycetes**	0%	0%	0%	41%	0%
Bacteria	**Proteobacteria**	24%	0%	79%	0%	34%
Fungi	**Ascomycota**	24%	0%	4%	10%	28%
Fungi	**Basidiomycota**	0%	0%	0%	3%	0%
Other Eukaryote	**Streptophyta**	2%	0%	0%	36%	4%
Prokaryote /Eukaryote	**Other**	2%	0%	0%	0%	1%
Unassigned	**Not assigned**	1%	100%	3%	3%	25%

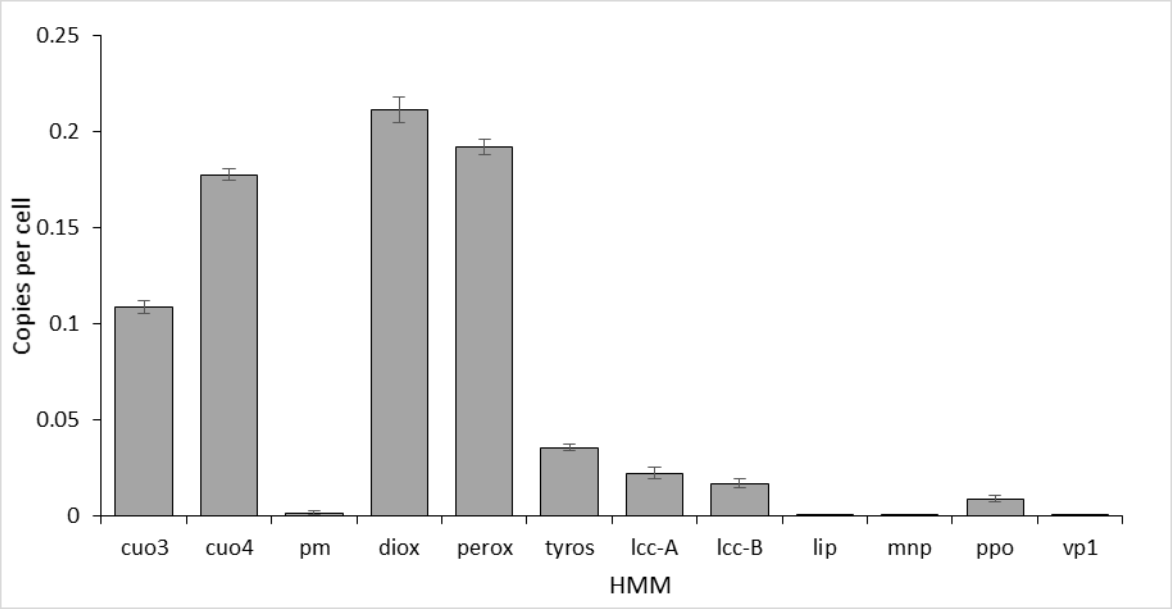

Figure 4.20: Mean abundance of HMMs representing genes involved in degradation of phenolic compounds, normalised by dividing number of hits by number of hits for rpoB, a universal single-copy gene. Gene/family names abbreviated as follows: cuo3 = copper oxidase type 3, cuo4 = copper oxidase type 4, pm = phenol mono-oxygenase, diox= dioxygenase, perox = peroxidase, tyros= tyrosinase, lcc-A = ascomycete laccase, lcc-B = basidiomycete laccase, lip = lignin peroxidase, mnp = Mn peroxidase, ppo = polyphenol oxidase, vp1= versatile peroxidase.

4.3.7 Abundance of genes involved in degradation of phenolic compounds and anaerobic metabolism

HMMs taken from PFAM yielded much higher numbers of hits than those taken from FunGene (Figure 4.20). The most abundant oxidase families were dioxygenase and peroxidase, closely followed by Cu-oxidase type 4. Taxonomic assignment of the matches to each PFAM HMM revealed that each oxidase gene had a different taxonomic distribution (Table 4.10). Acidobacteria, Proteobacteria and Ascomycota possessed the largest proportion of potential phenol oxidase domains, while Planctomycetes and Streptophyta possessed most peroxidase domains. A large proportion of reads within the phenol oxidases could not be assigned to phylum level: in particular, all copper oxidase type 4 genes and 25% of tyrosinase genes remained unassigned. Four HMMs were only present at very low abundances (pm, lip, mnp, vp1), and so were not analysed further. Of the remaining genes and gene families, none were significantly affected by drought (Table 4.11).

Table 4.11: Results of general linear models applied to test for the effect of drought on the number of oxidase genes per cell. Gene numbers per cell were calculated by dividing abundance of the gene in question by the abundance of *rpoB*, a universal single-copy gene. This gave a proportion which was arcsine transformed prior to fitting of a general linear model. Random effects were chosen by fitting the maximal model using two random effects terms (~1|core or ~core|time) as well as with fixed effects only, and comparing likelihood ratios of each.

Gene	Random	Factor	F	d.f.	p-	Bonferroni
lcc-A	**~time|core**	**Treatment**	0.8	1,8	0.4	1.0
		Time	2.3	4,32	0.08	0.6
		Treatment:Time	0.8	4,32	0.5	1.0
lcc-B	**~time|core**	**Treatment**	0.7	1,8	0.4	1.0
		Time	2.3	4,32	0.08	0.6
		Treatment:Time	0.8	4,32	0.6	1.0
ppo	**~time|core**	**Treatment**	1.5	1,8	0.3	1.0
		Time	2.6	4,32	0.05	0.4
		Treatment:Time	2.4	4,32	0.07	0.6
cuo3	**~1|core**	**Treatment**	0.2	1,8	0.6	1.0
		Time	0.8	4,32	0.5	1.0
		Treatment:Time	0.7	4,32	0.6	1.0
cuo4	**~1|core**	**Treatment**	1.0	1,8	0.3	1.0
		Time	0.3	4,32	0.9	1.0
		Treatment:Time	1.1	4,32	0.4	1.0
diox	**None**	**Treatment**	0.2	1	0.6	1.0
		Time	1.0	4	0.4	1.0
		Treatment:Time	0.4	4	0.8	1
perox	**~time|core**	**Treatment**	0.7	1,8	0.4	1.0
		Time	3.2	4,32	0.02	0.2
		Treatment:Time	2.7	4,32	0.04	0.4
tyros	**None**	**Treatment**	1.8	1,40	0.2	1.0
		Time	1.0	4,40	0.4	1.0
		Treatment:Time	1.0	4,40	0.4	1.0

Of the anaerobic metabolism genes investigated, by far the most abundant was *hydA* which is involved in H_2-evolving fermentation (Figure 4.21). The drought response of the four most abundant anaerobic metabolism genes (*dsrA*, *hydA*, *nirK*, *nosZ*) was investigated. Of these four genes, only *hydA* showed a significant interaction effect of time and treatment (Table 4.12), although a post-hoc test detected only a single marginally significant treatment effect at time point 9 (t_8 = -2.1, p= 0.07). Mean abundance of *hydA* (normalised by dividing by number of *rpoB* copies) is shown in Figure 4.22.

The number of hits to the *alkB* HMM was not significantly affected by time point or treatment, or by the interaction between time point and treatment (Table 4.13).

Table 4.12: Results of general linear models applied to test for the effect of drought on the copy numbers of selected anaerobic metabolism genes per cell. Gene numbers per cell were calculated by dividing abundance of the gene in question by the abundance of *rpoB*, a universal single-copy gene. This gave a proportion which was arcsine transformed prior to fitting of a general linear model. Random effects were chosen by fitting the maximal model using two random effects terms (~1|core or ~core|time) as well as with fixed effects only, and comparing likelihood ratios of each.

HMM	Random	Factor		F	d.f.	p-	Bonferroni
dsrA	**~time|core**	Time Point		0.4	4,32	0.8151	1.0
		Treatment		0.2	1,8	0.6917	1.0
		Time	Point:	0.7	4,32	0.6007	1.0
hydA	**~1|core**	Time Point		3.4	4,32	0.0208	0.08.
		Treatment		1.0	1,8	0.3572	1.0
		Time	Point:	3.8	4,32	0.0121	0.048*
nirK	**~1|core**	Time Point		2.8	4,32	0.7596	1.0
		Treatment		0.5	1,8	0.1482	0.6
		Time	Point:	0.9	4,32	0.4963	1.0
nosZ	**~time|core**	Time Point		0.8	4,32	0.5208	1.0
		Treatment		2.4	1,8	0.1603	0.6
		Time	Point:	1.1	4,32	0.3769	1.0

Table 4.13: Results of general linear models applied to test for the effect of drought on the copy number of *alkB* per cell. Gene numbers per cell were calculated by dividing abundance of *alkB* by the abundance of *rpoB*, a universal single-copy gene. This gave a proportion which was arcsine transformed prior to fitting of a general linear model. Random effects were chosen by fitting the maximal model using two random effects terms (~1|core or ~core|time) as well as with fixed effects only, and comparing likelihood ratios of each.

HMM	Random	Factor	F	d.f.	p-
alkB	**~1|Core**	**Treatment**	1.2	1, 8	0.3
		Time	0.3	4, 32	0.9
		Treatment:	0.5	4, 32	0.7

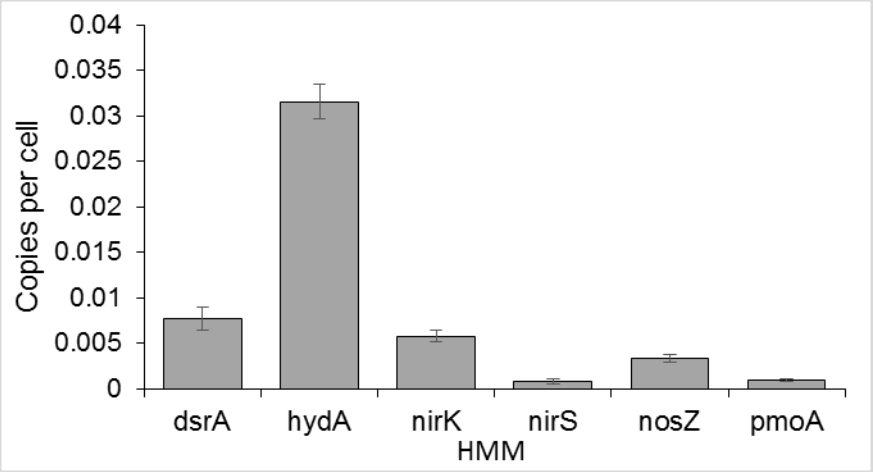

Figure 4.21: Mean abundance of HMMs representing genes involved in anaerobic metabolism, normalised by dividing number of hits by number of hits for *rpoB*, a universal single-copy gene. Gene/family names abbreviated as follows: *dsrA* = dissimilatory sulphite reductase; *hydA* = subunit of hydrogen dehydrogenase (NADH-dependent hydrogenase); *nirK* = nitrite reductase (copper containing); *nirS* = nitrite reductase (heme-containing); *nosZ* = nitrous oxide reductase; *pmoA* = particulate methane monooxygenase.

4.4 Discussion

4.4.1 Read Counts and Coverage

Only a small proportion of predicted genes could be annotated, and of these the majority of hits were of low quality and thus likely represented either distant homologues or erroneous hits. The low proportion of reads which could be annotated is consistent with earlier metagenomic studies of the soil microbiome (Delmont *et al.* 2012; Lin *et al.* 2014a). Soil communities are highly diverse (Curtis and Sloan 2004; Frisli *et al.* 2013) and often dominated by specialist and "conditionally rare" taxa (Shade *et al.* 2014; Mariadassou *et al.* 2015), meaning that global soil microbial diversity is almost unimaginably vast. The sparse annotation of metagenomes against current databases illustrates the fact that only a fraction of microbial life is currently catalogued.

Mean coverage estimated by Nonpareil (Rodriguez-R and Konstantinidis 2013) was approximately 53%. While the obtained coverage may seem like a relatively low proportion of the community, the majority of microbial diversity consists of rare species (Curtis and Sloan 2004; Quince *et al.* 2008) and so abundant species will likely be well-represented even in datasets with relatively low overall coverage values. Previous peat metagenomic studies have

obtained consistent results using datasets with similar coverage values to the current study (Lin *et al.* 2014a), suggesting that complete coverage of soil metagenomes is not necessary. Using current methodologies, complete sequencing of soil metagenomes is rarely affordable. Nonpareil estimated that 6.6 gigabases of sequence data would be required to reach 95% coverage of the community in these samples: this estimate is considerably lower than earlier estimates of the coverage required to achieve 95% coverage of permafrost and active layers of tundra soils (Rodriguez-R and Konstantinidis 2013), but still represents a very large quantity of sequence data. This volume of sequencing is rarely, if ever, reached consistently even with modern high-throughput sequencing methods (Delmont *et al.* 2012; Lin *et al.* 2014a; Leff *et al.* 2015; Souza *et al.* 2015), although as sequencing throughput continues to increase reaching near-complete coverage of soil microbial communities will become more achievable.

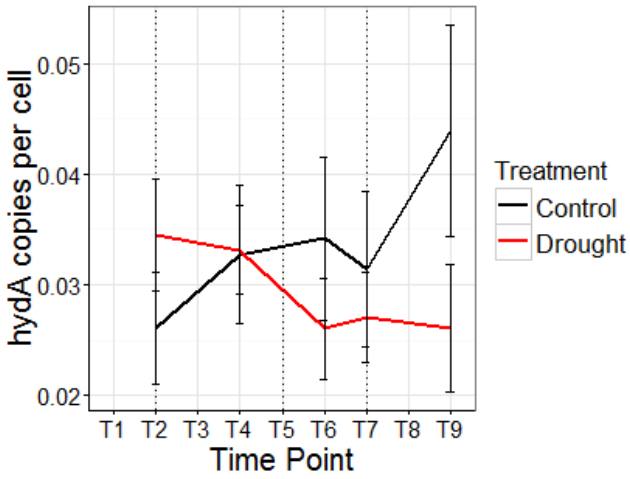

Figure 4.22: Mean counts of *hydA* gene, normalised using number of *rpoB* copies, in drought and control points at each time point sequenced. Error bars represent standard errors.

4.4.2 Undisturbed Community Composition

Bacteria made up the majority of annotated reads in the metagenomes (Figure 4.1). Bacteria almost invariably dominate soil metagenomes (Delmont *et al.* 2012; Tveit *et al.* 2013; Luo *et al.* 2014), but the proportion of bacterial reads in soil metagenomes is usually much higher than in the present study (often at least 90% of reads, compared to 61% in the current study) and a previous metagenomic study of two peatlands found approximately 90% of reads to belong to prokaryotes (Lin *et al.* 2014a). The relatively low proportion of bacterial sequences in the

current study was caused in part by the removal of the negative control: bacteria made up an average of 70% of annotated sequences prior to removal of potential contaminants, but this fell to 61% once sequences present in the negative control were removed. Removal of sequences matching to the negative control was considered to be an important step due to the near-ubiquitous nature of contaminant sequences in metagenomic data (Salter *et al.* 2014), but may appear to have a disproportionate effect on the metagenome annotations if contaminant sequences belong to better-studied organisms than genuine peatland micro-organisms, as is likely. However, removal of the negative control does not fully explain the high proportion of eukaryotic sequences: prior to removal of potential contaminants the ratio of eukaryotic to bacterial reads was still much higher than earlier studies.

The high ratio of eukaryotic to bacterial sequences suggests fungi may be important members of the microbial community in the bog, since the majority of eukaryotic sequences belonged to fungi (Figure 4.2). A number of studies have suggested that bacteria, rather than fungi, dominate peat metagenomes (Winsborough and Basiliko 2010; Lin *et al.* 2014a), biomass (Peltoniemi *et al.* 2015) and activity (Winsborough and Basiliko 2010). However, studies carried out across both bog and fen habitats find that fungi may dominate in bogs while bacteria dominate fens (Golovchenko *et al.* 2007; Amha *et al.* 2015). Certain aspects of the bog environment might be expected to favour a fungal-dominated ecosystem: in particular, low pH and a high organic matter content are characteristic of peat bogs, and both are associated with fungal dominance (Rousk *et al.* 2010; Rousk and Frey 2015). Nonetheless, waterlogging and low redox potentials could severely limit fungal growth and diversity in wetlands (Seo and DeLaune 2010a; Golovchenko *et al.* 2013). In the current study, bog mesocosm cores had considerably higher redox potentials than fen mesocosm cores (Chapter 2), and if strongly reducing conditions are characteristic of fen habitats then there is potential for redox potential to be a strong limiting factor for fungal growth in fen environments.

Alongside fungi, Streptophyta were the only other group to make up a significant proportion of the eukaryotic community, with the majority of Streptophyta sequences likely originating from undecomposed plant material. The proportion of Protozoa (Stramenopiles, Rhizaria and Alveolata) in the current dataset was very low, corresponding to marker gene analysis (MGA) of the same samples (Chapter 3). However, Protozoa have been poorly studied and so it is likely that SSU rRNA gene databases for this group are incomplete (Pawlowski *et al.* 2012;

Burki and Keeling 2014). Given the high proportion of reads which could not be classified, it is possible that many protist OTUs were missed.

Within the bacteria, the community composition was strongly affected by the methodology used to assign the taxonomic composition of the community (Figure 4.3). Although Proteobacteria were the most abundant phyla regardless of the method used for annotation, the proportion of the community comprised by Proteobacteria was much higher for MGRAST-M5NR annotations than other methods (Figure 4.3). Proteobacterial dominance when all genes were used for annotation is likely due to bias within genome databases: Proteobacteria make up almost half of all sequenced bacterial genomes while far fewer members of the Acidobacteria and Verrucomicrobia have been fully sequenced (http://www.ncbi.nlm.nih.gov/genome/browse/, accessed 15/06/16). Given that only BLAT hits with percentage similarity values of at least 97% were included in analyses, only organisms with a close relative in the SILVA database will have been considered and this likely led to a bias towards more intensively-studied phyla such as the Proteobacteria. Taxonomic bias is likely to be less problematic for taxonomic assignment of SSU rRNA genes due to the larger number of available SSU rRNA gene sequences. Annotation of SSU rRNA genes alone found higher proportions of other phyla, including Acidobacteria, Actinobacteria and Verrucomicrobia. Despite a great deal of variation between bacterial communities in different bogs, the dominant phyla in the current study are similar to earlier studies of peatland microbial communities, although with a slightly higher proportion of Proteobacteria and lower proportion of Acidobacteria (Lin *et al.* 2012; Serkebaeva *et al.* 2013; Lin *et al.* 2014b).

Within the fungi, Ascomycota was by far the dominant phylum (Figure 4.4), consistent with previous MGA (Lin *et al.* 2012; 2014b) and culture-based studies (Thormann 2006). Annotation of reads belonging to SSU rRNA genes indicates that Basidiomycota was the next most abundant phylum, comprising 25% of fungal reads, which is again consistent with previous results (Lin *et al.* 2014b). Each of the abundant fungal classes in the current study was well-represented in a global survey of fungi in soil habitats (Tedersoo *et al.* 2014), suggesting that peatlands are not qualitatively different from other soils. However, the proportion of Basidiomycetes in peat is much lower than the global average described by Tedersoo *et al.* (2014): in the latter study Agaricomycetes (a single class within the Basidiomycota) made up an average of 50% of soil fungi across all habitats surveyed. The relative rarity of members of

phylum Basidiomycota within peatlands (Thormann and Rice 2007; Lin *et al.* 2012; 2014b) is potentially relevant to the slow rates of decomposition observed in peatlands. Class Agaricomycetes in particular contains a wide diversity of decomposer taxa, including the majority of known brown-rot fungi (Riley *et al.* 2014) and are almost exclusively responsible for lignin peroxidases (Treseder and Lennon 2015). However, care should be taken when interpreting phylum-level trends: the ability to degrade lignin is not universal to all Agaricomycetes, and other decomposition-related traits (such as cellulose decomposition) are present in both Ascomycota and Basidiomycota (Treseder and Lennon 2015).

Taxonomic assignment using Phylosift indicated that Blastocladiomycota and Microsporidia made up a significant proportion of the fungal community (5% and 7%, respectively), although both phyla were barely detected by MG-RAST. Both Blastocladiomycota and Microsporidia have been poorly studied, but it is plausible that that both would be present in peatlands: Blastocladiomycota were recently split from the Chytridiomycota (James *et al.* 2006), a phylum which is limited to aquatic or waterlogged habitats (Freeman *et al.* 2009), while Microsporidia are obligate parasites on either terrestrial or aquatic hosts (Vossbrinck and Debrunner-Vossbrinck 2005). The poor representation of Blastocladiomycota and Microsporidia in MG-RAST outputs may in part be due to relatively small numbers of reference sequences present in databases in comparison to the more effectively characterised Ascomycota and Basidiomycota. A threshold similarity of 97% was used for MG-RAST annotations, meaning that only organisms with close relatives in the database were able to be assigned. Conversely, Phylosift is more flexible: reads are aligned to protein sequences on a reference tree prior to calculation of the best 'point of attachment' to the tree, meaning that reads may be assigned based on more distant relatives.

4.4.3 Effect of Drought on Taxonomic Composition (SILVA Annotations)

Bacterial OTU richness was significantly affected by treatment and time point (Table 4.2; Figure 4.5), representing a fall in the number of OTUs during the rewetting period and lower OTU richness at the end of the experiment than the beginning. This fall in OTU richness corresponds to the findings of a recent study using marker gene analysis (MGA), which found that prokaryotic diversity falls following drying-rewetting cycles (Nunes *et al.* 2015). However, the latter study is not truly comparable: Nunes *et al.* (2015) carried out a water table manipulation using peat cores collected at 50cm depth, where the community is very different

to the community at 5cm depth (Lin *et al.* 2012), and which would be expected to respond negatively to drying. Fungal OTU richness in the current study was not significantly affected by drought. However, care should be taken in interpreting OTU richness data from the current data: only reads which could be assigned to species in the SILVA database with a high degree of confidence were included in the current analyses. Therefore, the OTU richness values obtained represent the number of known species rather than the total number of OTUs. Given the high proportion of reads which could not be annotated (Section 4.4.1), OTU richness based on *de novo* OTU clustering is likely a more accurate measure of total diversity and was found not to respond to drought (Chapter 3).

None of the phyla tested showed a significant response to time point or treatment (Table 4.3), corresponding to the results of similar tests on phylum abundances within the MGA dataset (Chapter 3). In the MGA dataset, cyanobacteria/chloroplasts were the only phylum to respond to time point, but in the metagenomic dataset cyanobacteria were not one of the most abundant phyla and so were not analysed. However, it should be noted that both MGA and metagenomic analyses were limited by the difficulty of assigning taxonomy to soil bacteria: a high proportion of metagenomic SSU reads were not amenable to annotation, and a high proportion of the OTUs in the metagenomic dataset could likewise not be assigned to phylum level at the chosen confidence level (Chapter 3). Therefore, there is potential for community changes at the phylum level to have been missed.

Despite the compounding effects of core, 'envfit' found a weakly significant effect of treatment on the bacterial community composition (Table 4.5). While samples did not obviously cluster by treatment, the community composition within each droughted mesocosm core shifted in the same direction between pre-drought (T2) and later time points (Figure 4.6), and the direction of change follows the direction of maximum correlation between the NMDS ordination and the direction of water table change (Figure 4.7). However, PERMANOVA did not find treatment to significantly affect community composition (Table 4.4), likely because the differences between the two treatments were small.

Bacterial OTUs belonging to the same phyla exhibited weak clustering on the NMDS plot (Figure 4.7B), and a switch in phylum-level community composition occured along the first axis of the NMDS plot: many OTUs within the Actinobacteria had positive values on this axis, while Bacteroidetes had negative values. Positive values on the first axis also correspond

weakly to the drought treatment. Previous studies have demonstrated a strong link between Actinobacteria and water table depth, with Actinobacteria usually responding positively to water table drawdown (Jaatinen *et al.* 2007; Barnard *et al.* 2013) and negatively to rewetting (Placella *et al.* 2012; Barnard *et al.* 2015). Many Actinobacteria are aerobes and are highly sensitive to high CO_2:O_2 ratios which often occur in poorly aerated soils (Goodfellow and Williams 1983). An increase in the relative abundance of Actinobacterial OTUs may be relevant to carbon cycling within peat, as it has been suggested that Actinobacteria are important degraders of recalcitrant organic polymers such as lignocellulose (Goodfellow and Williams 1983) and in permafrost communities a large proportion of gene transcripts encoding cellulases, hemicellulases and debranching enzymes are assigned to Actinobacteria (Tveit *et al.* 2014). Actinobacteria appear to be of less importance in polysaccharide degradation in bogs and fens (Lin *et al.* 2014a), but this may in part be due to their relatively low abundance in peatlands compared to other soils. However, the number of reads belonging to each of these phyla was not significantly affected by time point or treatment (Table 4.3), suggesting that the effect of drought on the abundance of each phylum as a whole was weak and that any conclusions about the response of different bacterial phyla to drought should be interpreted with caution.

Enzyme activities (phenol oxidase and β-glucosidase; Chapter 2) were strongly related to bacterial community composition (Table 4.5; Figure 4.7). Bacteria possess many of the genes for phenol oxidase and β-glucosidase enzymes in peatlands (Lin *et al.* 2014a), and so it is likely that bacterial community composition has an impact on enzyme activities. Conversely, both enzyme activity and bacterial communities may be controlled by similar suites of environmental variables, which were unmeasured in the current study. However, it should be noted that given the large number of environmental variables fitted with envfit there is a high probability of spurious p-values, and so such relationships should be interpreted with caution.

To test for relationships between environmental variables and within-phyla community composition, NMDS ordination followed by 'envfit' analysis was carried out within each of the five most abundant bacterial phyla: Proteobacteria, Acidobacteria, Actinobacteria, Verrucomicrobia and Firmicutes. Community composition was significantly related to at least one environmental variable for every phyla except Acidobacteria. The weak response of Acidobacteria to environmental variables may be a result of the low diversity within the

Acidobacteria. Acidobacteria in peatlands commonly consist of a small number of highly abundant OTUs (Serkebaeva *et al.* 2013), and this appeared to be the case in the current study: out of a mean of 103 OTUs per sample, there were only 3-5 OTUs belonging to the Acidobacteria per sample (after rarefaction and removal of rare OTUs). Serkebaeva *et al.* (2013) suggest that the relatively low diversity of Acidobacteria in peatlands occurs because peat-inhabiting Acidobacteria all share very similar lifestyles (i.e. acidophilic chemo-organotrophs with weak hydrolytic capabilities). While members of the Acidobacteria do show differences in pH optima (Jones *et al.* 2009) and substrate preferences (Pankratov *et al.* 2008), their responses to the environment are relatively uniform in comparison to certain other phyla (Fierer *et al.* 2007), and this may result in weak within-phylum changes in community composition. Additionally, Acidobacteria are underrepresented in sequence databases, likely resulting in many members of this phylum not being recognised by the current methodology.

'Envfit' analyses found that the environmental variables which were significant for the highest number of phyla were redox potential, mesocosm core and the plant community. Each of these variables was significantly related to community composition within the majority of phyla tested (Table 4.5). This echoes results from MGA, where community differences between different mesocosm cores were strongly significant and related to vegetation differences between the cores (Chapter 3). The effect of redox potential is particularly relevant to the aims of this chapter and thesis: species-level community composition within three of the five most abundant phyla (Actinobacteria, Firmicutes and Proteobacteria) was significantly correlated with redox potential. However, treatment did not have a significant effect on community composition within any of the dominant phyla, suggesting that the drought did not cause redox-driven community changes. Redox potential likewise has a strong impact on community composition in permafrost (Lipson *et al.* 2015), in particular because fermentative bacteria flourished at low redox potentials. Redox potential is additionally a strong driver of bacterial community composition in general. For example, Winogradsky columns demonstrate the development of distinct communities driven in part by a redox gradient (e.g. Rundell *et al.* 2014). However, the large number of variables fitted with 'envfit' means that the results should be interpreted with caution, as the large number of tests leads to a high likelihood of a type I error. Nonetheless, the fact that redox potential, mesocosm core and plant community composition are consistently significant across a number of phyla makes the chances of a false positive less likely.

Although PERMANOVA detected a significant effect of treatment on fungal community composition, the fact that there was no significant effect of time point or of the interaction between time point and treatment suggests that the effect of treatment was due to pre-existing differences between mesocosm cores rather than to the drought itself. However, fungal community composition was significantly related to redox potential, but not to treatment, water table or water content (Table 4.5; Figure 4.10). Anoxic conditions restrict fungal biomass in wetlands (Seo and DeLaune 2010a) and so it can be hypothesised that conditions of low redox potential might favour fungi which are able to utilise alternative electron accepters such as nitrate (Hayatsu *et al.* 2008; Seo and DeLaune 2010b). Additionally, there was some evidence for phylum-level responses to redox potential: OTUs belonging to the Basidiomycota appear to cluster with samples that had high redox potential values (Figure 4.10B). Redox-driven selection for Basidiomycota has large potential implications for decomposition rates: the most abundant class within phylum Basidiomycota was the Agaricomycetes, members of which are highly important for the degradation of complex polymers and particularly for the degradation of lignin (Riley *et al.* 2014; Treseder and Lennon 2015).

Although the abundances of certain OTUs from the MGA dataset were significantly affected by the interaction between time point and treatment (Chapter 3), none of the OTUs within the shotgun sequencing data were. Far fewer OTUs were affected by drought in the bog, which was chosen for shotgun sequencing, than in the fen. Additionally, a much smaller number of 16S rRNA genes were present in the shotgun sequencing dataset (16S rRNA data was rarefied to 70,000 reads in the MGA dataset compared to 2,286 for shotgun sequencing data) and the number of OTUs was consequently lower. Many of the species which responded to drought in the MGA dataset were novel (few could be assigned to genus level with a high confidence value), meaning that OTU assignment by alignment against known species would have missed out important taxa. MGA analysis has the advantage of allowing *de novo* OTU clustering (as all reads come from the same region and thus can be aligned together), which may mean that MGA is better able to detect community changes in diverse and poorly studied habitats such as soils.

4.4.4 Effect of Mesocosm Core on Taxonomic Composition (SILVA Annotations)

Different mesocosm cores had different microbial community compositions (Figure 4.6; Table 4.6). The abundance of all three domains and many phyla was significantly different between

the different mesocosm cores (Table 4.6), with core ‘B10’ showing the most obvious differences at domain level (Figure 4.11). The within-core homogeneity is interesting in the light of a recent study which found considerable differences between bacterial communities in adjacent centimetre squares (O'Brien *et al.* 2016). However, community differences between mesocosm cores were likely related to differences in vegetation and biogeochemistry observed between cores. Plant functional groups had a strong effect on microbial communities (Table 4.5), corresponding to numerous previous studies which have found plant functional groups to exert a strong influence on belowground microbial communities (Jassey *et al.* 2014; Lange *et al.* 2014; Legay *et al.* 2014). The impact of plant functional groups on soil microbes is unsurprising: plants are responsible for the majority of organic matter inputs to soils, both as plant litter and as root exudates. Different plant functional groups differ in litter quality (Lang *et al.* 2009), and litter microbial communities may be adapted to the litter of a specific plant species (Ayres *et al.* 2009; Strickland *et al.* 2009). In addition, while vascular plants act as a source of root exudates, mosses do not (Hornibrook 2009; Kao-Kniffin and Zhu 2013). The effect of soil biogeochemistry was also important: for example, core ‘B1’ has a very different community composition to the other cores, potentially related to its high redox potential (Figure 4.6).

4.4.5 Effect of Drought on Taxonomic Composition (EMIRGE Assemblies)

In order to include novel bacterial species which were absent from the SILVA database in analyses, full-length 16S rRNA genes were assembled using EMIRGE and the resulting assemblies (hereafter “EMIRGE-assemblies”) used as a reference database for closed-reference OTU-picking of metagenomic reads (“EMIRGE-community”). The taxonomic composition of both EMIRGE-assemblies and EMIRGE-communities were very different to the taxonomic composition obtained by MG-RAST SSU annotation (Figure 4.13). For example, both Proteobacteria and Acidobacteria comprised a larger proportion of the EMIRGE-community than of the MG-RAST annotations, and Acidobacteria were much more diverse in the EMIRGE-community than the MG-RAST annotations. EMIRGE additionally assembled 16S rRNA sequences belonging to rarer phyla such as candidate divisions OD1, OP11, OP3 and TM7, although candidate divisions made up a very small proportion of the community. Conversely, Firmicutes, Actinobacteria and Bacteroidetes made up a smaller proportion of the EMIRGE-community than of the MG-RAST annotations. It is likely that

many members of the candidate phyla and the Acidobacteria are missing from existing 16S rRNA databases, as these phyla were only discovered relatively recently (Ludwig *et al.* 1997; Hugenholtz *et al.* 2001) and lack cultured representatives (Hugenholtz *et al.* 1998; Kantor *et al.* 2013). Proteobacteria are relatively well studied, but are also highly diverse and so it is likely that many members remain to be discovered and added to sequence databases.

Unlike ordination of the MGRAST-SSU annotations, ordination of EMIRGE bacterial communities did not reveal even a weak effect of treatment (Figure 4.14; Table 4.7). Although 'envfit' did detect a significant relationship between community composition and peat water content, this relationship was not significant after removal of a single 'outlier' sample. Interestingly, OTUs belonging to the Actinobacteria appear to exhibit low values on the second axis, clustering with samples with low water content: this corresponds with the results of the ordination of SILVA-annotated reads, in which Actinobacteria appeared to cluster with droughted samples. The weaker effect of treatment on the EMIRGE-community compared to the community annotated using the MGRAST-SSU database seems to suggest that better-studied bacterial taxa contained within the MGRAST-SSU database may respond more strongly to drought than do novel taxa whose 16S rRNA sequences were assembled using EMIRGE.

4.4.6 Functional Composition

Overall, protein metabolism was by far the most abundant SEED subsystem for both eukaryotic and bacterial genes, containing over 50% of annotated genes within both domains (Figure 4.15; Table 4.8). Within bacterial annotations, 60% of genes were involved in protein metabolism and 14% were involved in carbohydrate metabolism, with the remaining subsystems each comprising less than 3% of annotations. The extreme dominance of protein metabolism in functional annotations represents a very different pattern to previous soil metagenomic studies in which there is a more even spread of genes between subsystems and the most abundant SEED subsystems contain less than 20% of total annotations (Delmont *et al.* 2012; Bai *et al.* 2014; Souza *et al.* 2015). The high proportion of functional annotations involved in protein metabolism could be indicative of nitrogen limitation, as microbial communities typically invest more in nitrogen-acquiring enzymes under conditions of nitrogen limitation (Sinsabaugh and Moorhead 1994) and there is some evidence that microbial carbon mineralisation in peatlands is nitrogen limited (Keller *et al.* 2006; Bragazza *et al.* 2012). However, other sources

suggest that phosphorus limitation may be more important (Hill *et al.* 2014). Alternatively, the high proportion of genes involved in protein metabolism could be due to a paucity of known genes in current databases. The majority of genes within the protein metabolism subsystem were SSU rRNA genes, and the importance of SSU rRNA as a marker gene in microbial ecology means that a larger number of SSU rRNA genes have been sequenced than functional genes or complete genomes. Within the five most abundant phyla, the proportion of genes within the protein metabolism subsystem increases as the number of genomes in the NCBI Genome Database (http://www.ncbi.nlm.nih.gov/genome/browse/; accessed 15/06/16) decreases. For example, 90.5% of annotated proteins within the Acidobacteria were assigned to the protein metabolism subsystem in comparison to 46.6% of annotated Proteobacteria genes; the NCBI Genome Database holds 34 and 31,547 genomes belonging to these two phyla, respectively. Quality filtering of annotations in the current study was much more stringent than many of the aforementioned studies, which used threshold identity cut-offs as low as 60% (e.g. Souza *et al.* 2015) rather than the 90% threshold used in the current study, and lower thresholds likely meant that genes belonging to unsequenced organisms could be assigned to distant homologues. Metagenomic annotation thus currently represents a trade-off between including poor quality and potentially erroneous annotations in analyses, or only being able to annotate a small proportion of each dataset. Such a trade-off highlights the need for continuing expansion of curated sequence datasets.

Within the bacteria, carbohydrate metabolism was the next most abundant SEED subsystem after protein metabolism. Carbohydrate polymers dominate the carbon content of upper layers of peat (Lin *et al.* 2014a) and become degraded with increasing depth, although in bogs the process of decomposition is incomplete (Tfaily *et al.* 2013). In the current study, the most abundant carbohydrate metabolism subsystems at hierarchy level 2 were those involved in organic acid metabolism and CO_2 fixation. In contrast, an earlier peat metagenomic study found genes for monosaccharide degradation to make up the majority of genes for carbohydrate metabolism (Lin *et al.* 2014b), implying that differences in peat chemistry between individual sites may drive differences in bacterial functional potential. Organic acids are likely present in bogs as a by-product of fermentation, which plays an important role in carbon flow in peatlands (Drake *et al.* 2009). The high proportion of genes for CO_2 fixation was the result of genes involved in photosynthesis, and thus likely originated either from cyanobacteria DNA or from chloroplast DNA in undecomposed plant matter: chloroplasts originated as cyanobacterial

symbionts, and so many of the genes involved in photosynthesis within plant cells remain homologous to cyanobacteria (Martin *et al.* 2002). As photosynthesis is only feasible at the surface of the peat, this result might appear to suggest that DNA may be highly stable in peat ecosystems. However, a number of cyanobacteria are capable of heterotrophic growth (Smith 1982), which would enable them to survive in underground peat.

Few genes for nitrogen metabolism were found in the dataset, possibly reflecting the fact that phosphorus, rather than nitrogen, is the primary limiting nutrient in most peatlands (Hill *et al.* 2014; Lin *et al.* 2014b). Of the nitrogen metabolising genes which were present, genes involved in ammonium assimilation were most abundant: ammonium is the dominant form of inorganic nitrogen in anoxic conditions (Vepraskas and Faulkner 2001). Genes for the utilisation of allantoin (a form of organic nitrogen which higher plants accumulate in tissue or release as root exudates) were even more abundant than genes for ammonia assimilation. Allantoin is rapidly degraded by microbial communities in rice paddy soil (Wang *et al.* 2007), but the relevance of allantoin in other soils and plants is currently poorly understood.

Very few SEED subsystems passed abundance filtering prior to testing for the effects of time point and treatment, probably as a result of incomplete community coverage and of the limited number of reads which could be annotated (Section 4.3.1). Of the subsystems tested, only the gene for S12p (a ribosomal protein which is a part of the bacterial 30S subunit) showed a significant response to drought: abundances of the S12p gene were lower in droughted than control mesocosm cores at the fourth time point. The S12p gene was the only ribosomal gene to pass abundance filtering, and thus it is not possible to compare this result with other SSU genes. Unlike ribosomal RNA genes, ribosomal protein genes are rarely present in multiple copies (Yutin et al. 2012), and so it is possible that a change in the number of S12p genes in the rarefied data represents an increase in the mean genome size at this time point (although in this case similar patterns would be expected for other universal single-copy bacterial genes). However, the standard errors of the mean numbers of S12p genes at each time point were very large, making it difficult to draw firm conclusions about the behaviour of this gene.

Significant relationships were found between the functional composition within the carbohydrate metabolism subsystem of bacteria and both redox potential and phenol oxidase activity (Figure 4.18; Table 4.9). It is possible that phenol oxidase activity acts as an indicator of the quality or degree of decomposition of organic matter: as decomposition proceeds, the

aromaticity and lignin content of litter increases as the content of labile carbon falls (Bray *et al.* 2012) and higher lignin content results in higher phenol oxidase activity (DeAngelis *et al.* 2011). Meanwhile, the relationship between carbohydrate metabolism and redox potential could be caused by a reduction in anaerobic carbohydrate metabolism systems under oxic conditions: both methanogenesis and fermentation are included within the category of carbohydrate metabolism in the SEED hierarchy. There was no relationship between water table and the composition of the carbohydrate metabolism subsystem, but the significant effect of drought on redox potential (Chapter 2) means that changes to the gene composition within the carbohydrate metabolism subsystem could be linked to drought-driven changes in carbon release. However, the relationship between the composition of carbohydrate metabolising genes and phenol oxidase was only marginally significant ($p=0.48$), meaning that further investigation would be required before drawing firm conclusions about the effect of aromaticity on community functional potential.

There are several possible explanations for the lack of changes in functional potential during drought. Firstly, changes to the taxonomic community composition during drought were weak (Section 4.4.3), and thus it seems that in the current study drought did not have a large effect on microbial communities. The weak effect of drought either indicates that peatland microbial communities are relatively resilient to short-term drought, or that the current methodology was unable to detect changes which did occur. A recent study suggests that a large proportion of DNA in soil is extracellular, and likely is a 'relic' of dead organisms (Carini *et al.* 2016). It is unknown how long relic DNA persists in the environment, but it may obscure changes occurring over short term experiments such as the current study. The problem of relic DNA is likely compounded by the effect of dormant cells: many bacteria are able to become dormant in order to survive drought and other environmental stresses (Jones and Lennon 2010; Manzoni *et al.* 2014). Future studies could potentially incorporate transcriptomic methods: RNA has a more rapid turnover rate and thus is likely to give a better indicator of the 'active' community. (Moran *et al.* 2013). Additionally, difficulties in the annotation of metagenomic reads may have obscured changes in taxonomy and functional potential within the dataset. Only a third of predicted proteins were able to be annotated by the MG-RAST webserver, and only a fraction of these annotations met the minimum threshold values for percentage identity and coverage. In addition, these annotations are likely to be biased towards genes found in either model

organisms or micro-organisms of economic or medical importance (such as plant or human pathogens).

4.4.7 Abundance of genes involved in phenol degradation

Phenol oxidase enzymes in peatlands are of particular interest due to the role they play in degradation of the phenolic compounds, which are one of the factors hypothesised to inhibit decomposition in peat (Freeman *et al.* 2001; Fenner and Freeman 2011). Other enzymes which may play a role in the aerobic decomposition of phenolic compounds include peroxidases, which are involved in the depolymerisation of lignin (Sinsabaugh 2010), and dioxygenases (Bugg 2003), a category which includes catechol dioxygenase. The role of phenol oxidases in the decomposition of peat organic matter is well known, while the role of other phenol-degrading enzymes in peatlands has been less well-studied. However, peroxidases often exhibit high activities in peat (Jassey *et al.* 2012; Gittel *et al.* 2014), and are known to exert a strong influence on organic matter decomposition in non-peat soils (Tian and Shi 2014). Peroxidase activity is strongly positively correlated to the concentration of phenolic compounds in porewater, suggesting that peroxidase enzymes may play a role in the regulation of phenolic compounds in peatlands (Romanowicz *et al.* 2015). However, it should be noted that peroxidases are also abundant in plants and play a role in lignin biosynthesis (Passardi *et al.* 2005).

In the current study dioxygenases were the most abundant oxidoreductase domain: dioxygenases play important roles in the degradation of phenolic compounds, including the cleavage of catechol rings, but also catalyse a wide range of other reactions. Peroxidase and Cu-oxidase type 4 (a family including laccases) domains were also abundant (Figure 4.20). However, while peroxidase domains were abundant very few hits were found for lignin peroxidase (lip) and manganese peroxidase (mnp; Figure 4.20), two peroxidases which are important in fungal lignin degradation (Sinsabaugh 2010). Therefore, it is likely that the majority of peroxidase genes identified in the current study play non-degradative roles, a conclusion supported by the high proportion of peroxidase domains which were assigned to the Streptophyta (Table 4.10): plant peroxidases play a wide variety of roles including cell wall formation, protection from oxidative stress and regulation of signalling pathways (Passardi *et al.* 2005). A previous peatland metagenomic study similarly found Cu-oxidase type 4 to be by far the most abundant oxidase domain in a North American bog (Lin *et al.* 2014a), suggesting

that this enzyme family is important across a wide variety of peatlands. However, no study has yet combined metagenomic analyses with assays for both peroxidases and phenol oxidase, and so it is not possible to draw conclusions about whether the number of genes for these enzymes relates directly to their expression and activity.

Drought did not significantly affect the abundance of oxidative genes in peat (Table 4.11), although prior to the application of corrections for multiple comparisons there was a significant effect of drought on peroxidase enzymes. However, in the current experiment no significant change in phenol oxidase activities was found (Chapter 2), suggesting that the degradation of phenolic compounds was not the main driver of drought-driven carbon dioxide release in the mesocosms used for the current study. A previous study found that drought significantly increased the abundance and diversity of catechol 2,3-dioxygenase as measured by community fingerprinting (Fenner *et al.* 2005), suggesting that the effect of drought on phenol oxidase genes may be context dependent.

4.4.8 Abundance of genes involved in anaerobic metabolism

Under normal conditions, the majority of the peat profile in a wetland is anoxic as oxygen is unable to penetrate more than a few centimetres into waterlogged peat (Askaer *et al.* 2010). Waterlogging implies that organisms must rely on alternative electron acceptors to oxidise organic matter, which may be either inorganic (anaerobic respiration) or organic (fermentation). Of the marker genes for anaerobic metabolism investigated in the current study, by far the most abundant was *hydA*, a hydrogenase involved in the H_2-evolving, NADH-regenerating step of fermentation (Figure 4.21). Previous research suggests that fermentation is an important pathway in peatlands (Keller and Bridgham 2007; Drake *et al.* 2009; Keller *et al.* 2009), and fermentation genes were also found to be abundant in an earlier peat metagenomic study (Lin *et al.* 2014a). Dissimilatory sulfite reductase (*dsrA*) was only present at a slightly higher relative abundance than copper-containing nitrite reductase (*nirK*) genes, contrary to previous results which have found *dsrA* to be much more abundant than *nirK* in peatlands (Lin *et al.* 2014a). Nitrate concentrations are normally extremely low in peatlands, presumably limiting the rate of denitrification (Knorr *et al.* 2009; Palmer *et al.* 2010). However, in the present study mean nitrate concentration in the bog at 5 cm was 8 µmol L^{-1} (Chapter 2), much higher than previous studies, likely enabling higher rates of denitrification than are usually observed. Low counts of particulate methane monooxyganase (*pmoA*) genes were also

uncovered in this study. Coupled with low methane fluxes (Chapter 2), low numbers of *pmoA* genes suggest that the bog mesocosm cores had comparatively low rates of methanogenesis. Measured redox potential values were considerably above the expected redox potential at which methanogenesis occurs (McBride 1994).

The only anaerobic metabolism gene to show a significant effect of treatment was the hydrogenase *hydA*. *HydA* was less abundant in droughted than control mesocosm cores at the final time point, potentially suggesting that fermentative micro-organisms were outcompeted by aerobes during drought. However, a post-hoc Tukey test found only a marginally significant difference between the two treatments, which appeared to be due in part to a rise in copy numbers in the control treatment as well as a fall in the drought treatment (Figure 4.22). Further work would therefore be required to draw conclusions about the effect of drought on fermentation in peatlands.

Despite the fact that two of the drought-affected OTUs described in Chapter 3 were affiliated with alkane-degrading bacteria, *alkB* (a gene involved in alkane degradation) did not respond to drought. However, the potentially alkane-degrading OTUs only responded to drought in the fen habitat, so it is possible that shotgun sequencing in the fen would have detected a significant effect of drought on the abundance of alkane hydroxylase genes.

4.5 Conclusions

1. The microbial community at 5cm in the Marchlyn Mawr bog was dominated by bacteria, but bacterial dominance was weaker than previous metagenomic studies of peatlands (Lin *et al.* 2014a). Abundant bacterial phyla were Proteobacteria, Acidobacteria, Actinobacteria, Firmicutes and Verrucomicrobia.

2. The taxonomic composition of bacterial and fungal communities differed between mesocosm cores as a result of differences in the plant communities. The proportion of Archaea was also affected by mesocosm core.

3. There was a weak effect of the drought treatment on the taxonomic composition of bacterial communities, and drought responses were seemingly conserved at phylum level: NMDS ordination plots show Actinobacteria and Proteobacteria to cluster with droughted samples, while Bacteroidetes clustered with control samples. However, there was no significant effect of drought on the abundances of any of these phyla. The functional potential of microbial

communities did not respond to drought, suggesting either that a large degree of functional redundancy occurs in peatland microbial communities or that the methodology employed was unable to detect changes.

4. Neither genes for phenol oxidase nor genes for peroxidase enzymes exhibited significant changes in abundance during drought. However, the abundance of *hydA* (a hydrogenase involved in H_2-evolving fermentation) was lower in droughted than control mesocosm cores at the final time point, potentially representing a reduction in the importance of fermentation under oxic conditions.

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Chapter 5

Assembly and analysis of metagenome-assembled genomes from a Welsh peat bog

5.1 Introduction

For much of the history of microbiology, the cultivation of microorganisms has been the primary method of studying the properties of bacteria and fungi. However, it has long been known that easily cultivable microorganisms make up only a tiny fraction of overall microbial diversity: only an estimated 0.01-1% of microorganisms observed under the microscope can be cultured on traditional media (Garza and Dutilh 2015). The development of high-throughput sequencing and its subsequent application to the development of culture-independent methodologies for the study of microbial communities has thus been hugely beneficial to our knowledge of microbial diversity. As explored in previous chapters of the current work, both marker gene analysis (Chapter 3) and metagenomics (Chapter 4) can be used to determine the taxonomic composition of microbial communities, both prokaryotic and eukaryotic; while metagenomics can additionally be used to infer functional potential. A further application of metagenomic sequencing is the ability to assemble genome sequences from uncultivable organisms.

Given that many bacterial and archaeal taxa have no cultured representatives (McDonald *et al.* 2012; Kantor *et al.* 2013), genome assembly from metagenomes presents an opportunity to understand the functional potential of these organisms. For example, a recent study was able to obtain a number of archaeal genomes through metagenomic sequencing of groundwater and sediment taken from an aquifer, including the first complete genomes to be obtained from two phyla (Castelle *et al.* 2015). Other studies have obtained genomes from uncultured bacteria in a wide variety of habitats, including aquifer sediment (Wrighton *et al.* 2012; Kantor *et al.* 2013), cow rumens (Hess *et al.* 2011) and brackish water bodies (Hugerth *et al.* 2015). Information from genome assemblies could additionally be used to guide targeted culturing efforts (Garza and Dutilh 2015). For example, analysis of genomes from the SAR11 clade of plankton suggested that members of this group required a source of reduced sulfur for growth, and addition of reduced sulfur sources greatly increased the number of SAR11 cells in culture (Tripp *et al.* 2008).

The assembly of partial or complete genomes from metagenomes involves several steps: raw reads are first assembled into ‘contigs’ (‘contiguous consensus sequences’), and contigs are then binned (i.e. grouped into clusters, or ‘bins’, based on their characteristics). Finally, the contigs within each genome may be extended and joined if possible. Each genome assembled

in this way represents a population rather than an individual (Sangwan *et al.* 2016). In recent years a great deal of progress has been made on the assembly of short metagenomic reads into contigs: a large number of assembly algorithms have been developed specifically for use on metagenomic datasets, with examples including MetaVelvet (Namiki *et al.* 2012), Ray Meta (Boisvert *et al.* 2012) and IDBA_UD (Peng *et al.* 2012). The rapid rate of change in the field of bioinformatics means that online forums such as blogs are additionally of interest, providing rapid comparisons of new assemblers (e.g. http://ivory.idyll.org/blog/2014-how-good-is-megahit.html; accessed 11/03/2016). Algorithms for binning contigs into genomes are also improving: many early studies binned contigs based solely on tetranucleotide frequencies (e.g. Hess *et al.* 2011; Kantor *et al.* 2013) or similarity in coverage within one or few samples (e.g. Tyson *et al.* 2004), while more recent methods combine between-sample variations in coverage with sequence composition. The addition of detailed coverage information can significantly improve genome bins: contigs from the same genome are expected to show similar patterns of abundance across multiple-sample datasets (Sangwan *et al.* 2016). A number of algorithms now exist to carry out genome binning based on both patterns of abundance and sequence information: one of the earliest was CONCOCT (Alneberg *et al.* 2014). Comparisons of these algorithms are currently lacking (Sangwan *et al.* 2016), but undoubtedly will soon begin to appear in the literature.

In peatlands, two areas of particular interest are anaerobic metabolism and the degradation of phenolic compounds. The mean redox potential of bog mesocosm cores in the current study varied between +223 and +355mV under control (non-drought) conditions, while the redox potential under oxic conditions is roughly +350-+600mV at pH 7 and slightly higher at lower pH values (McBride 1994), such as those measured in bog mesocosm cores. While these redox potentials are on the borderline between oxic and anoxic metabolism, bog mesocosm cores exhibited a positive methane flux under control conditions (Figure 2.6). Methanogenesis only occurs at very low redox potentials (typically -200mV or below), and so the presence of a positive methane flux may suggest that the bog habitat contained 'micro niches' with much lower redox potential than that measured (Blodau and Moore 2003; Knorr and Blodau 2009; Askaer *et al.* 2010). Additionally, there was a rise in concentrations of sulfate and nitrate during droughted conditions (Figure 2.15; Figure 2.16), potentially suggesting that sulphur and nitrogen are present in reduced forms under submerged conditions. Therefore, it is likely that at least some of the micro-organisms inhabiting peat would have pathways for anaerobic

metabolism, such as anaerobic respiration or fermentation. Reduction of nitrate, sulfate and ferric iron have all been observed to occur in peatlands (Keller and Bridgham 2007; Knorr and Blodau 2009), and fermentation also accounts for a large fraction of anaerobic metabolism in peatlands (Vile *et al.* 2003; Hamberger *et al.* 2008)

The degradation of phenolic compounds is of interest due to the role played by phenolic compounds in inhibiting the degradation of organic matter from peatlands (Freeman *et al.* 2001), and it has been shown that phenol oxidase enzymes play a role in the release of carbon dioxide from peatlands during drought (Freeman *et al.* 2001; Fenner and Freeman 2011). The cleavage of catechol rings is also an important step in the degradation of phenol by soil micro-organisms, with catechol being a frequent intermediate in the degradation of phenol (Varga and Neujahr 1970). Cellulose degradation is also of potential interest: as an important structural compound of plant cell walls, cellulose is a highly abundant polymer in plant matter (Paul and Clark 1989). A highly simplified model of cellulose degradation involves the breakdown of polymeric cellulose into cellobiose (a disaccharide) by endo- and exocellulases. Cellobiose may then be taken up by microorganisms or broken down into glucose monomers by extracellular β-glucosidase enzymes. β-glucosidase commonly increases in activity following drought, and may thus be involved in increased microbial carbon cycling and carbon dioxide release following peatland water table drawdown (Fenner and Freeman 2011).

Aims and Objectives of Chapter

The aim of this chapter was to assemble prokaryotic genomes from the metagenomes described in Chapter 4 in order to better understand the metabolic potential contained within metagenome-assembled genomes in the bog mesocosm cores taken from Marchlyn Mawr, both under normal conditions and during a simulated drought. Specific objectives were:

1. To infer the metabolic potential of prokaryotic populations inhabiting the bog environment, with particular focus on anaerobic metabolism and the degradation of phenolic compounds and cellulose
2. To identify whether functional potential is conserved at phylum level in peatland environments
3. To identify metagenome-assembled genomes (MAGs) which respond to drought

5.2 Methods

5.2.1 Sequence data

The shotgun metagenome sequence dataset described in the current chapter is the same as that in Chapter 4. The dataset represents a replicated time series: ten bog mesocosm cores were sampled regularly over a period of approximately six months, with half subjected to a simulated drought and half maintained at high water table (Chapter 2). DNA extracted from samples taken at five time points was shotgun sequenced on an Illumina HiSeq 2500 in Rapid Run mode (Chapter 4).

5.2.2 Contig Assembly and Binning

Contigs were assembled from paired-end reads using MEGAHIT (Li *et al.* 2015) with the 'meta' parameter combination (recommended for general assembly from metagenomes). Next, the CONCOCT algorithm (Alneberg *et al.* 2014) was used to bin contigs into genomes. CONCOCT combines information about sequence composition (tetranucleotide frequencies) with coverage information across multiple samples, as contigs originating from the same genome will share similar distribution patterns. The current dataset is highly appropriate for CONCOCT, as it represents a time series of samples taken from discrete mesocosm cores: this experimental design is expected to generate variations in coverage between both different sampling time points and different mesocosm cores. To achieve optimum results when binning with CONCOCT, short contigs (<2000bp) were first removed. Next, contigs of 20,000 base pairs or longer were fragmented into sections 10,000 base pairs long, with the exception of the final section of each contig which remained appended to the previous section to avoid generating contigs <10,000bp long. Secondly, the raw reads from each sample were mapped onto the contig sections using BWA-MEM with default parameters (Li and Durbin 2009). The BAM files generated by BWA-MEM were used to create a coverage file for input into CONCOCT. The CONCOCT algorithm was then run with default parameters in order to cluster contigs into what will hereafter be termed 'metagenome-assembled genomes' or MAGs.

In order to estimate the completeness of each MAG, copy numbers of 36 single copy, universally conserved COGs (Single Copy Genes; hereafter SCGs) within each MAG were calculated as follows. Predicted protein-coding regions were extracted and translated using

List 5.1: Single copy, universally conserved COGs (SCGs) which were used to assign the completeness of each MAG. List taken from https://github.com/BinPro/CONCOCT/blob/master/scgs/scg_cogs_min0.97_max1.03_unique_genera.txt; accessed 05/04/2016.

COG0016
COG0048
COG0049
COG0051
COG0052
COG0060
COG0072
COG0080
COG0081
COG0087
COG0088
COG0089
COG0090
COG0091
COG0092
COG0093
COG0094
COG0096
COG0097
COG0100
COG0102
COG0103
COG0130
COG0184
COG0185
COG0186
COG0197
COG0198
COG0200
COG0201
COG0244
COG0256
COG0504
COG0532
COG0541
COG0552

Prodigal (Hyatt *et al.* 2010). Domains belonging to COGs were found within predicted protein sequences using RPS-BLAST v2.3.0 (Camacho *et al.* 2009) and the "Cog_LE" database of conserved domains (ftp://ftp.ncbi.nih.gov/pub/mmdb/cdd/little_endian/). The number of SCGs were then counted. A list of COGs considered to be SCGs is given in List 5.1.

As the majority of the current analysis focused on complete and uncontaminated MAGs (i.e. those which contained single copies of all 36 SCGs), complete MAGs were visualised using anvi'o (Eren *et al.* 2015). Abundance profiling was carried out for all contigs belonging to near-complete MAGs based on the same BAM files as those used for binning in CONCOCT. Results of CONCOCT clustering (described above) were imported using the command 'anvi-import-collections', and both mean coverage and variability of all MAGs was visualised using the 'anvi-interactive' interface.

5.2.3 Taxonomy, Distribution and Functional Potential of MAGs

To assign MAGs taxonomically, all MAGs containing 33 or more SCGs (i.e. MAGs that were >90% complete) were used as input for phylogenetic tree reconstruction in PhyloPhlAn (Segata *et al.* 2013), using the reference database within PhyloPhlAn as the reference. Given the large number of MAGs which were assigned to the Acidobacteria and the relatively small number of Acidobacteria in the PhyloPhlAn reference database, an additional tree was generated for this phylum using twenty-eight genomes downloaded from the NCBI

genome database as reference sequences. Protein-coding amino acid sequences within the downloaded genomes were predicted with Prodigal (Hyatt *et al.* 2010), and the tree was generated using PhyloPhlAn.

The mean coverage across all contigs within each MAG in each sample was calculated using the script "ClusterMeanCov.pl" which is packaged with CONCOCT (Alneberg *et al.* 2014). The coverages of all MAGs were used to generate an NMDS ordination of all samples (command 'metaMDS' within R package VEGAN (Oksanen *et al.* 2015)). Additionally, PERMANOVA was carried out (command 'adonis'). To examine relationships between the distributions of near-complete MAGs which shared functional characteristics, NMDS ordination was additionally carried out using the coverages of only near-complete MAGs as input.

The MAGs which contained single copies of all SCGs were selected for further analysis of genome-scale functional potential. Gene annotation was carried out using blastx in DIAMOND v0.7.11 (Buchfink *et al.* 2015) with default parameters against the KEGG gene database (Ogata *et al.* 1999). KEGG gene annotations were used to generate a list of the KEGG ontology identifiers present in each MAG, which was then used as input to the KEGG Mapper webserver (http://www.genome.jp/kegg/tool/map_pathway1.html). Pathways of interest were inspected manually.

In the current chapter, particular emphasis was given to pathways of anaerobic metabolism, including three types of anaerobic respiration: dissimilatory nitrate reduction to ammonium (DNRA), dissimilatory sulfate reduction and iron reduction. The presence of pathways for dissimilatory nitrate and sulfate reduction, as well as fermentation, were inferred based on the output of the KEGG mapper. However, as the KEGG Mapper does not specifically cover iron reduction, a BLAST search was carried out to identify homologues of two outer membrane bound cytochromes involved in the reduction of ferric iron, *omcB* and *omcS* (Weber *et al.* 2006). Proteins belonging to *omcB* and *omcS* were downloaded from the UniProt database and used to generate a BLAST database. Translated predicted protein-coding sequences output by Prodigal (Section 5.2.2) were searched against the database of *omcB* and *omcS* sequences using blastp (v2.3.0; Camacho *et al.* 2009).

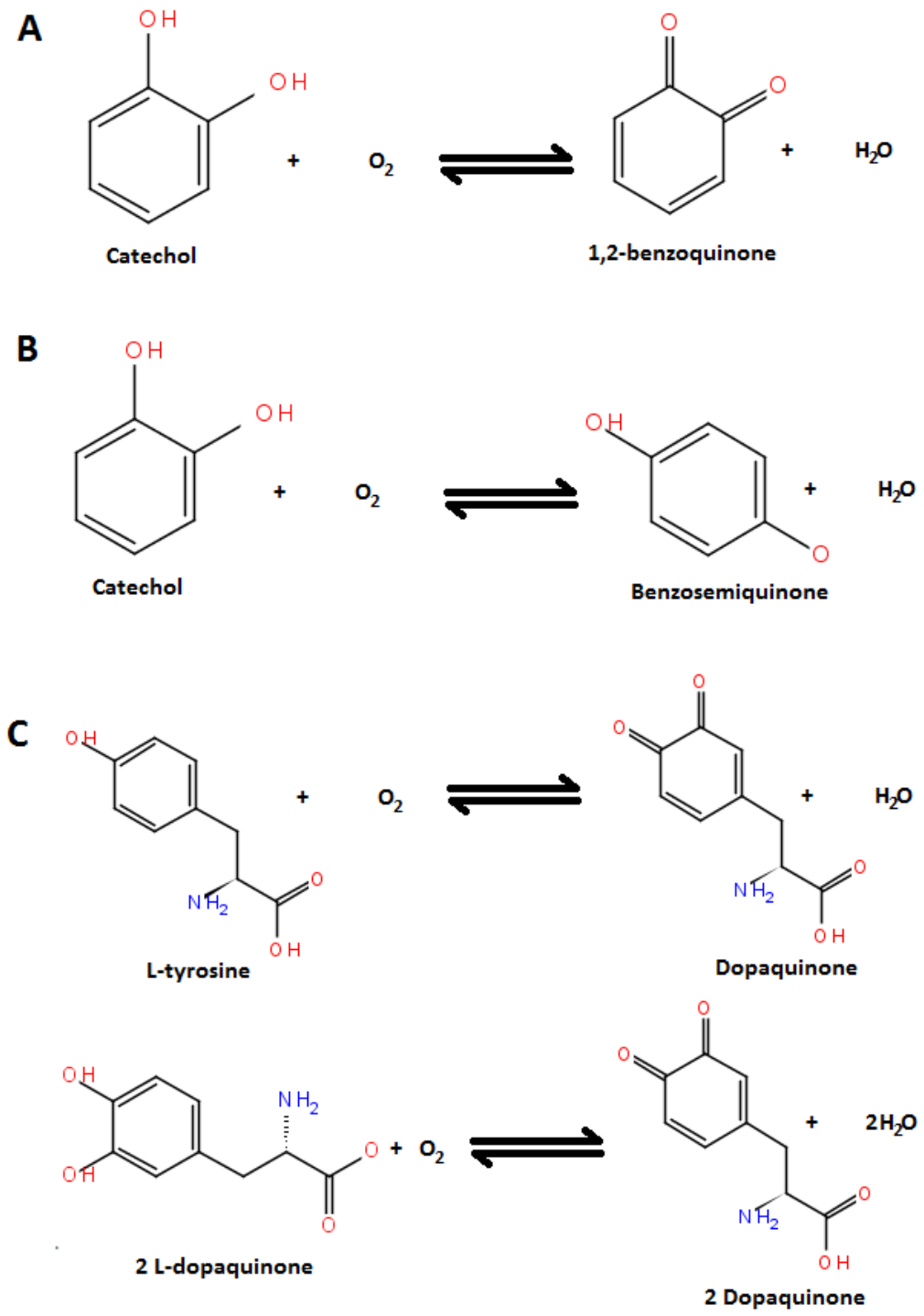

Figure 5.1: Diagrams showing the biochemical reactions catalysed by catechol oxidase (A), laccase (B) and tyrosinase (C). Molecules were drawn using R package 'rcdk' v3.3.2 (Guha 2007).

Due to the importance of phenol oxidase and glycoside hydrolase enzymes in decomposition, protein sequences corresponding to both these categories were downloaded from the UniProt

database and used to generate custom BLAST databases (Consortium 2015). Phenol oxidase protein sequences were downloaded if they were taxonomically assigned to bacteria and corresponded to one of the following EC numbers: EC 1.10.3.1 (catechol oxidase), EC 1.10.3.2 (laccase) and EC 1.14.18.1 (tyrosinase). These three enzymes were chosen as they represent well-characterised phenol oxidases, and the reactions catalysed by each of these enzymes are shown in Figure 5.1. In order to find potential cellulase genes, glycoside hydrolase protein sequences were identified as those which were cross-referenced to glycoside hydrolase families in the CAZy database (Lombard *et al.* 2014). Blastp (v2.3.0; Camacho *et al.* 2009) was used to find matches to the phenol oxidase and glycoside hydrolase protein sequences in translated predicted protein sequences (output by Prodigal, section 5.2.2). Following Berlemont & Martiny (2013), β-glucosidases were defined as genes belonging to families GH1 and GH3 within the CAZy database, and endo- and exocellulases were defined as genes belonging to families GH5, GH6, GH9, GH12, GH44, GH45 and GH48. To estimate the subcellular location of the obtained phenol oxidase genes, protein sequences which were identified as phenol oxidase genes were input into the Gneg-mPloc webserver if they came from an MAG assigned to another phyla (Shen and Chou 2010). Gneg-mPloc infers the subcellular location of proteins based on homology to other proteins in the SwissProt database where possible. Gneg-mPloc is limited to the gram-negative bacteria, but all MAGs that contained hits to the phenol oxidase genes were assigned to phyla of gram-negative bacteria. Where significant homology to other proteins in the SwissProt database does not exist, the subcellular location is inferred based on the subcellular locations of matching functional domains and modelling of the evolutionary history of a protein (Shen and Chou 2010).

5.2.4 Functional Potential of Phyla

To examine functional differentiation between phyla, an NMDS plot was generated using a matrix with the number of proteins assigned to each KEGG orthologue (KO) as the 'species' and each near-complete MAG as a 'sample'. NMDS plots were based on Bray-Curtis dissimilarity (function 'metaMDS' in R package VEGAN (Oksanen *et al.* 2015)). PERMANOVA was then applied to test the significance of between-phylum differences (function 'adonis' from R package VEGAN).

Table 5.1: Properties of all MAGs which contained single copies of 35 or 36 SCGs (out of a total of 36). Tax.Conf.: confidence level given to taxonomic assignment by PhyloPathia. Cov. (coverage) refers to total coverage across all samples.

MAG	No. of Contigs	No. of SCGs	Taxonomy	Tax. Conf.	Cov.
C100	441	36	Bacteria; Nitrospirae; Nitrospira; Nitrospirales; Nitrospiraceae	High	30.6
C106	315	36	Bacteria; Bacteroidetes	Low	11.2
C153	303	36	Bacteria; Verrucomicrobia; Verrucomicrobiae; Verrucomicrobiales	High	18.0
C155	310	36	Bacteria; Bacteroidetes	Medium	12.9
C184	365	36	Bacteria; Acidobacteria; Acidobacteria; Acidobacteriales; Acidobacteriaceae	High	20.5
C239	303	36	Archaea; Euryarchaeota; Methanomicrobia; Methanomicrobiales; Methanoregulaceae	High	9.3
C33	256	36	Bacteria; Bacteroidetes	Medium	16.5
C351	410	36	Bacteria; Acidobacteria; Acidobacteria; Acidobacteriales	High	12.6
C364	462	36	Bacteria; Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae	Low	17.3
C398	154	36	Bacteria; Firmicutes; Negativicutes; Selenomonadales; Veillonellaceae	High	13.3
C399	151	36	Bacteria; Acidobacteria; Acidobacteria; Acidobacteriales; Acidobacteriaceae	High	22.7
C417	308	36	Bacteria; Acidobacteria; Acidobacteria; Acidobacteriales	High	24.9
C443	211	36	Bacteria; Proteobacteria; Betaproteobacteria	Low	20.6
C78	626	36	Bacteria; Proteobacteria; Deltaproteobacteria; Myxococcales	High	16.3
C84	387	36	Bacteria; Acidobacteria	High	19.6
C104	187	35	Bacteria; Acidobacteria; Acidobacteria; Acidobacteriales; Acidobacteriaceae	High	22.7
C129	662	35	Bacteria; Acidobacteria; Acidobacteria; Acidobacteriales	High	65.0
C207	259	35	Bacteria; Bacteroidetes	High	10.4
C285	346	35	Bacteria; Acidobacteria; Acidobacteria; Acidobacteriales; Acidobacteriaceae	High	11.6
C291	546	35	Bacteria; Acidobacteria	High	18.8
C303	425	35	Bacteria; Nitrospirae; Nitrospira; Nitrospirales; Nitrospiraceae	High	12.7
C330	510	35	Bacteria; Acidobacteria; Acidobacteria; Acidobacteriales; Acidobacteriaceae	High	18.9
C344	286	35	Bacteria; Acidobacteria; Acidobacteria; Acidobacteriales	High	17.0
C355	506	35	Bacteria; Acidobacteria; Acidobacteria; Acidobacteriales	High	14.4
C437	736	35	Bacteria; Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae	Low	22.1
C52	409	35	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales	High	10.4
C87	562	35	Bacteria; Chloroflexi; Ktedonobacteria; Ktedonobacterales	Low	11.7

To find out which KOs were significantly different between the three phyla which contained the most MAGs (Acidobacteria, Proteobacteria and Bacteroidetes), generalised linear models with Poisson distributions were fitted on the abundances of each KO that was present in at least five MAGs (KOs which were present in fewer MAGs were excluded in order to avoid spurious relationships). Significant differences were determined based on analysis of deviance, and corrected for multiple comparisons using Benjamini-Hochberg corrections. Where significance was found, Tukey's post-hoc tests were carried out and only cases where post-hoc significance was found were examined further.

5.2.5 Effect of Drought on MAGs

Mean coverage values for all MAGs, regardless of the number of SCGs they contained, were calculated based on BWA mapping of reads to contigs (Section 5.2.2). MAG coverage values were used to generate NMDS plots using R package 'VEGAN' (command 'metaMDS') with default parameters. Two separate PERMANOVA tests were run (command 'adonis'): one to test for an interaction effect between time point and treatment, and one to test for the main effect of core. Next, generalised linear models with Poisson distributions were fitted to test for the effect of drought on individual MAGs: in each case, both core and the interaction effect between time point and treatment were included as independent variables. Model statistics were extracted and the resulting p-values adjusted using Benjamini-Hochberg corrections.

5.3 Results

5.3.1 Contig Assembly and Binning

Assembly with MEGAHIT generated a total of 9,875,881 contigs ranging in size from 200bp to 192,198bp with an N50 of 832. Of these, a total of 419,522 contigs were >2,000bp in length, representing a total of 1.75 billion base pairs. Clustering of all contigs >2,000bp in CONCOCT gave 445 clusters in total. When thirty-six SCGs were used to assess the completeness of each MAG (List 5.1), 15 MAGs contained a single copy of all SCGs and a further 12 contained single copies of all but one (Table 5.1). Hereafter, the 27 MAGs containing 35 or 36 SCGs will be referred to as 'near-complete MAGs'. The coverage patterns of contigs within a given near-complete MAG were highly similar (Figure 5.2), although there were exceptions: for example, a particular subset of contigs assigned to C84 showed high coverages in sample T2-B9 while

the coverage of other contigs within this MAG was much lower (leading to anvi'o clustering these contigs with C443 and C78 rather than C84). Overall coverage values for each near-complete MAG are given in Table 5.1. Variability in coverage within contigs was low in almost all cases (Figure 5.3), suggesting that assembly with MEGAHIT was successful in yielding non-chimeric contigs.

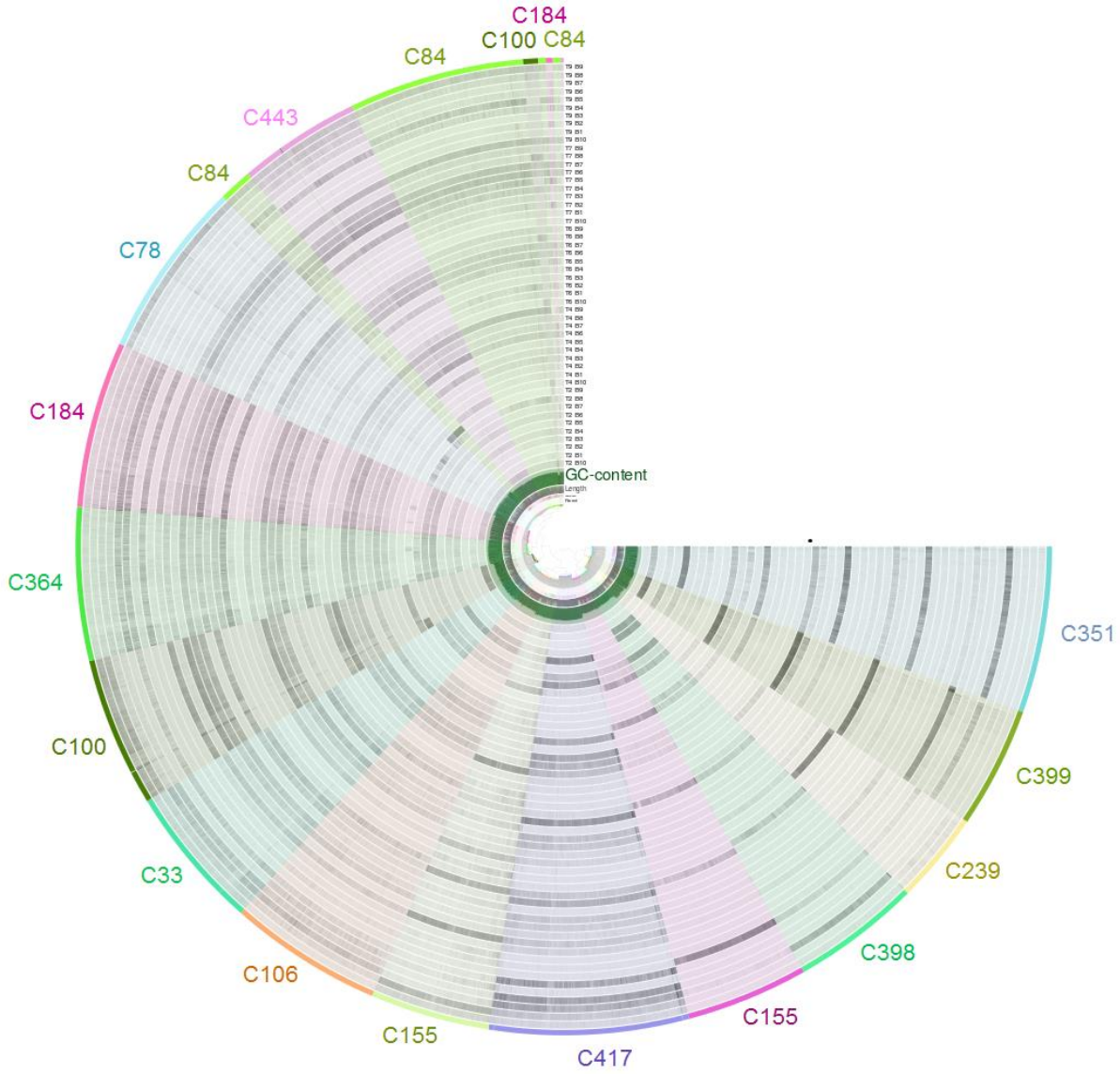

Figure 5.2: Anvi'o plot showing mean coverage across each contig in all samples within the dataset as well as mean GC content. Each radius (line from the centre to the outside of the circle) represents the properties of a single contig. In the outer fifty rings, colour intensity of a given radius represents coverage of that contig within each of the fifty samples sequenced, while in the ring labelled 'GC-content', the height of the green bar within each radius represents average GC content within each contig. Ordering of contigs is based upon hierarchical clustering within anvi'o, based on coverage and GC content, and this clustering is shown in the tree in the centre. Different coloured 'slices' represent different MAGs, each consisting of numerous contigs.

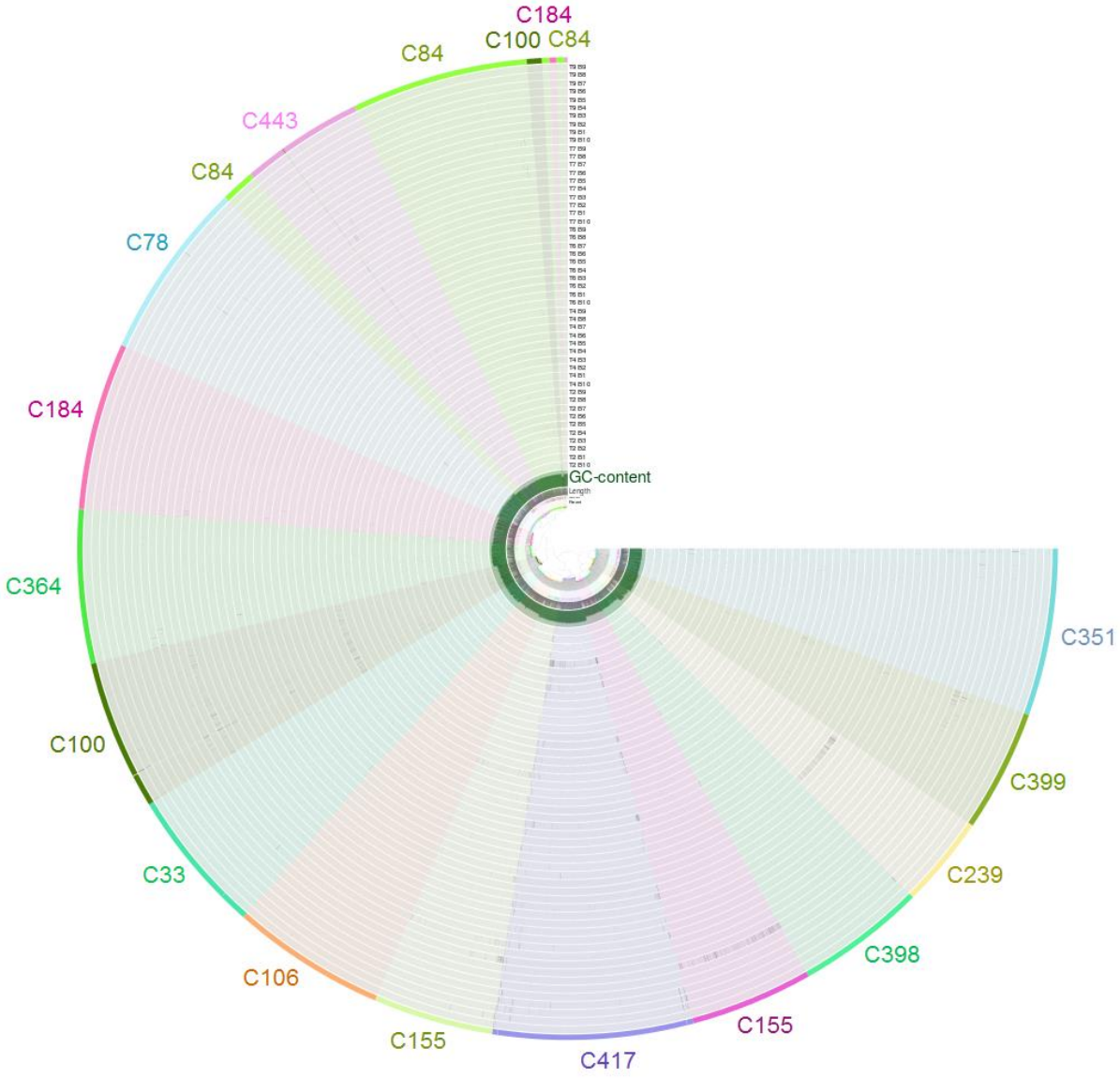

Figure 5.3: Anvi'o plot showing mean variance in coverage across each contig in all samples within the dataset as well as mean GC content. Each radius (line from the centre to the outside of the circle) represents the properties of a single contig. In the outer fifty rings, colour intensity of a given radius represents the level of variation in coverage of that contig within each of the fifty samples sequenced, while in the ring labelled 'GC-content', the height of the green bar within each radius represents average GC content within each contig. Ordering of contigs is based upon hierarchical clustering within anvi'o, based on coverage and GC content, and this clustering is shown in the tree in the centre. Different coloured 'slices' represent different MAGs, each consisting of numerous contigs.

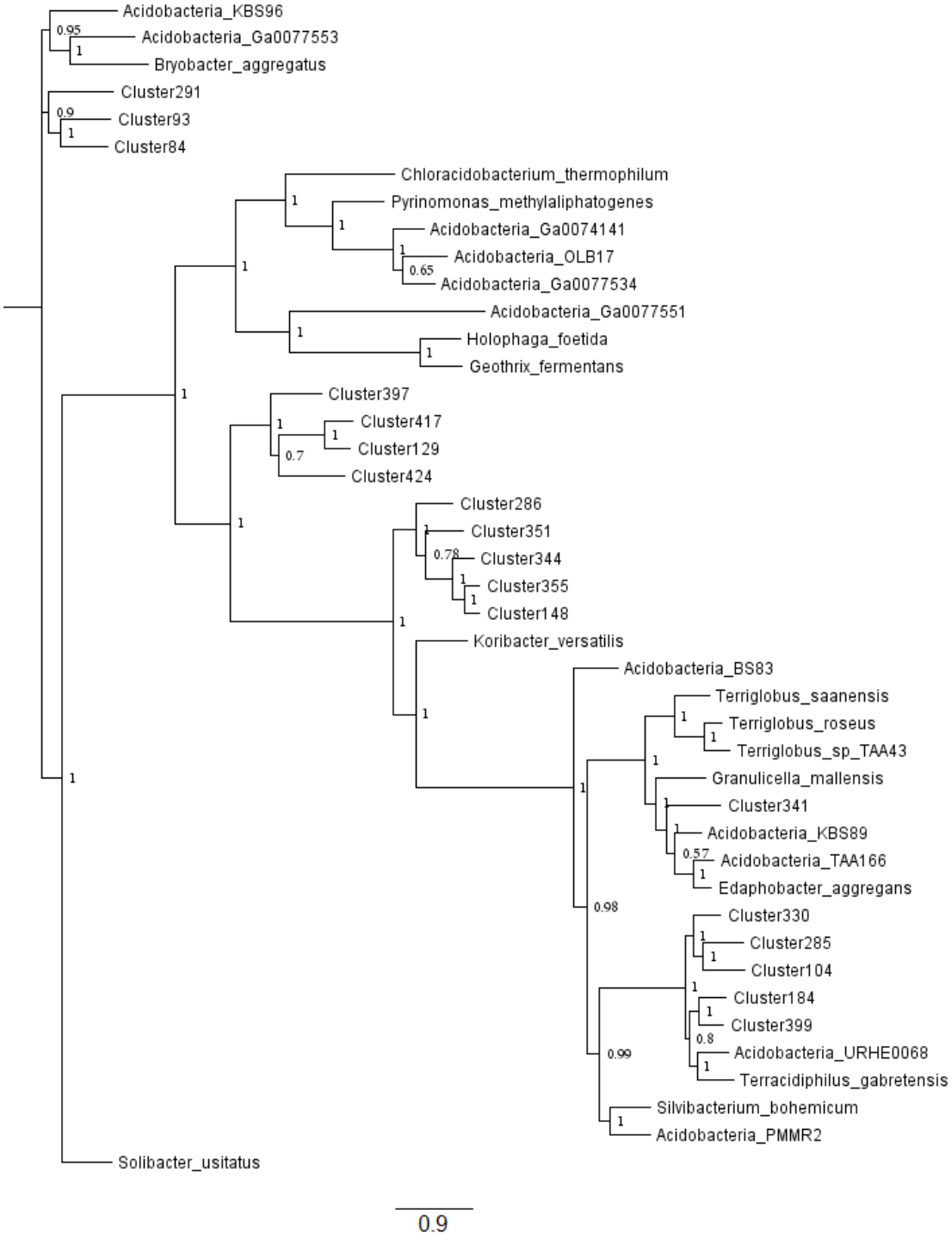

Figure 5.4: Phylogenetic tree of phylum Acidobacteria, depicting both Acidobacteria taken from the NCBI Genome database and all near-complete MAGs that were assigned to Acidobateria. All MAGs which were assigned to Acidobacteria and which contained 33 or more SCGs were included in the tree, meaning that the total number of MAGs (clusters) is greater than that shown in Table 5.1. The phylogeny was generated by PhyloPhlAn (Segata *et al*. 2013) and visualised in FigTree (http://tree.bio.ed.ac.uk/software/figtree/). The scale bar represents the estimated number of changes per site for a unit of branch length.

5.3.2 Taxonomy, Distribution and Functional Potential of MAGs

The majority of near-complete MAGs were assigned to domain Bacteria, although a single MAG was assigned to the Archaea (C239; Table 5.1). A large proportion of near-complete MAGs were assigned to phylum Acidobacteria: a total of 12 MAGs. Other near-complete MAGs were assigned to phyla Bacteroidetes (5 MAGs), Proteobacteria (4), Nitrospirae (2), Firmicutes (1), Verrucomicrobia (1) and Chloroflexi (1). Of the MAGs assigned to Acidobacteria, several formed clusters distinct from all previously sequenced genomes on the phylogenetic tree, suggesting that they may represent novel diversity within phylum Acidobacteria (Figure 5.4).

NMDS ordination based on the coverage of all MAGs suggested a strong effect of core (Figure 5.5A), but with no noticeable effect of treatment or time (Figure 5.5B). Likewise, PERMANOVA found no significant effect of the interaction between time point and treatment (F_4=0.3, p=1.0), and a second PERMANOVA test found that community composition was significantly different between mesocosm cores (F_9=8.9, p=0.001, R^2 =0.7).

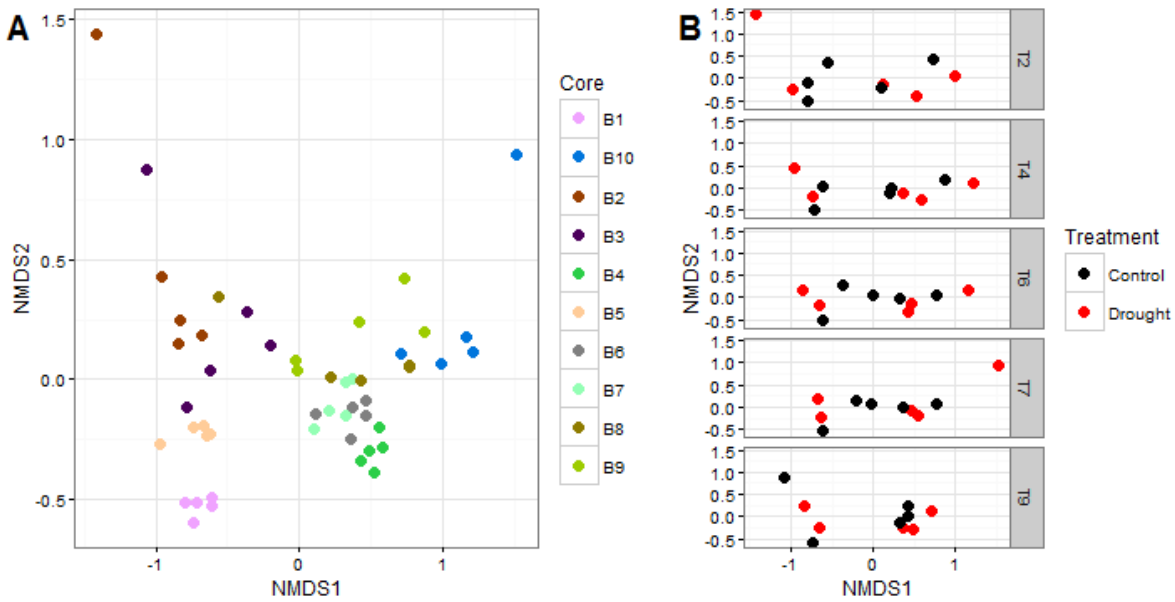

Figure 5.5: NMDS ordination of samples based on mean coverage of all MAGs, with colours representing (A) core and (B) treatment.

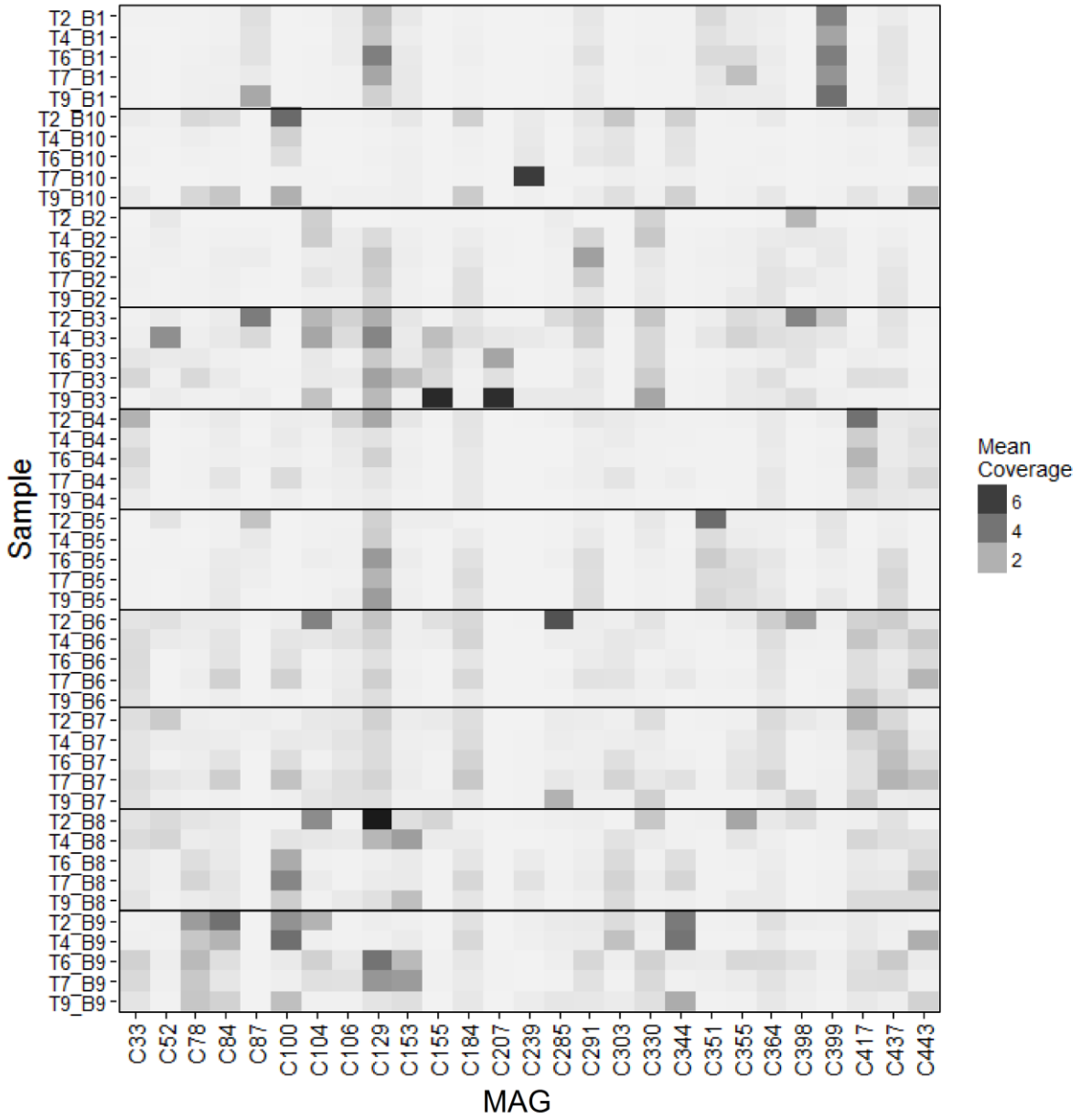

Figure 5.6: Heatmap depicting mean coverage of each near-complete MAG within each sample.

The 27 near-complete MAGs were selected for further analysis of their distribution patterns and genome-scale functional potential. Each near-complete MAG showed a unique distribution pattern (Figure 5.6). Some MAGs were present at high abundances in a single sample but were absent or rare in other samples: for example, C239 was only abundant in sample T7-B10 and C52 was only abundant in sample T4-B3 (Figure 5.6). Others appeared to be most abundant in a single core: in particular, C399 was abundant in core B1 but only present at low abundances elsewhere.

Table 5.2: Functional potential for nitrogen metabolism within each near-complete MAG, as predicted by the KEGG Mapper using KO annotations.

	Assimilatory Reduction		Dissimilatory Reduction		Denitrification	Nitrogen Fixation
MAG	**Nitrate**	**Nitrite**	**Nitrate**	**Nitrite**		
C78	Y	N	N	N	N	N
C84	N	N	N	Y	*norBC*, *nosZ*	N
C100	N	Y	Y	Y	*narGHIJ*, *napAB*, *nirK*	nifK, nifH
C106	N	N	N	N	*norBC*	N
C153	N	N	N	N	N	N
C155	N	N	N	Y	N	*nifD*, *nifH*, *nifK*
C184	N	N	N	Y	*nirK*	*nifD*, *nifH*, *nifK*
C239	N	N	N	Y	N	nifH
C33	N	N	N	N	*nirK*	N
C351	N	N	N	Y	*nosZ*	N
C364	N	N	N	Y	*nirK*	N
C398	Y	N	N	Y	N	*nifD*, *nifH*, *nifK*
C399	N	N	N	Y	N	N
C417	N	N	N	Y	N	N
C443	Y	N	Y	Y	*narGHIJ*	*nifD*, *nifH*, *nifK*
C104	N	Y	N	Y	*norBC*	*nifD*, *nifH*, *nifK*
C129	N	N	N	N	N	N
C207	N	N	N	N	N	*nifD*, *nifH*, *nifK*
C285	N	N	N	N	N	*nifD*, *nifH*, *nifK*
C291	N	N	N	Y	N	N
C303	N	N	Y	Y	*narGHIJ*, *norBC*	*nifD*, *nifH*, *nifK*
C330	N	N	N	N	N	*nifD*, *nifH*, *nifK*
C344	Y	N	Y	Y	*narGHIJ*, *napAB*, *norBC*	N
C355	N	N	N	Y	N	N
C437	N	N	N	N	N	N
C52	N	N	N	N	*norBC*	*nifD*, *nifH*, *nifK*
C87	N	N	N	Y	*nirK*	N

Near-complete MAGs represented a wide variety of lifestyles. One MAG, C239, was a putative methanogen: C239 lacked genes for proteins in both the electron transport chain and the TCA cycle and possessed a complete pathway for hydrogenotrophic methanogenesis, as well as for acetoclastic methanogenesis. Numerous other MAGs possessed genes for pathways of anaerobic respiration (Table 5.2-5.4) or fermentation (Table 5.5): four contained genes for all enzymes in pathways of dissimilatory nitrate reduction to ammonium (DNRA) and dissimilatory sulfate reduction/oxidatation (C100, C443, C303, C344), and one contained genes for a complete pathway of dissimilatory sulfate reduction but not DNRA (C104). Additionally, sixteen MAGs contained at least one pathway for fermentation (Table 5.5). Six

non-methanogenic MAGs were missing one to two genes for enzymes involved in the TCA cycle (also known as the Krebs or citric acid cycle): these MAGs were C100, C398, C443, C129, C207 and C303. Five of the MAGs which were lacking genes from the TCA cycle contained pathways for DNRA and/or dissimilatory sulfate reduction.

Table 5.3: Details of hits to *omcB* and *omcS*, two outer-membrane cytochromes involved in reduction of ferric iron. Protein sequences of all known *omcB* and *omcS* sequences were downloaded from UniProt, and predicted protein sequences within each MAG were searched using blastp (see main text).

Cluster	Number of Hits	Top Hit UniProt ID	% ID	E-value	Gene
C129	1	A0A0M2HVV5	31.1	4.19E-35	*omcB*
C303	7	B5EHF0	40.0	7.24E-18	*omcS-3*
		A0A0A8WLR3	27.7	3.41E-06	*omcB*
		Q74A86	40.8	3.28E-18	*omcS*
		B5EHE9	43.6	3.75E-73	*omcS-2*
		Q74A86	40.7	4.94E-92	*omcS*
		A0A0K6L084	28.7	9.03E-24	*omcB*
		Q749K5	30.2	1.10E-54	*omcB*
C153	3	A0A0F2C7U7	36.8	3.47E-08	*omcB*
		A0A0F2C7U7	26.3	1.27E-07	*omcB*
		Q749K5	25.7	4.91E-06	*omcB*
C351	3	A0A0F2C7U7	35.4	5.51E-09	*omcB*
		A0A0F2C7U7	36.9	4.11E-06	*omcB*
		A0A0F2C7U7	32.6	1.82E-64	*omcB*
C417	3	A0A0M2HVV5	33.1	5.24E-06	*omcB*
		A0A0M2HVV5	36.6	3.26E-11	*omcB*
		A0A0M2HVV5	33.5	9.40E-35	*omcB*
C78	1	A0A0F2C7U7	31.0	8.94E-08	*omcB*
C104	2	Q749K5	30.7	2.11E-07	*omcB*
		Q749K5	24.3	2.11E-08	*omcB*
C344	2	A0A0A8WLR3	33.3	7.39E-06	*omcB*
		A0A0F2C7U7	34.3	2.03E-09	*omcB*
C437	1	A0A0F2C7U7	38.5	1.27E-13	*omcB*

The genetic potential of each MAG for nitrogen metabolism is summarised in Table 5.2. Of particular note was the fact that seventeen MAGs possessed pathways for dissimilatory reduction of nitrite to ammonia but only four possessed the ability to reduce nitrate to nitrite. Relatively few MAGs possessed genes for assimilatory reduction of nitrate or nitrite to ammonia: four contained genes for assimilatory nitrate reduction and two contained genes for

assimilatory nitrite reduction. No MAG was found to contain genes for assimilatory reduction of both nitrate and nitrite. Twelve MAGs possessed at least one gene involved in denitrification, but none contained the complete pathway for denitrification. Ten MAGs possessed homologues to all three genes (*nifD*, *nifH* and *nifK*) coding for the subunits of the nitrogenase molybdenum-iron protein (Table 5.2).

Table 5.4: Functional potential for sulfur metabolism within each near-complete MAG, as predicted by the KEGG Mapper using KO annotations. N = not present, Y = present, and P = partially present. 'Dissimilatory reduction/oxidation' refers to a pathway of three genes: *sat*, *aprAB* and *dsrAB*, and this pathway was considered present if at least one gene for each enzyme were found. The 'assimilatory reduction' pathway refers to a pathway of four steps, catalysed by the enzymes *PAPSS*/*sat*/*cysND*, *PAPSS*/*cysC*, *cysH* and *cysJI*/*sir* (where '/' indicates that either enzyme may catalyse a relevant step), and was considered present if at least one gene for an enzyme catalysing each step was found.

MAG	Assimilatory reduction	Dissimilatory reduction/oxidation	Sulphur oxidation
C78	P: APS -> PAPS	N	*soxB*, *soxD*
C84	Y	N	*soxD*
C100	P: Sulfate -> PAPS	Y	N
C106	P: Sulfate -> PAPS	N	*soxD*
C153	Y	N	*soxD*
C155	N	N	N
C184	Y	N	*soxD*
C239	P: PAPS -> sulfite	P: APS -> sulfite	N
C33	Y	N	*soxD*
C351	Y	N	*soxC*
C364	Y	N	*soxY*, *soxD*
C398	P: Sulfate -> PAPS	P: APS -> sulfide	N
C399	Y	N	*soxD*
C417	Y	N	*soxD*
C443	N	Y	*soxA*, *soxB*, *soxD*, *soxY*, *soxZ*
C104	P: Sulfate -> PAPS	Y	N
C129	P: Sulfite -> sulfide	N	*soxD*
C207	Y	N	N
C285	P: Sulfite -> sulfide	N	*soxD*
C291	Y	N	*soxC*, *soxD*
C303	Y	Y	*soxD*
C330	P: PAPS -> sulfite	N	N
C344	N	Y	*soxD*
C355	Y	N	*soxD*
C437	Y	N	*soxD*
C52	Y	N	*soxD*
C87	Y	N	N

Table 5.5: Functional potential for fermentation of pyruvate to lactate, acetate and propionate within each near-complete MAG, as predicted by the KEGG Mapper using KO annotations. N = not present, Y = present, and P = partially present. Following reactions within the MetaCyc database (Caspi *et al*. 2014), fermentation reactions were defined as follows. Fermentation of pyruvate to lactate was defined as EC 1.1.1.27; fermentation of pyruvate to acetate was defined as EC 2.3.1.8 + EC 2.7.2.1/EC 3.6.1.7 or EC 1.2.7.1 + EC 6.2.1.13; fermentation of pyruvate to propionate was defined as EC 1.1.1.37 + EC 4.2.1.2 + EC 1.3.5.4 + EC 2.8.3.- or EC 2.8.3.- + EC 4.2.1.54 + EC 1.3.1.95 + EC 2.8.3.1.

Cluster	Pyruvate -> Lactate	Pyruvate -> Acetate	Pyruvate -> Propionate
C100	N	Y	N
C104	Y	Y	P: Pyruvate -> Succinate
C106	N	N	N
C129	N	N	N
C153	N	Y	P: Pyruvate -> Succinate
C155	Y	Y	N
C184	N	Y	N
C207	Y	Y	P: Pyruvate -> Succinate
C239	Y	N	P: Pyruvate -> Succinate
C285	Y	N	P: Pyruvate -> Succinate
C291	Y	N	N
C303	N	N	P: Pyruvate -> Succinate
C33	N	N	N
C330	N	N	P: Pyruvate -> Succinate
C344	Y	Y	N
C351	N	N	N
C355	N	N	P: Pyruvate -> Succinate
C364	N	N	N
C398	Y	Y	Y
C399	N	Y	N
C417	Y	N	N
C437	N	Y	N
C443	N	N	N
C52	N	Y	N
C78	N	N	N
C84	N	Y	N
C87	N	N	P: Pyruvate -> Succinate

As *omcB* and *omcS* are both outer membrane bound cytochromes involved in iron reduction, a database comprising both genes was generated and predicted protein-coding sequences from

each MAG were searched against the database using blastp. A number of hits were obtained (Table 5.3), but the percentage identity of all hits was weak.

Table 5.6: Functional potential for two steps of methanotrophy (methane oxidation) within each near-complete MAG, as predicted by the KEGG Mapper using KO annotations.

Cluster	Methane => Methanol	Methanol => Formaldehyde
C78	N	N
C84	N	N
C100	N	N
C106	N	Y
C153	N	N
C155	N	N
C184	N	N
C239	N	N
C33	N	N
C351	N	N
C364	N	Y
C398	N	N
C399	N	N
C417	N	N
C443	N	N
C104	N	N
C129	N	N
C207	N	N
C285	N	N
C291	N	Y
C303	N	N
C330	N	N
C344	N	N
C355	N	N
C437	N	Y
C52	N	N
C87	N	Y

All but three MAGs contained genes for the full or partial pathway of assimilatory sulfate reduction, while seven contained genes for dissimilatory sulfate reduction/oxidation and five contained the complete pathway for dissimilatory reduction/oxidation of sulfate (Table 5.4). Nineteen MAGs contained at least one gene within the *sox* operon, of which the most common was *soxD*. However, only C443 contained the full *sox* operon (Table 5.4).

No MAG contained the genetic potential for methane oxidation: none contained genes for enzymes to convert methane to methanol, although five the contained genetic potential for conversion of methanol to formaldehyde (Table 5.6).

Table 5.7: Details of hits to the phenol oxidase genes laccase and tyrosinase. Protein sequences of known omcS sequences were downloaded from uniptot, and predicted protein sequences within each MAG were searched using blastp (see main text). Cellular locations of proteins which were matched to phenol oxidase genes were predicted using Gneg-mPLoc.

MAG	Total	Cellular Location
C106	1	Periplasm (1)
C153	0	
C78	1	Periplasm (1)
C84	2	Periplasm (1), cell inner membrane (1)
C100	0	
C155	0	
C184	4	Periplasm (4)
C239	0	
C33	0	
C351	4	Periplasm (4)
C364	5	Periplasm (4), cell inner membrane (1)
C398	0	
C399	0	
C417	9	Periplasm (9)
C443	1	Periplasm (1)
C104	0	
C129	1	Periplasm (1)
C207	0	
C285	0	
C291	2	Periplasm (2)
C303	2	Periplasm (1), cell inner membrane (1)
C330	0	
C344	5	Periplasm (5)
C355	2	Periplasm (2)
C437	3	Periplasm (3)
C52	1	Cell inner membrane (1)
C87	1	Cell inner membrane (1)

An NMDS ordination of sites based on only the near-complete MAGs showed a gradient of roughly increasing redox potential moving from right to left on the first axis. At low values on the first axis, there was also a trend of increasing redox potential when moving from low to high values on the second axis (Figure 5.7A). In addition, there was strong clustering of samples by core (data not shown). The MAGs themselves clustered by lifestyle in some cases: for example, four MAGs (C344, C100, C443, and C303) which contained complete pathways for both dissimilatory nitrate and sulfate reduction clustered together at the right hand side of the plot (Figure 5.7B; Figure 5.7C). The sole MAG containing the potential for methanogenesis

(C239) was plotted in the top right-hand corner, distinct from all other MAGs. However, the MAGs containing complete pathways for fermentation did not form obvious clusters (Figure 5.7D; Figure 5.7E).

The total number of glycoside hydrolase genes (a group which contains cellulases) in each MAG varied from 11 to 169. All MAGs contained β-glucosidase genes (Figure 5.8A), but four were lacking in genes for the breakdown of endo- and exocellulases (Figure 5.8B). All four of the MAGs which contained no predicted endo- or exocellulase genes possessed pathways for anaerobic respiration or fermentation (Figure 5.8B). The MAGs with the most genes for β-glucosidases did not necessarily correspond to the MAGs which contained the most endo- and exocellulase genes or the most phenol oxidase genes.

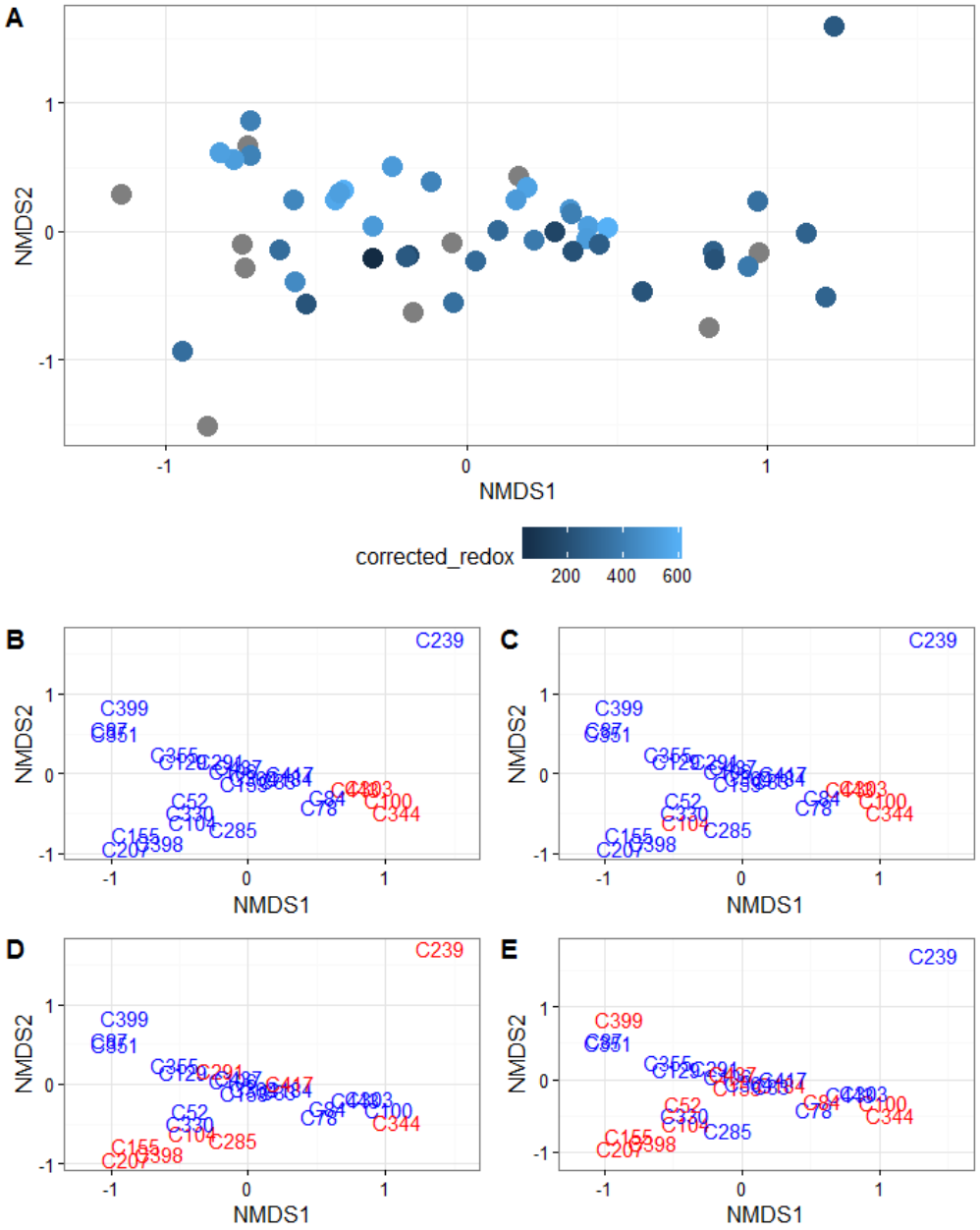

Figure 5.7: NMDS ordination of (A) samples and (B-E) MAGs, based on near-complete MAGs only. In plots B-E, MAG names are coloured ordering to the presence (red) or absence (red) of the fuctional potential for various traits: (B) complete dissimilatory nitrate reduction pathway; (C) complete dissimilatory sulfate reduction pathway; (D) fermentation of pyruvate to lactate; (E) fermentation of pyruvate to acetate. In plot (A), grey points represent samples where redox potential was not measured (i.e. samples taken at time point 2).

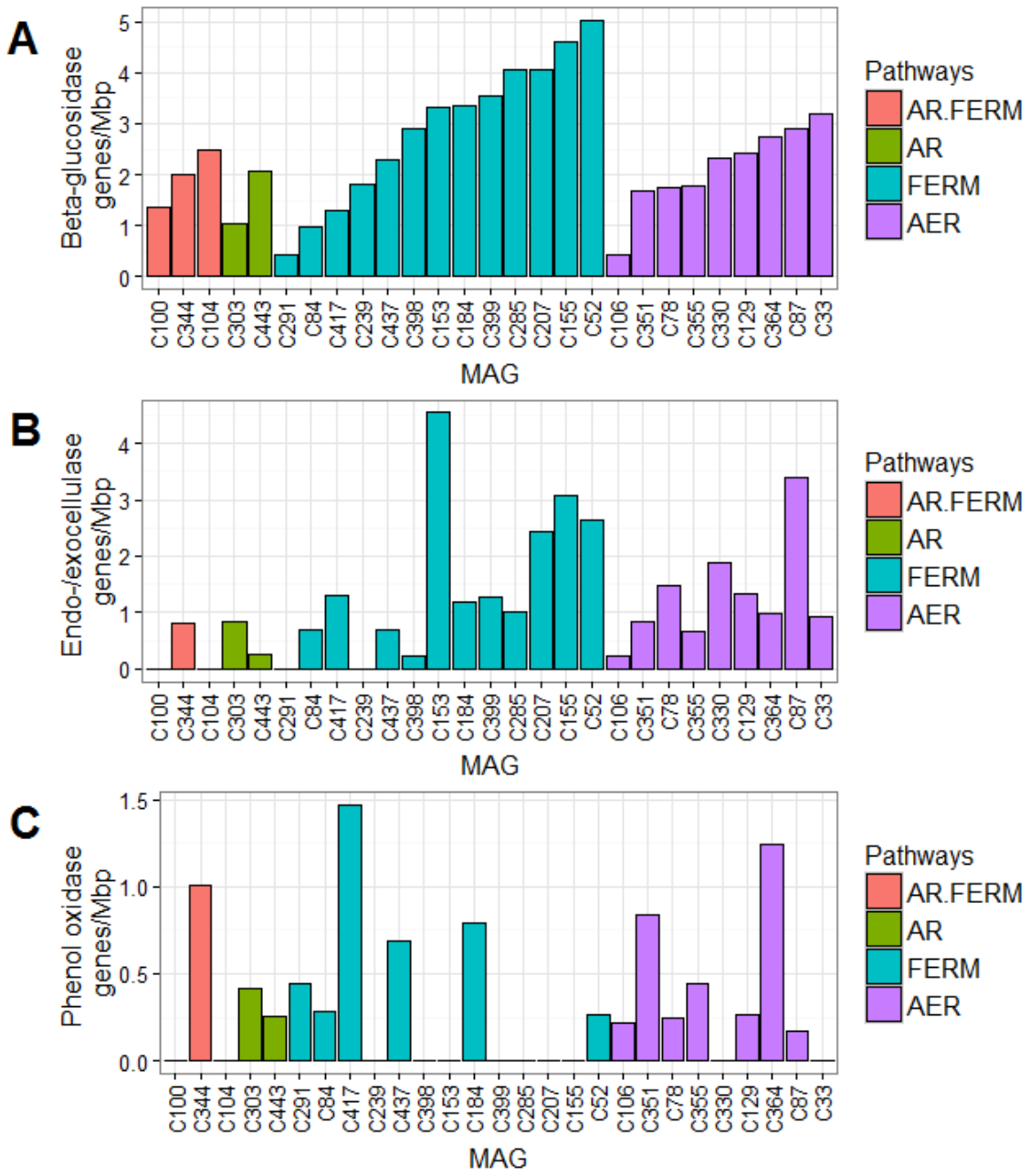

Figure 5.8: Number of predicted genes for (A) β-glucosidase enzymes, (B) endo- and exocellulase enzymes and (C) phenol oxidase per million base pairs (Mbp) of sequence in each MAG. The colour of each bar represents whether the MAG contains genes for anaerobic respiration (AR), fermentation (FERM), both (AR.FERM), or neither (AER). Denitrification is not included in anaerobic respiration, as no MAG contained a complete pathway for this function.

Table 5.8: Details of KEGG ontology categories which were significantly different between Acidobacteria, Bacteroidetes and Proteobacteria (three phyla which contained the majority of MAGs). B = Bacteroidetes; A = Acidobacteria; P = Proteobacteria; BCAA = branched-chain amino acid; p.adj = adjusted p-value.

KO	χ^2	p.adj	Post-hoc	Function/Gene	Pathway
K10385	15.2	0.040	B > A	Loricrin	Eukaryotic cytoskeleton
K12323	15.2	0.040	B > A	ANPRA, NPR1	Purine metabolism
K05989	29.0	0.000	B > A	*ramA*; alpha-L-rhamnosidase	Glycosidases
K07491	42.2	0.000	B > A & P	putative transposase	
K09607	30.8	0.000	B > A	*ina*	Immune inhibitor
K01187	15.6	0.036	B > P	*malZ*; alpha-glucosidase	Glycosidases
K01212	22.8	0.002	B > A	Levanase	Glycosidases
K01387	15.2	0.040	B > A	*colA*	Collagenase; microbial toxin
K01728	17.1	0.022	B > A	Pectate lyase	Pentose and glucuronate interconversion
K04485	15.3	0.039	P > A	*radA*	DNA repair
K06045	24.4	0.001	A > P	*shc*	Metabolism of terpenoids and polyketides
K07093	20.3	0.006	B & P > A	uncharacterized protein	
K09667	32.7	0.000	A & P > B	OGT; O-GlcNAc transferase	Glycan biosynthesis and metabolism; transcriptional regulation
K02453	23.1	0.002	A > B	gspD	Type II secretion
K02482	21.2	0.004	A > B & P	two-component system, NtrC family	
K00114	14.4	0.049	A > B	Alcohol dehydrogenase	
K00995	18.7	0.012	B > A	*pgsA*	Lipid metabolism
K00010	16.6	0.024	A > B & P	*iolG*	Streptomycin biosynthesis; inositol phosphate metabolism
K10947	90.9	0.000	A & P > B	*padR*	Transcription regulator
K12308	16.9	0.023	A > P	beta-galactosidase	Glycosidases
K13587	37.4	0.000	A > P	*cckA*	Cell cycle signalling
K02035	14.6	0.048	A > B	ABC. PE.S	Peptide/nickel transport system
K02050	14.6	0.048	A > B	ABC.SN.P	NitT/TauT transport system
K02488	35.4	0.000	A & P > B	*pleD*	Cell cycle signalling
K03408	17.7	0.018	A & P > B	*cheW*	Purine-binding chemotaxis protein
K03412	16.9	0.023	A > B	*cheB*	Two-component system, chemotaxis family
K03413	20.8	0.005	A & P > B	*cheY*	Two-component system, chemotaxis family
K00479	18.6	0.012	P > A	Rieske 2Fe-2S family protein	
K01996	27.6	0.000	P > A	*livF*	BCAA transport system
K01997	22.5	0.003	P > A & B	*livH*	BCAA transport system
K01998	20.3	0.006	P > A	*livM*	BCAA transport system
K01999	25.0	0.001	P > A & B	*livK*	BCAA transport system
K07112	16.7	0.024	P > A	uncharacterized protein	
K00799	137.5	0.000	P > A & B	Glutathione S-transferase	Glutathione metabolism
K11179	27.6	0.000	P > A	*tusE, dsrC*	tRNA synthesis

K08086	15.2	0.040	P > A & B	*fimV*	Pilus assembly
K00108	27.6	0.000	P > A	Choline dehydrogenase	Betaine biosynthesis
K00119	15.9	0.033	P > A	Unknown	
K08086	15.2	0.040	P > A & B	*fimV*	Pilus assembly
K13924	15.5	0.037	P > A & B	*cheBR*	Two-component system, chemotaxis family
K14986	21.8	0.003	P > A & B	*fixL*	Two-component system, LuxR family

Due to the important role played by phenol oxidases in peatland carbon cycle, all MAGs were searched for phenol oxidase genes using blastp. Over half of MAGs were found to contain at least one hit to either laccase or tyrosinase (Table 5.7; Figure 5.8C), and laccase genes were far more common than tyrosinase genes (Table 5.7). No matches to catechol oxidase were found. Gneg-mPloc suggested that the majority of predicted phenol oxidase proteins occurred in the periplasm (the space between the inner and outer cell membranes in gram-negative bacteria; Table 5.7). Mapping of metabolic pathways with the KEGG mapper found that nine MAGs possessed the ability to cleave catechol rings: these were C33, C153, C184, C351, C364, C87, C129, C291, C355 and C437. MAGs with the potential to cleave catechol did not correspond to the MAGs with the most phenol oxidase genes found using blastp (Table 5.7), and several MAGs with the ability to cleave catechol did not contain any phenol laccase or tyrosinase genes (C33, C153; Table 5.7). Many MAGs did not contain any predicted phenol oxidase genes (Table 5.7). However, a number of MAGs containing phenol oxidase genes also possessed predicted pathways of anaerobic respiration and fermentation (Figure 5.8).

5.3.3 Functional Potential of Phyla

An NMDS plot of MAGs based on KEGG orthologues (KOs) showed that MAGs clustered by phylum (Figure 5.9). Significant differences in functional potential between phyla were confirmed by PERMANOVA (F_6=2.8, R^2=0.47, p = 0.001).

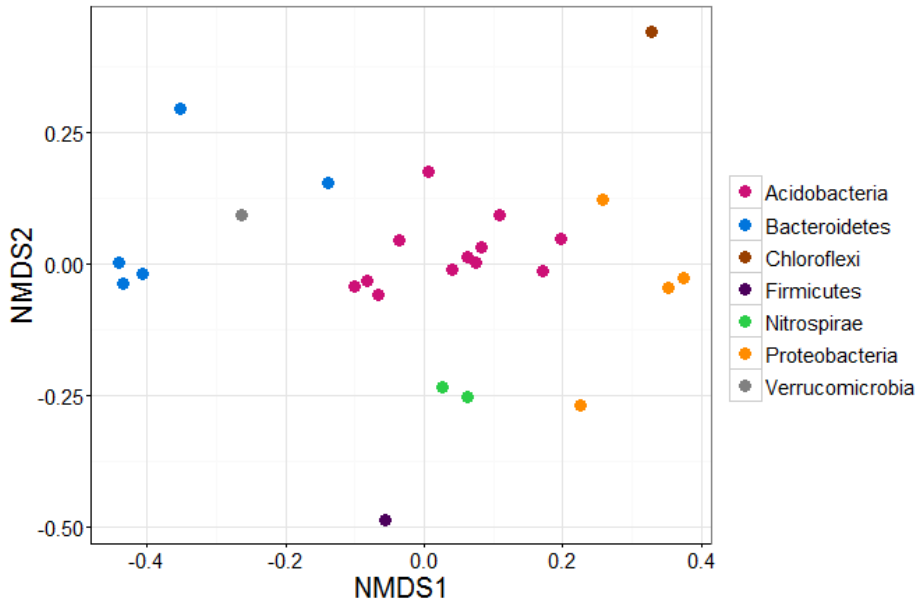

Figure 5.9: NMDS ordination of near-complete MAGs based on the abundance of KEGG ontology categories. The colour of each point represents the phylum to which that MAG was assigned by PhyloPhlan.

The abundances of a number of KOs were significantly different between the Acidobacteria, Bacteroidetes and Proteobacteria (Table 5.8; Figure 5.10). Bacteroidetes contained higher numbers of genes for three different glycosidase enzymes (K05989, K001187, K01212), while Acidobacteria contained higher numbers of genes for beta-galactosidase (K12308). Several genes from the *che* operon (*cheB*, *cheW*, *cheY*) were more abundant in Proteobacteria and Acidobacteria than Bacteroidetes. Conversely, genes from the *liv* operon (*livF*, *livH*, *livM*, *livK*) were more abundant in Proteobacteria than either of the other phyla.

5.3.4 Effect of Drought on MAGs

Generalised linear models with Poisson distributions were fitted to test for the effect of drought on individual MAGs, regardless of the number of SCGs they contained. Prior to the application of corrections for multiple comparisons, the mean overall coverage of seventeen MAGs was significantly affected by the interaction between time point and treatment (Table 5.9; hereafter drought-affected MAGs), suggesting an effect of drought. However, following application of Benjamini-Hochberg corrections for multiple comparisons, the effect of the interaction between time point and treatment was not significant for any MAG (Table 5.9). Plotting the overall mean coverage of each drought-affected MAG revealed that in many cases, the

interaction effect is likely driven by the effect of a few cores with especially high coverage values (e.g. C353, Figure 5.11G; C299, Figure 5.11M). In other cases, MAG coverage changes with time in multiple cores (e.g. C137, Figure 5.11E; C150, Figure 5.11F). None of the near-complete MAGs were affected by drought, and the majority of putatively drought-affected MAGs contained few or no SCGs (Table 5.9).

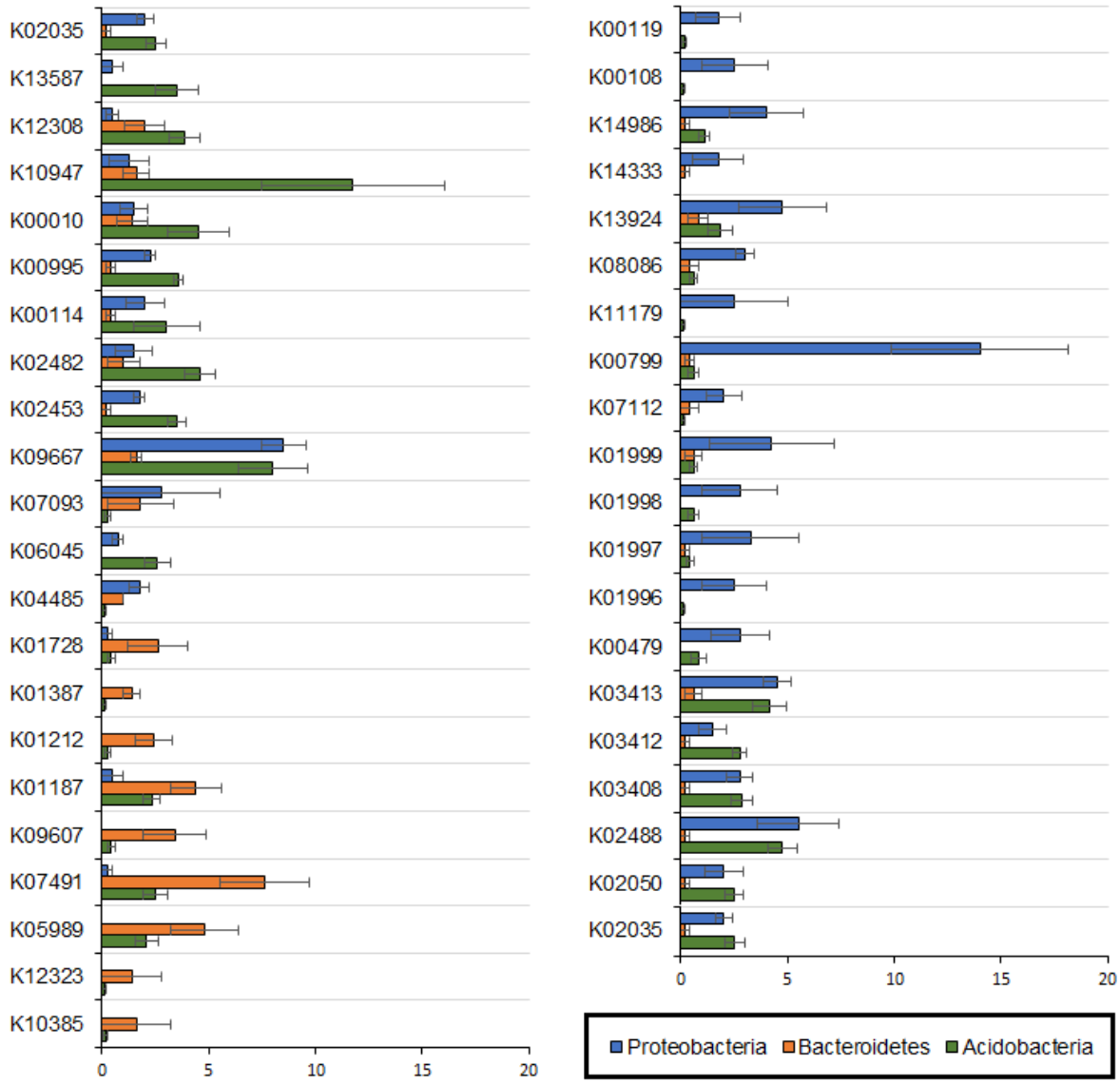

Figure 5.10: Mean number of proteins within near-complete MAGs that were assigned to each protein that was differentially abundant between Acidobacteria, Proteobacteria and Bacteroidetes. Acidobacteria, Bacteroidetes and Proteobacteria are depicted as these three phyla contained the highest proportion of MAGs.

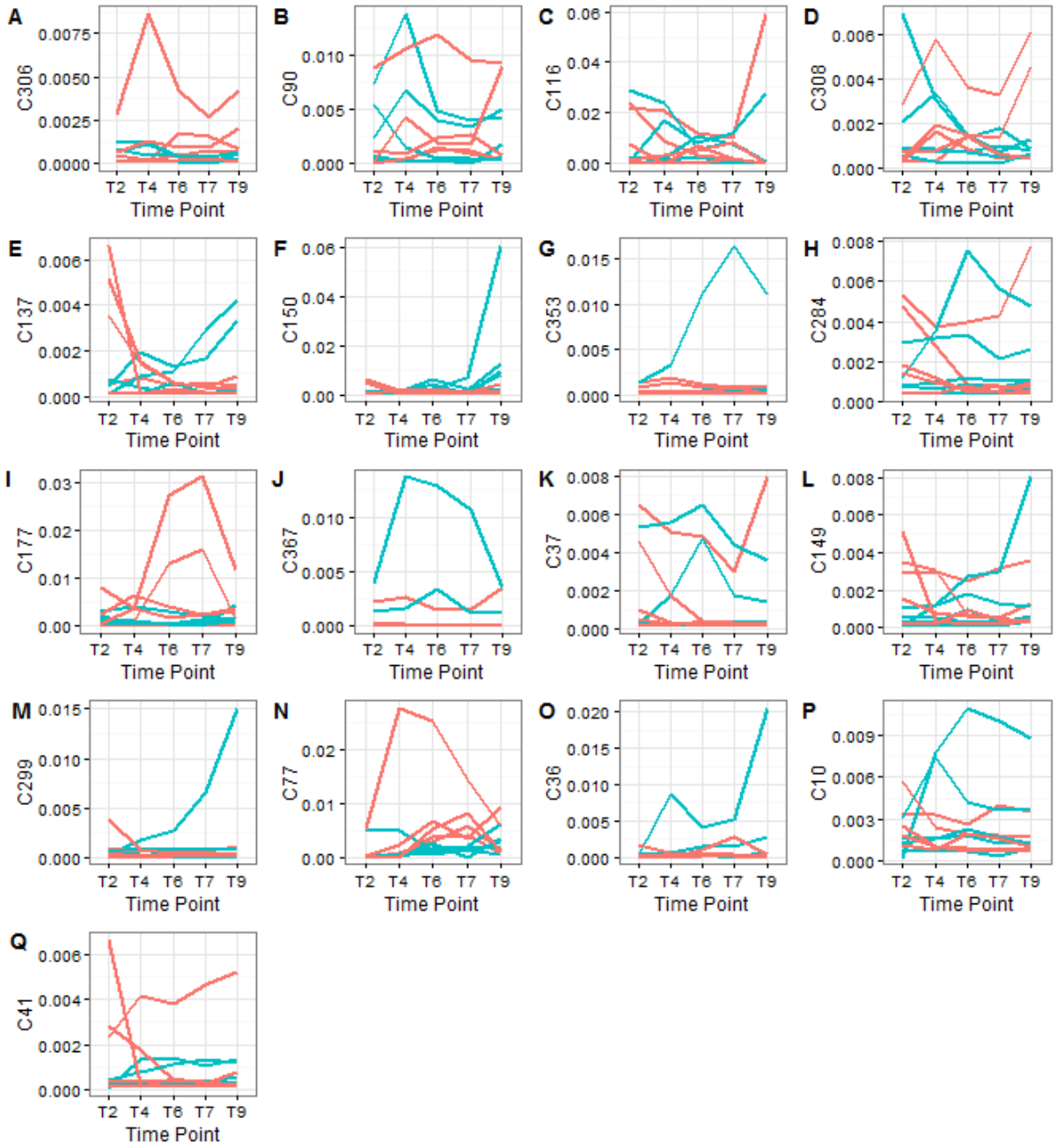

Figure 5.11: Mean coverage of each MAG whose coverage was significantly affected by the interaction between time point and treatment. Pink lines represent control cores and blue lines represent droughted cores. Coverages within each core are plotted separately in order to convey differences between cores.

Table 5.9: Details of cases where the effect of the interaction between time point and treatment on coverage of an MAG was significant. Number of multi-copy SCGs means the number of SCGs of which there were 2 or more copies. P.adj = adjusted p-value. Mean coverage of each MAG by core and time point is depicted in Figure 5.11.

Cluster	F	p	p.adj	Number of SCGs	Number of multi-copy SCGs
D306	5.1	0.003	0.7	0	0
D90	4.6	0.005	0.7	9	0
D116	4.6	0.005	0.7	0	0
D308	4.0	0.010	1.0	4	0
D137	3.7	0.014	1.0	15	0
D150	3.6	0.016	1.0	0	0
D353	3.5	0.018	1.0	15	0
D284	3.4	0.020	1.0	0	0
D177	3.4	0.020	1.0	0	0
D367	3.2	0.025	1.0	1	0
D37	3.1	0.031	1.0	27	7
D149	2.9	0.038	1.0	24	0
D299	2.9	0.039	1.0	4	0
D77	2.9	0.039	1.0	0	0
D36	2.8	0.043	1.0	0	0
D10	2.8	0.044	1.0	25	2
D41	2.7	0.046	1.0	11	0

5.4 Discussion

5.4.1 Contig Assembly and Binning

Although clustering of contigs by CONCOCT was based on coverage across multiple samples as well as sequence composition (i.e. tetranucleotide frequency), coverage patterns within each MAG were not always consistent (Figure 5.2), and in some cases hierarchical clustering within anvi'o was not consistent with clustering by CONCOCT (e.g. C100, Figure 5.2). The discrepancy between methods was likely a result of differing clustering methodologies within each algorithm: CONCOCT incorporates both coverage and compositional information into PCA to reduce the number of dimensions in the data, extracts the minimum amount of principal components required to explain 90% of the variation, and then extracts clusters based on clustering within the PCA. Therefore, some relevant information about coverage or sequence composition may be lost during the PCA clustering step. Conversely, anvi'o uses hierarchical

clustering rather than a PCA step, but is primarily used for visualisation of contigs: it does not categorize the contigs into discrete clusters. Anvi'o includes functionality to manually 'refine' bins where coverage patterns are inconsistent. However, clustering and visualisation of contigs in anvi'o is highly computationally intensive and thus it is not currently possible to manually refine groupings for large numbers of contigs. Additionally, variation between the coverages of different contigs existed within all MAGs, even those with the most consistency in coverage values, suggesting that noise is likely unavoidable when generating MAGs from complex metagenomes such as soil.

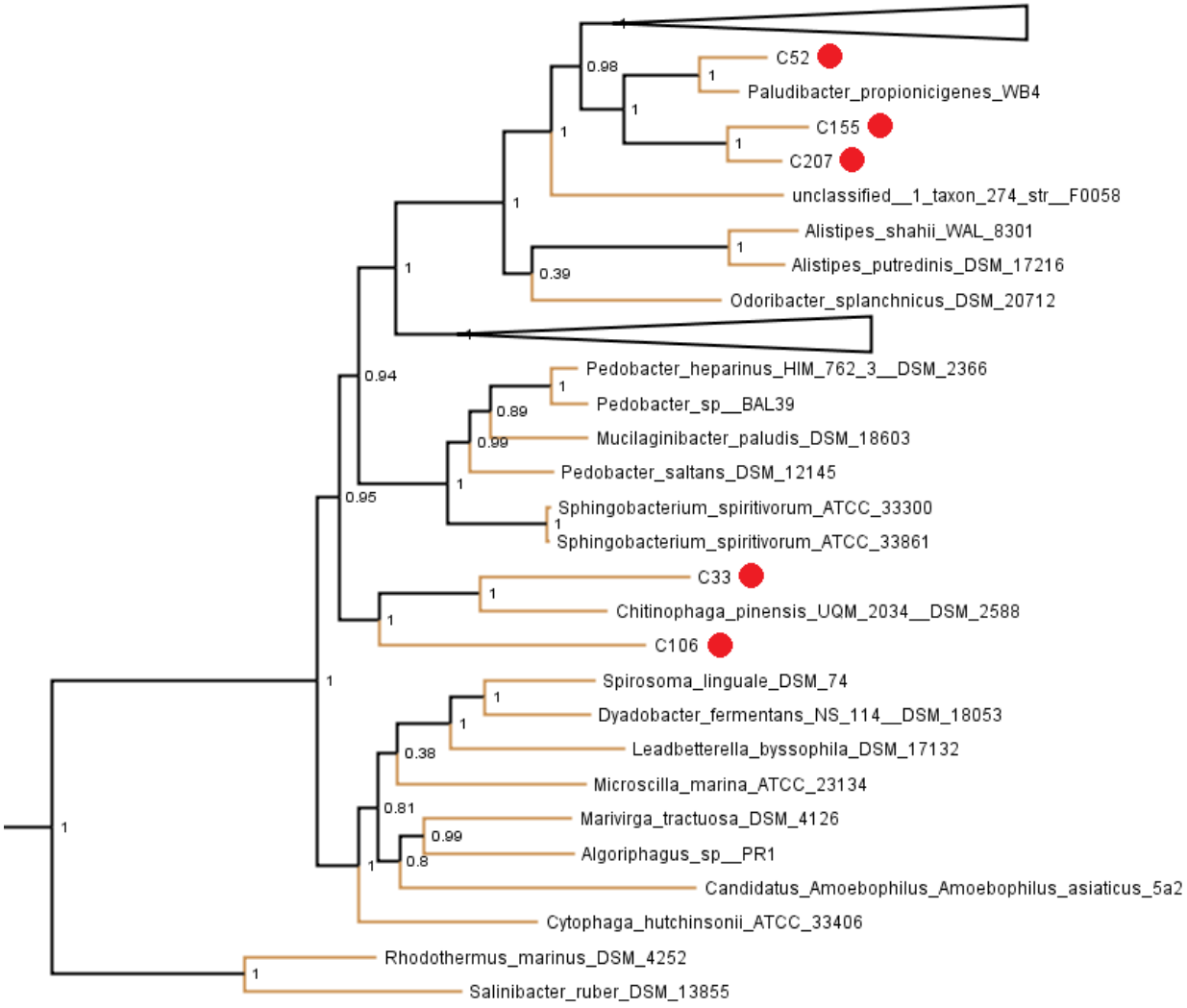

Figure 5.12: Subsection of the full PhyloPhlAn phylogeny containing phylum Bacteroidetes. The phylogeny was based on the PhyloPhlAn database of genomes. Red dots depict near-complete MAGs.

5.4.2 Distribution and Functional Potential of MAGs

Each near-complete MAG was assigned to at least phylum level based on a phylogeny generated in PhyloPhlAn. Phylogeny generation in PhyloPhlAn is based on a set of >400 conserved and highly informative proteins, and has been shown to provide accurate classifications (Segata *et al.* 2013).

Of the 27 near-complete MAGs analysed in depth, twelve were assigned to Acidobacteria. The high proportion of MAGs assigned to Acidobacteria echoed the dominance of Acidobacteria in both marker gene analysis (Chapter 3) and in unassembled metagenomic reads (Chapter 4). Acidobacteria are both abundant and widely distributed in soils (e.g. Felske *et al.* 2000; Jones *et al.* 2009; O'Brien *et al.* 2016), including in peat (Lin *et al.* 2012; Lin *et al.* 2014), and additionally contain a large degree of morphological and taxonomic diversity (e.g. Hugenholtz *et al.* 1998; Jones *et al.* 2009). Nonetheless, relatively few genomes belonging to members of this phylum have been sequenced to date: the NCBI Genome database listed just 34 at the time of writing (http://www.ncbi.nlm.nih.gov/genome/browse/; accessed 5/5/2016). A number of the Acidobacteria-derived MAGs in the current study form branches on the phylogeny that are distinct from the genomes downloaded from the NCBI Genome database, suggesting that many of the Acidobacteria in peat belong to poorly studied lineages. It is possible that differences in methodology caused MAGs from the current study to cluster separately from the downloaded genomes. However, prediction of protein-coding regions was carried out using Prodigal for all genomes and the downloaded reference genomes encompass a wide variety of assembly methods and sequencing technologies. Novel diversity was also detected within other phyla: for example, many of the MAGs within phylum Bacteroidetes could not be assigned below domain level, although inspection of the phylogeny generated by PhyloPhlAn suggests that several may be related to *Paludibacter propionicigenes* (Figure 5.12) which is an obligate anaerobe isolated from a rice paddy soil (Ueki *et al.* 2006).

The mesocosm core from which samples were taken had a clear and significant effect on overall community composition, while drought did not (Figure 5.5). This strong effect of mesocosm core on prokaryotic communities is similarly observed in both marker gene analysis of 16S rRNA (Chapter 3) and analysis of unassembled 16S rRNA gene reads from the shotgun sequencing data (Chapter 4), and is probably related to differences in biogeochemistry and vegetation between mesocosm cores (Chapter 3; Chapter 4). In particular, samples from core

B1 formed a distinct cluster (pink shapes; Figure 5.5) and core B1 had the highest redox potential of any non-droughted mesocosm core (Figure 5.13). Although redox potential was not measured until time point 4 (i.e. after the commencement of drought), it likely that the redox potential in the droughted cores only rose for a relatively short time period (i.e. during water table drawdown), while the redox potential in B1 may have been elevated for a longer period. Thus, while the persistence of dormant bacteria and relic DNA might obscure the short-term effects of redox potential on the bacterial community in the droughted cores, the effect of redox potential on a longer timescale may be visible in the differences between the community in B1 and the other cores. However, confirming this link would require monitoring of redox potential over a longer period.

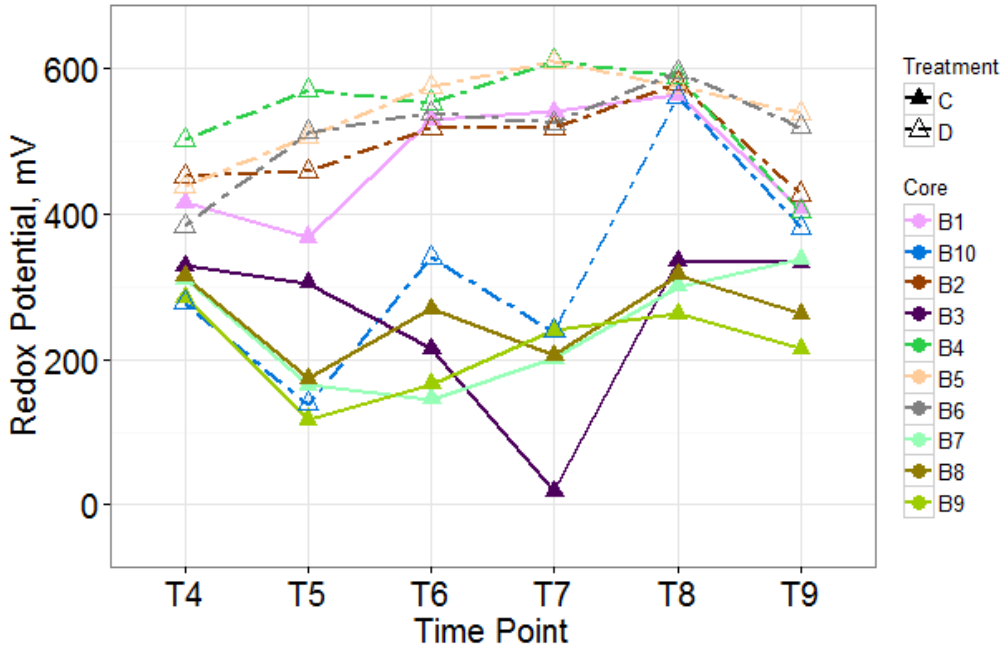

Figure 5.13: Redox potential (mV) within each bog mesocosm core at all time points where it was measured. Note that the redox potential was not measured until time point T4. For further discussion of redox potential, see Chapter 2. C= control, D = drought.

Ordination of all near-complete MAGs found that distribution patterns were related at least in part to redox potential: an NMDS plot depicting sites shows a gradient of approximately increasing redox potential when moving from high to low values on the first axis. Accordingly, visualisation of the MAGs themselves within the same ordination showed some clustering by lifestyle: the MAGs with the highest values on the first axis were the single methanogen (C239) and four MAGs with the potential for dissimilatory reduction of nitrate and sulfate (Figure

5.7B; Figure 5.7C). This suggests that populations possessing the potential to carry out anaerobic respiration may be selected for in environments with lower redox potentials, as would be expected. However, MAGs containing the potential for fermentation of pyruvate to lactate and acetate were less obviously clustered (Figure 5.7D; Figure 5.7E) and did not show clearly redox-driven patterns of distribution.

Metagenome-assembled genomes contained the genetic potential for a variety of lifestyles, including both anaerobic and aerobic respiration. The majority of non-droughted mesocosm cores (with the exception of B1) exhibited redox potentials which were suggestive of anoxic conditions (Figure 5.13), and so it is unsurprising that many MAGs possessed the ability for anaerobic metabolism. Seven MAGs were lacking the complete set of genes involved in the TCA cycle: of these, one was likely a methanogen (C239), while five contained pathways for fermentation (C100, C398, C207; Table 5.5) and/or anaerobic respiration (C100, C443, C303; Table 5.5). While the TCA cycle does not require oxygen, it generates three molecules of NADH for each molecule of pyruvate entering the cycle, and these must be re-oxidised to NAD^+ to allow glycolysis and the TCA cycle to continue. Therefore, the lack of a complete TCA cycle may in some cases suggest that MAGs represent organisms with obligately fermentative lifestyles (e.g. Wrighton *et al.* 2012). However, some anaerobic sulfate-reducing bacteria do contain a complete TCA cycle (Brandis-Heep *et al.* 1983), and so an incomplete pathway for the TCA cycle is a poor indicator of an anaerobic lifestyle in many cases. In addition, one of the MAGs which lacked a complete set of genes for the TCA cycle also contained no complete pathways for anaerobic metabolism (C129), suggesting that in this case the lack of genes for the TCA cycle may have resulted from incomplete binning or assembly. Conversely, several MAGs did not contain genes for the pathways of dissimilatory nitrate or sulfate reduction, denitrification or fermentation, and thus were potentially obligate aerobes (e.g. C330). Given the anoxic/microaerobic conditions which prevailed in non-droughted bog mesocosm cores (Chapter 2), it is possible that obligate aerobic members of the community persisted in a dormant state under anaerobic conditions (e.g. Lim *et al.* 1999) or that they inhabited aerobic microhabitats.

Many of the MAGs contained partial pathways for denitrification and dissimilatory nitrate reduction to ammonium (DNRA), but few contained full pathways for either. No MAG contained a complete pathway for denitrification, while four contained full pathways for DNRA (Table 2). DNRA is expected to be more important than denitrification under constant

anoxic conditions: DNRA is typically the preserve of obligate anaerobes, while denitrification occurs in aerobes under conditions of temporary oxygen limitation (Tiedje 1988). Therefore, if oxygen concentrations in the bog were consistently low, as would be expected, low redox potential may have selected for organisms with the potential for DNRA rather than denitrification. Alternatively, the dominance of DNRA organisms in the current study could be an artefact of incomplete annotation of genes involved in denitrification. Interestingly, all MAGs containing full dissimilatory nitrate reduction pathways were also sulfate reducers, suggesting that anaerobic micro-organisms in peat may switch between electron acceptors depending on the conditions.

It is unclear whether the large number of incomplete pathways for DNRA and denitrification was a result of missing annotations, or are genuinely a result of MAGs carrying genes for only certain steps in the nitrate reduction or denitrification processes. It is possible for organisms to contain incomplete pathways for denitrification: for example, it is common for denitrifiers to lack *nosZ* (Philippot *et al.* 2011) or to begin denitrification with nitrite rather than nitrate (Philippot *et al.* 2007), while some non-denitrifying bacteria possess functional *nosZ* without other genes in the denitrification pathway (Sanford *et al.* 2012). However, the proportion of MAGs containing incomplete pathways for denitrification is extremely high (13 of 27 near-complete MAGs), and thus may result from incomplete annotation or assembly. A large proportion of the genes for denitrification in peatland soils are only distantly related to known sequences (Palmer *et al.* 2012). Unexpectedly, many MAGs which contained the genetic potential for dissimilatory reduction of nitrite to ammonium did not contain nitrate reductases: nitrite is rapidly degraded in acidic soils and thus is unlikely to be a viable substrate for anaerobic respiration in peat bogs.

Iron reduction is another potentially important method of facultative anaerobic respiration, occurring at redox potentials between approximately +100 and -100 mV (McBride 1994). The importance of iron reduction in anaerobic peat ecosystems appears to be variable. One study found that iron reduction accounted for only a very small proportion of anaerobic carbon mineralisation across a gradient from bog to fen (Keller and Bridgham 2007), while other studies suggest that iron reduction may play an important role in fens (Knorr and Blodau 2009; Knorr *et al.* 2009) and permafrost (Lipson *et al.* 2010). In the current study, a number of matches were found to *omcB* and *omcS* (Table 5.3), two outer-membrane-bound cytochromes which are involved in oxidation of ferric iron in iron-reducing micro-organisms (Weber *et al.*

2006). This could suggest that some MAGs contain the genetic potential to reduce ferric iron. However, interpretation of these findings is further complicated by the fact that *omcB* also acts as an adhesin mediating host binding in intracellular parasites such as those within genus *Chlamydia* (Fadel and Eley 2007). Other enzymes involved in dissimilatory iron reduction, such as c-type cytochromes, also play roles in other redox reactions and so are similarly weak indicators that MAGs contain the genetic potential for the reduction of ferric iron. For example, *cymA* is involved in reduction of nitrate, iron(III), fumarate and manganese(IV) in *Shewanella* (Myers and Myers 1997). Therefore, we conclude that analysis of genetic potential for the reduction of ferric iron remains difficult, but future research into the genes underlying these pathways will make it easier to draw conclusions about ferric iron reduction from genetic data.

Five MAGs contained genes for all enzymes in the KEGG pathway for dissimilatory sulfate reduction/oxidation: *sat*, *aprAB* and *dsrAB*. Of these, C443 was a likely sulfate oxidiser (see next paragraph) while the remaining four MAGs (C100, C104, C303 and C344) were potential sulfate reducers. Taxonomically, two of the four sulfate reducers were assigned to Acidobacteria (C104, C344) and two to Nitrospirae (C100, C303). To date, sulfate reduction has not been described in any cultured Acidobacteria. However, a large number of *dsrAB* genes (a commonly used marker gene of dissimilatory sulfate reduction) in wetlands and other habitats are not related to the *dsrAB* genes in known sulfate reducers (Pester *et al.* 2012), and it is possible to speculate that some of the unknown diversity in *dsrAB* genes could belong to Acidobacteria. However, it should be noted that possessing the genetic potential for dissimilatory sulfate reduction does not prove that organisms carry out sulfate reduction. For example, a number of bacterial species possess *dsrAB* but are not capable of dissimilatory sulfate reduction (Pester *et al.* 2012). Therefore, further research would be required to explore the role of Acidobacteria in peatland sulfur cycling.

In addition to the five MAGs containing the *dsrAB* pathway, a single MAG (C443) was found to contain the genetic potential for sulphur oxidation through the *sox* operon (Table 5.4). Sulfur oxidation is potentially of great importance for sulfur cycling in wetlands: sulfate concentrations in wetlands are typically low, but sulfur oxidation in micro-niches with higher redox potentials can provide a constant supply of electron acceptors for sulfate reducers (Pester *et al.* 2012). C443 additionally contained full pathways for DNRA and dissimilatory sulfate reduction, and lacked two genes necessary for enzymes in the TCA cycle. Further clues to the lifestyle of C443 may come from the fact that it's closest relative in the PhyloPhlAn database

is *Thiobacillus denitrificans* (Figure 5.14), a facultatively anaerobic chemolithotroph which is able to couple sulfur oxidation to reduction of molecular oxygen, nitrate, nitrite or nitrous oxide (Ghosh and Dam 2009). Unlike *T. denitrificans*, C443 does not contain a complete denitrification pathway and instead contains genetic potential for DNRA, raising the possibility that C443 may potentially be able to link DNRA to sulfur oxidation. However, without isolation and characterisation of the organism in question, such suggestions remain speculative. While most MAGs contained the gene *soxD* (Table 5.4), the KEGG orthologue for this gene is also involved in apoptosis, meaning it is not a good indicator of sulfur oxidation.

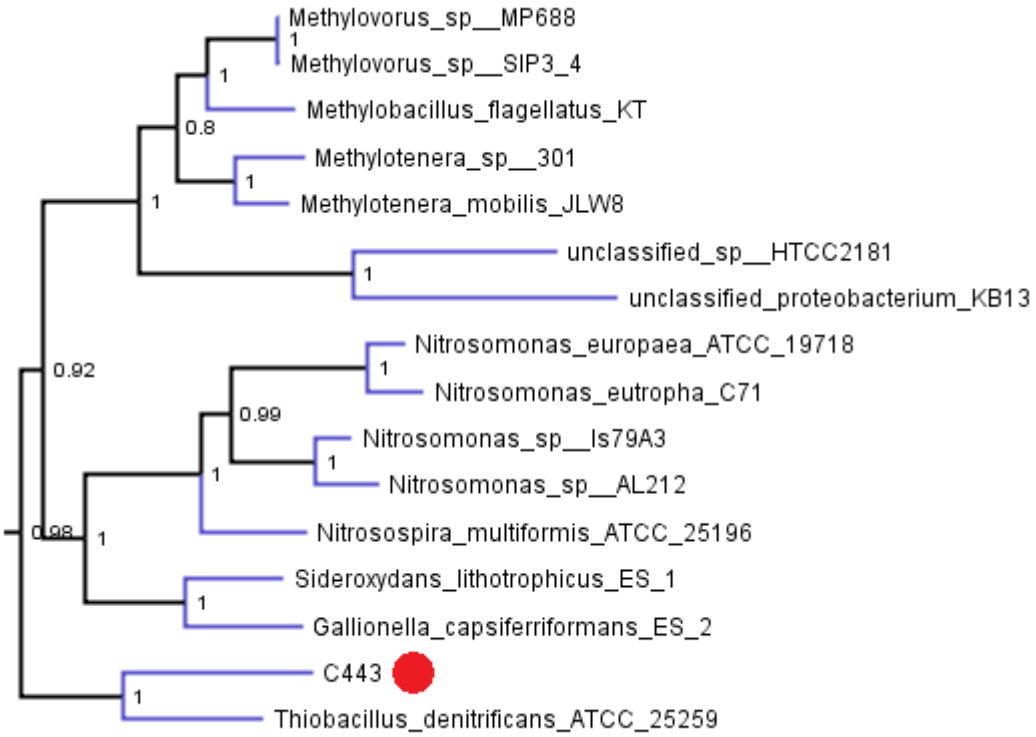

Figure 5.14: Subsection of full PhyloPhlAn phylogeny a section of Proteobacteria which includes C443 and Thiobacillus denitrificans. The phylogeny was based on the PhyloPhlAn database of genomes. Red dots depict near-complete MAGs.

One of the MAGs, C239, belonged to a putatively methanogenic Archaea. Of the genomes included in the PhyloPhlAn tree, C239 was most closely related to *Methanoregula boonei*: a hydrogenotrophic methanogen adapted to the oligotrophic, low pH conditions present in peat bogs (Bräuer *et al.* 2015). Likewise, C239 appeared to have the ability to carry out

hydrogenotrophic methanogenesis. It also contained *cdhC*, a gene for acetyl-CoA decarbonylase synthase (ACDS), which is a crucial enzyme in the pathway of acetoclastic methanogenesis (methanogenesis which uses acetate as a substrate). However, homologues of *cdhC* with non-methanogenic functions are widespread amongst anaerobic Bacteria and Archaea (Ferry 2010), and all known acetoclastic methanogens belong to genera *Methanosarcina* or *Methanosaeta* (Liu and Whitman 2008). Given the placement of C239 within genus *Methanoregula*, which contains hydrogenotrophic archaea, it is unlikely that it carries out acetoclastic methanogenesis. The assembly of a methanogen MAG from the dataset was unexpected: methanogenesis occurs at negative redox potentials (McBride 1994), while the redox potentials of bog mesocosm cores were typically positive (Figure 5.13; Chapter 2). However, methane production can occur in 'microniches' with especially low oxygen concentrations (Blodau and Moore 2003; Knorr and Blodau 2009; Askaer *et al.* 2010), meaning that overall redox potential of the peat may be a misleading measure of the potential for methanogenesis in a given environment. Alternatively, it is possible that disturbance during sampling allowed oxygen to access otherwise anoxic areas of the peat and led to misleadingly high measurements of redox potential. Mean methane fluxes from the bog mesocosm cores were much lower than those from the fen but were invariably positive (Chapter 2), suggesting that methanogenesis did occur in this habitat.

In addition to the putative nitrate- and sulfate-reducers described above, a large number of MAGs contained the functional potential for fermentation: sixteen of the 27 MAGs contained pathways for fermentation of pyruvate to lactate or acetate. Fermentation pathways were present in MAGs both with and without pathways of anaerobic respiration, and occurred in a wide variety of taxa: MAGs which contained genes for complete fermentation pathways belonged to phyla Nitrospirae, Acidobacteria, Verrucomicrobia, Bacteroidetes, Euryarchaota, Firmicutes, Proteobacteria (γ-class) and Chloroflexi (Table 5.1; Table 5.5). Fermentation is an important pathway of anaerobic carbon metabolism in peatlands (Vile *et al.* 2003; Keller and Bridgham 2007; Hamberger *et al.* 2008), and so a high prevalence of fermenters among assembled MAGs is what would be expected. Pathways for fermentation of pyruvate to acetate were most common among the MAGs. Whilst research into the partitioning of organic carbon between pathways of fermentation have been limited, acetate production is an important pathway in many peatlands (Hines *et al.* 2001; Hamberger *et al.* 2008; Galand *et al.* 2010) and

at low hydrogen pressure fermentation of glucose to acetate is the most efficient pathway of ATP production via fermentation (Schink 1997).

None of the assembled MAGs contained pathways for methanotrophy (methane oxidation). However, *pmoA* genes were detected within the unassembled shotgun sequencing data (Chapter 4), suggesting that methanotrophs were present in the environment but that individual mehanogenic species or strains were not abundant enough for contig assembly and binning. Methanotrophs decrease in abundance with decreasing pH and under anoxic conditions (Le Mer and Roger 2001). Therefore, bogs would be expected to represent a poor habitat for them, given the low pH and redox potential found in these habitats. However, a large proportion of the methane produced in bogs is oxidised before it is released (Freeman *et al.* 2002; Freitag *et al.* 2010), suggesting that many bogs nonetheless contain methanotrophic populations. Methane oxidation is typically limited to the aerobic layers of the peat (Krumholz *et al.* 1995; Freitag *et al.* 2010), while the redox potential of many of the current samples was slightly anoxic (Chapter 2). Therefore, assembly of the genomes of methane oxidisers would likely require sequencing of peat taken immediately below the surface, as oxygen does not typically penetrate more than a few centimetres into waterlogged peat (Askaer *et al.* 2010).

All near-complete MAGs possessed β-glucosidase genes, suggesting that they are potentially able to use cellobiose as a carbon or energy source, and all but four contained genes for exo- or endocellulase enzymes. Therefore, a much higher proportion of the community than would be expected appeared to contain the genetic potential for cellulolysis. A previous study which looked at 5,123 bacterial genomes found that 20% possessed no β-glucosidase genes and 56% possessed β-glucosidase genes but not exo- or endocellulase genes, with only 24% containing exo- and endocellulases as well as β-glucosidase (Berlemont and Martiny 2013). The widespread presence of genes for cellulose utilisation in peat-inhabiting bacteria likely results from the importance of plant-derived carbon to the peat ecosystem: the genomes surveyed by Berlemont & Martiny (2013) represented a variety of lifestyles, including parasitism and autotrophy, both of which were associated with low numbers of cellulose genes. Conversely, subsurface peatland microbial communities are primarily based around the degradation of plant material, of which cellulose makes up a significant proportion. The proportion of genomes with the potential for cellulose degradation was nonetheless higher than expected. In the reindeer rumen, a habitat which is likewise dependent on plant material, the proportion of cultivable bacteria able to survive on cellulose is only 15-35% (Orpin *et al.* 1985), while in agricultural

soils only 17-40% of bacteria can degrade soluble cellulose (Ulrich and Wirth 1999). However, the peat microbiome is highly diverse and the MAGs presented in the current study represent only a small proportion of the community. Therefore, the current results do not necessarily contradict previous studies which found a much smaller proportion of the community to be cellulolytic. While most of the dominant members (i.e. those which were present at high enough abundances to be assembled) contained the functional potential for cellulolysis, it is possible that many of the unassembled community members could lack the potential for complete cellulose degradation. It should also be noted that micro-organisms containing the apparent genetic potential for cellulolytic activity will not necessarily exhibit cellulolytic activity in the environment. Some of the predicted genes may represent pseudogenes, some may only be expressed under particular conditions, and some may be involved in the degradation of polymeric compounds other than cellulose. A small percentage of genes contained within each GH family classified as containing cellulases are in fact involved in other processes (Berlemont and Martiny 2013).

The majority of phenol oxidase genes detected within MAGs were laccases, and were predicted to be located within the periplasm of the cell (Table 5.7) by Gneg-mPLoc (Shen and Chou 2010). The degradation of complex substrates in soil is typically attributed to extracellular enzymes (Sinsabaugh 2010), but genes for extracellular phenol oxidases were not detected in any of the MAGs analysed (Table 5.7). The role of periplasmic enzymes in the degradation of phenolic compounds remains unclear. Laccases from the periplasm may be involved in the degradation of environmental aromatic compounds (Ahmad *et al.* 1997; Rosconi *et al.* 2005) and small phenolic compounds are able to traverse the outer membrane, potentially exposing them to periplasmic enzymes (Sikkema *et al.* 1995). The periplasmic location of these enzymes may prevent competition for degradation products with 'cheaters', who use the products of degradation without themselves producing extracellular enzymes (Allison 2005). However, the primary purpose of many periplasmic phenol oxidases may be to protect cells from damage, for example by preventing the build-up of high levels of copper (Grass and Rensing 2001) or phenol-mediated damage to the cytoplasmic membrane (Sikkema *et al.* 1995).

Numerous MAGs possessed both phenol oxidase genes and genes for anaerobic respiration or fermentation (Figure 5.8). Since phenol oxidase genes require oxygen to function, this suggests that these organisms are either facultative anaerobes or are adapted to survive periods of oxygenation. Although a number of bacteria are able to degrade phenolic compounds and other

aromatic compounds under anaerobic conditions, anaerobic degradation of aromatics in sulfate reducers and fermenters proceeds via reductive attacks involving dehydrogenation or carboxylation, rather than oxidation (Schink *et al.* 2000). Phenol oxidases fulfil a number of roles in facultative anaerobes: for example, they can provide protection from oxygen intermediates and free radicals under oxic conditions (Shivprasad and Page 1989). In addition, phenol oxidases are involved in the production of melanin which acts as an electron shuttle between insoluble Fe(III) and dissimilatory metal-reducing bacteria in genus *Shewanella*, although melanin production only occurs during aerobic growth (Turick *et al.* 2002).

5.4.3 Functional Potential of Microorganisms with Assembled Genomes

Non-metric multidimensional scaling based on counts of functional genes produced groupings of MAGs which roughly corresponded to phylogeny (Figure 5.9), and the association between phylogeny and function was confirmed by PERMANOVA. A similar relationship between phylogeny and function is seen for MAGs assembled from a brackish metagenome (Hugerth *et al.* 2015) despite the very different phylogenetic composition of the MAGs assembled from the brackish habitat. Phylogenetic clustering of bacterial function has also been observed using other methodologies: for example, the response to labelled substrates is often phylogenetically conserved at phylum level (Goldfarb *et al.* 2011; Morrissey *et al.* 2016), although many traits are conserved only at lower taxonomic levels (Martiny *et al.* 2015).

Glycoside hydrolases were present at greater abundances in Bacteroidetes than other phyla, a finding that was also observed in brackish habitats (Hugerth *et al.* 2015) and river sediments (Baker *et al.* 2014), suggesting that Bacteroidetes may play an important role in carbohydrate degradation across a variety of habitats. Three genes within the *che* operon were significantly more abundant in Acidobacteria and Proteobacteria than Bacteroidetes. Each of these genes (*cheW*, *cheB* and *cheY*) is typically involved in controlling the response of bacterial flagellar movement to environmental stimuli. However, the *cheY* receiver domain is also a common regulatory protein for other genes (Hamer *et al.* 2010), and so is not necessarily linked to chemotaxis. Rather than indicating a lack of motility in Bacteroidetes within peat, this may indicate that the Bacteroidetes use different methods of motility, with different methods of regulation, than do Acidobacteria and Proteobacteria: for example, 'gliding' motility has been well studied within the Bacteroidetes, although it is also present in the Proteobacteria (Shrout 2015). However, homologues of the *che* genes, the *frz* genes, are present in some gliding

bacteria (Zusman *et al.* 2007). Copy numbers of *cheY* are commonly higher than those of other proteins in the *che* system (Hamer *et al.* 2010), and this was also the case in the current study: more copies of *cheY* (K03413) were detected than of *cheB* (K03412) or *cheW* (K03408; Figure 5.10). The three most abundant phyla also differed in the abundances of several transport proteins. For example, genes involved in the LIV-I transport system of branched-chain amino acids were significantly more abundant in the Proteobacteria than other phyla (Table 5.8). A peptide/nickel ABC transporter (K02035**)** was more abundant in Acidobacteria than Bacteroidetes, although was similarly abundant in both Proteobacteria and Acidobacteria (Figure 5.10). At least two of the KEGG ortholog groups found to differ between phyla corresponded to functions not found in bacteria: K10385 is described involved in the eukaryotic cytoskeleton, while K09607 is an immune inhibitor (Table 5.8). Therefore, both of these annotations are likely incorrect, or alternatively may represent distant homologues of eukaryotic genes that have not yet been catalogued in prokaryotes.

5.4.4 Effect of Drought on MAGs

Non-metric multidimensional scaling showed that the core from which samples were taken had a much greater effect on community composition than did drought; and there was no significant effect of the sampling time point or treatment on overall community composition (Section 5.3.2). The lack of an overall community response to drought echoes similar results from marker gene analysis (Chapter 3) and analysis of unassembled metagenomics reads (Chapter 4), and may be a result of dead or dormant microbial cells or of microbial resistance to drought. A recent preprint documented high levels of extracellular 'relic' DNA in soils, with the highest amounts of relic DNA found in soils that had a low pH and low concentrations of base cations (Carini *et al.* 2016). Both characteristics describe bog peat: peat slurry samples in the current experiment had mean pH values of 4-5 (Chapter 2) and while base cations were not measured directly, the concentration of extractable bases in bogs is typically low (Bridgham *et al.* 2000; Wang *et al.* 2015). Therefore, it is possible that peat taken from bogs contains high levels of relic DNA, obscuring the effects of drought. In addition, many bacteria may become dormant in response to unfavourable conditions, again leading to a weak drought response (Jones and Lennon 2010). To distinguish the effects of dormancy and relic DNA from the genuine community resistance to change, future studies should combine DNA-based analyses with

analysis of community RNA, which is less stable than DNA and therefore has a more rapid turnover time (Moran *et al.* 2013).

While a number of MAGs showed a significant response to drought, none were significant following the application of corrections for multiple corrections. Furthermore, very few MAGs showed consistent responses to treatment and time across all cores (Figure 5.11). The two MAGs showing the most consistent response to time point and treatment were C150 (Figure 5.11F) and C77 (Figure 5.11N). Both MAGs showed a similar response to time point as well as treatment across all mesocosm cores, suggesting that the effects observed were not due simply to differences between cores. The mean coverage of C150 decreased in control cores between the first two sampling time points but increased in droughted cores relative to control cores at time points T6 and T9. Conversely, the coverage of C77 rose with time in all control cores, but remained low in droughted cores. Both of these MAGs were incomplete, containing no SCGs (Table 5.9). The incomplete nature of both MAGs makes it difficult to draw conclusions about the functional potential of the populations to which they belong. C150 contained a gene for β-glucosidase, an enzyme which typically increases in activity during drought (e.g.Fenner and Freeman 2011), although this was not observed in the current study (Chapter 2). However, the overall effect of drought on the abundance of MAGs was weak or non-existent, and in most cases significant effects of the interaction between time point and treatment appeared to be caused by random variations between cores and time points.

5.5 Conclusions

1. Twenty-seven metagenome-assembled genomes (MAGs) were produced. Amongst these MAGs, the most abundant phyla were Acidobacteria (12 MAGs), Bacteroidetes (5 MAGs) and Proteobacteria (4 MAGs).

2. The MAGs contained the functional potential for a wide variety of lifestyles, spanning putatively obligate anaerobes, facultative anaerobes and obligate aerobes. Inference of metabolic pathways based on the KEGG mapper suggested that certain MAGs had the potential for dissimilatory nitrate and sulfate reduction, as well as fermentation of pyruvate to acetate and/or lactate. Analysis indicated that a single MAG was a putative chemolithotrophic sulfur oxidiser, while another was a methanogen related to *Methanoregula boonei*.

3. Phylogenetic patterns were observed in the functional potential of MAGs, similar to the pattern observed in brackish environments (Hugerth *et al.* 2015). In particular, Bacteroidetes appeared to contain genetic potential for the hydrolysis of polymeric carbohydrates.

4. The effect of drought on the coverage of MAGs was negligible, while the mesocosm core from which samples were taken had a strong effect on community composition.

5.4 References

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Chapter 6

General Discussion

6.1 Introduction

The application of nucleic acid-based methodologies has unveiled an extremely high level of diversity within soil microbial communities (Torsvik *et al.* 1990; Roesch *et al.* 2007), and led to increased interest in the role that underground microbial communities play in ecosystem processes/service delivery and in the effect of the environment on these communities. The effect of anthropogenic pressures, such as climate change, on ecosystem processes and microbial communities within the soil may be of particular concern. For example, there is potential for a positive feedback loop to be generated as warming and precipitation changes lead to an increase in soil respiration and CO_2 fluxes (Rustad *et al.* 2001). Alternatively, the effect of warming on net carbon fluxes may be countered by an increase in plant primary production driven by a rise in soil nitrogen mineralisation by micro-organisms (Melillo *et al.* 2002).

Environmental change may affect ecosystem processes either directly or indirectly via effects on soil microbial communities (Figure 1). In many cases it is difficult to separate direct effects of the environment (top-down effects) from microbially-mediated (bottom-up) changes, but several approaches have been used to demonstrate clearly that microbial communities are important in both normal soil functioning and in determining the response of soil function to environmental change. For example, Allison *et al.* (2013) used a reciprocal transplant experiment to demonstrate that microbial communities from nitrogen-enriched plots exhibited higher decomposition rates in nitrogen-enriched plots than did communities from control plots; conversely, microbial communities from droughted plots showed lower decomposition rates than those from control plots regardless of the conditions. Similarly, Matulich & Martiny (2014) exposed leaf litter mesocosms containing randomly-specified inoculums to changes in moisture, nitrogen and temperature, and observed that changes in respiration rate with environmental change were related to both the inoculum and to changes in the community which occurred over time. Thus, it is clear that in many cases the microbial community has the potential to strongly influence carbon cycling in soils. Conversely, there is evidence that in some cases, functional redundancy occurs in soil microbial communities (e.g. Rousk *et al.* 2009; Andert *et al.* 2012), weakening the interactions between environmental change, microbial community composition and soil functioning. Additionally, there is evidence that some functions may be affected by the environment but not by the microbial community: for example, the activity of β-glucosidase and phosphatase enzymes differed between peat types

but not between different inocula in a reciprocal transplant experiment (Dimitriu *et al.* 2010). Therefore, it is crucial that we understand the roles played by micro-organisms in the functioning of soil communities and the responses they will have to environmental change in order to predict and plan for the effects of climate change.

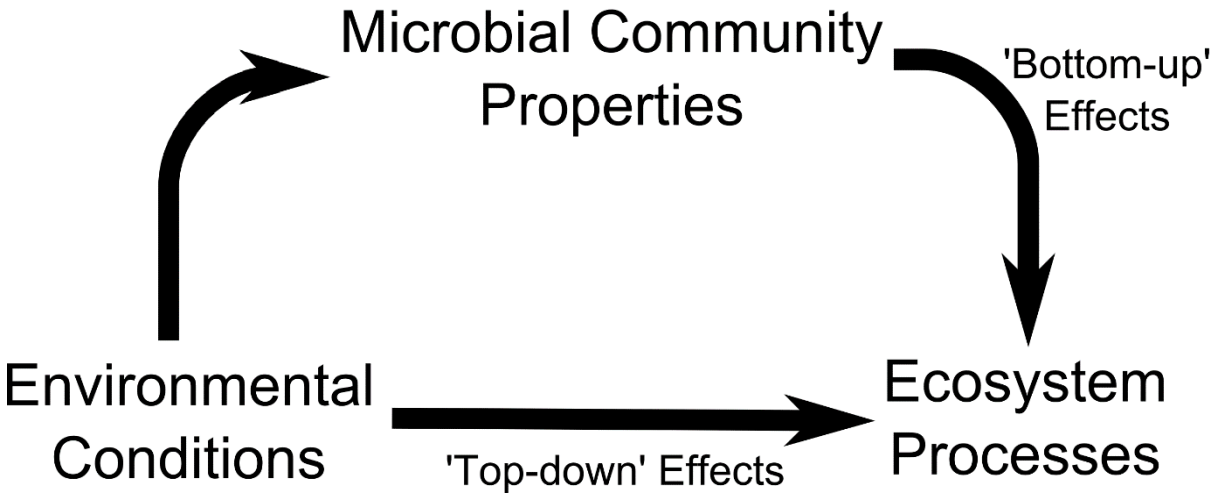

Figure 6.1: Diagram depicting direct and microbially-mediated effects of environmental change on ecosystem processes.

In peatlands, numerous links have already been demonstrated between anthropogenic environmental change and ecosystem processes. For example, peatland carbon fluxes are affected by warming (Silvola *et al.* 1996; Fenner *et al.* 2006; Kim *et al.* 2012), elevated carbon dioxide concentrations (Fenner *et al.* 2007; Ellis *et al.* 2009b) and water table (Silvola *et al.* 1996; Fenner and Freeman 2011). Interactions between different types of environmental change may additionally affect the response: for example, warming and elevated CO_2 concentrations have a synergistic effect on dissolved organic carbon exports (Fenner *et al.* 2007). Conversely, over longer time scales the effects of environmental change may cancel themselves out: for example, long-term water table drawdown in peatlands leads to an expansion of shrub growth, and the recalcitrant nature of shrub litter slows the decomposition rate (Wang *et al.* 2015). Each of these changes also has an effect on microbial communities (Mitchell *et al.* 2003; Kim *et al.* 2008; 2012; Wang *et al.* 2015), and given the known links between microbial communities and ecosystem processes it is reasonable to assume that microbial communities would play a key role in determining the effect of environmental

change on carbon fluxes and other response variables. However, a number of gaps remain in our knowledge of peatland microbial communities and the way they will respond to environmental change.

Drought-driven carbon dioxide release has been the focus of a large number of studies in recent decades (e.g. Freeman *et al.* 1993a; 1993b; 2001; Ellis *et al.* 2009a; Fenner and Freeman 2011; Romanowicz *et al*. 2015). However, the microbial mechanisms underlying this process remain poorly understood. More generally, the composition of microbial communities in peatlands is also of interest: peatlands contain a large proportion of global soil carbon stores (Gorham 1991) and the unusual conditions within the peat environment (including anaerobic conditions, highly recalcitrant organic matter, and often low pH values) may also yield novel and exciting diversity. As described in the introductory chapter, the overall aims of the current thesis were to use second-generation sequencing methodologies to expand on current knowledge of the taxonomy and function of the communities inhabiting peatland ecosystems, and to identify drought-driven changes in the taxonomic composition and functional potential of microbial communities in peatland soils. To reach these goals both marker gene analysis (MGA; Chapter 3) and metagenomic sequencing (Chapter 4; Chapter 5) were employed, alongside ARISA fingerprinting and biogeochemical assays (Chapter 2). The results obtained have provided insights into peatland microbial community composition and the effect of drought upon it, and generated numerous hypotheses to be tested in future research.

6.2 The taxonomic and functional composition of microbial communities in peatlands

Recently, several studies have emerged which use second-generation sequencing to characterise microbial communities in peat soils: Lin *et al.* (2012; 2014b) previously presented marker gene analyses of both bacterial and fungal communities at a variety of depths in bogs and fens, while Nunes *et al*. (2015) carried out marker gene analysis of the prokaryotic community in a French fen at 50cm depth. Lin *et al*. (2014a) additionally carried out shotgun sequencing of the peat profile in a North American bog. All MGA- and metagenomics-based studies to date indicate that peatland bacterial communities contain a high proportion of Acidobacteria and Proteobacteria, and in the current study both were highly abundant in MGA data (Chapter 3). In the bog at 5cm, Acidobacteria made up just over a third of reads in the MGA dataset but represented a much smaller proportion of metagenomic annotations when the

SILVA database was used as a reference (12%; Chapter 4). However, when assembled full-length 16S rRNA genes were used as a custom reference database against which metagenomic reads were annotated, the proportion of Acidobacteria was similar to that in the MGA dataset. Acidobacteria additionally dominated metagenome-assembled genomes (MAGs; Chapter 5), suggesting that the relatively low proportion of Acidobacteria in SILVA annotations was a consequence of the limited number of sequences from this phyla which are contained in sequence databases.

However, in other instances the results of the current study differ from those of Lin *et al.* (2012; 2014a; 2014b). For example, Lin *et al.* (2012; 2014a; 2014b) found much higher abundances of Verrucomicrobia than the current study, while a much higher proportion of OTUs and metagenomic reads in the current study remained taxonomically unassigned. The analysis in the current study was designed to avoid false positives, with a stringent percentage identity cut-off for annotations of SSU rRNA genes from shotgun sequencing reads (97% rather than 60%, the default) and the use of utax with a confidence threshold of 0.85 for the taxonomic assignment of OTUs in the marker gene datasets. However, these stringent thresholds likely prevented the annotation of many reads and OTUs, particularly those originating from poorly studied taxa. The high proportion of unassigned reads/OTUs in the current study therefore reflects the high proportion of microbial diversity which remains uncatalogued, as well as highlighting a trade-off between accuracy and completeness when annotating microbial sequence data. Within the MGA datasets, the proportion of unassigned reads was especially high for eukaryotes at 20cm depth: microbial eukaryote taxa likely contain a large amount of unknown diversity (Pawlowski *et al.* 2012; del Campo *et al.* 2016), and this may be especially true in anoxic habitats (Richards and Bass 2005). However, it is also possible that a number of the unassigned reads were a result of undetected sequencing errors or chimeric sequences.

Community composition was strongly affected by habitat and depth in both the amplicon sequencing and ARISA fingerprinting data (shotgun sequencing was limited to one habitat and one depth). Differences between bogs and fens and between different depths along the peat profile have similarly been observed in previous studies (Lin *et al.* 2012; 2014b). Additionally, the community composition within each habitat varied between different mesocosm cores, with differences between cores making up a significant proportion of community variation. However, differences between different mesocosm cores were strongly related to differences in vegetation and biogeochemical variables between cores (Chapter 3; Chapter 4).

Relationships between the community composition of plant and microbial communities in soils are well documented, both in peats (Jassey *et al.* 2014; Lin *et al.* 2014b) and in other soils (e.g. Bonito *et al.* 2014).

Although low phenol oxidase activity is thought to be one of the main causes of low rates of decomposition in peatlands (Freeman *et al.* 2001), the current study appears to suggest that a number of the micro-organisms present in peatlands may possess phenol oxidase genes. Predicted phenol oxidase genes were fairly widely distributed amongst metagenome-assembled genomes (MAGs) and numerous dioxygenase, copper oxidase and peroxidase domains were detected in unassembled shotgun sequencing data. A previous metagenomic analysis of bog microbial communities at a similar depth below the surface likewise found abundant copper oxidase and dioxygenase domains, with roughly 0.16 and 0.12 copies respectively of each gene for each copy of *rpoB*, a conserved single-copy gene (Lin *et al.* 2014a). However, these domains have a broad range of functions (Passardi *et al.* 2005), and so the current study used more specific hidden Markov models to additionally search for fungal laccases and for two fungal peroxidases commonly implicated in the degradation of lignin, a complex polyphenolic molecule (lignin and manganese peroxidases). Genes for lignin and manganese peroxidases were barely detected, suggesting that these enzymes are rarely present in peatlands. Low copy numbers of these enzymes may have important consequences for peat decomposition, as peroxidases are key for the degradation of lignin (which in peatlands may originate from grasses or dwarf shrubs, and potentially from *Sphagnum* moss in small quantities (Bland *et al.* 1968)) and the release of sugars and amino acids from humus encapsulation (Tian and Shi 2014). Laccases from both basidiomycetes and ascomycetes were more abundant than fungal peroxidases, but still made up only a small proportion of copper oxidases. Additionally, the presence of genes for a given function does not necessarily mean that these genes will be active in the environment, as enzyme activity requires transcription, translation, and appropriate conditions for enzyme activity: in particular, low redox potential and lack of oxygen in saturated peat would inhibit phenol oxidase activity.

Shotgun sequencing detected diverse and abundant potential for anaerobic metabolism in bacterial communities in the bog. Fermentation appeared to be the most widespread pathway: genes for complete pathways of fermentation were present in at least sixteen of the twenty-six near-complete MAGs, including both obligate and facultative anaerobes. Amongst unassembled shotgun metagenomic sequencing reads, *hydA* (a gene for an enzyme which

carries out the hydrogen-producing step in fermentation) was abundant in both the current study and that of Lin et al. (2014a). Earlier studies of carbon metabolism in peat likewise suggest that fermentation is an important pathway in peatlands (Hines *et al.* 2001; Hamberger *et al.* 2008; Galand *et al.* 2010). Amongst unassembled metagenomic reads, genes for denitrification (*nirK* and *nosZ*) were slightly more abundant than the sulfate reduction gene *dsrA*, corresponding to the results of Lin *et al.* (2014a). Conversely, no MAG contained a complete pathway for denitrification while several contained pathways for dissimilatory sulfate reduction and dissimilatory nitrate reduction to ammonia (DNRA). Therefore, the presence of dissimilatory sulfate reduction and DNRA, but not denitrification, in MAGs may suggest that the genome assembly and binning process is skewed towards certain organisms, and that the MAGs are not necessarily representative of the community as a whole. In particular, it is possible that the genetic material of anaerobic organisms was most likely to be assembled into MAGs: denitrification is limited to facultative anaerobes, while DNRA is found in obligate anaerobes (Tiedje 1988). Reasons for this skew are unclear, but may suggest that individual populations/strains of aerobes present in the bog are rarer and thus less likely to be assembled. Interestingly, two of the MAGs assigned to Acidobacteria contained complete predicted pathways for dissimilatory sulfate reduction. As yet, Acidobacteria are not associated with dissimilatory sulfate reduction, and so may account for a proportion of wetland *dsrA* genes which cannot be assigned taxonomically. However, this conclusion cannot be proven based on MAGs alone.

6.3 The effect of drought on microbial communities in peat soils

Climate change is predicted to alter precipitation regimes, leading to increased potential for droughts (Douville *et al.* 2002; Bates 2008). In turn, drought has a strong impact on greenhouse gas emissions from peatlands (e.g. Freeman *et al.* 1993b; Fenner and Freeman 2011). In particular, drought increases the release of carbon dioxide from peatlands, an effect which is mediated by increased degradation of phenolic compounds (Freeman *et al.* 2001). Prior to the current work, drought had been shown to increase the diversity of genes for a particular phenolic-degrading enzyme, catechol 2,3-dioxygenase, in a Welsh gully mire (Fenner *et al.* 2005); to decrease the abundance of 16S rRNA genes in both bogs and fens (Kim *et al.* 2008); and to have a negative impact on the abundance of *nirS*, a marker of denitrifying micro-organisms (Kim *et al.* 2008). Conversely, drought has been found to increase bacterial growth rate and cell numbers (Fenner and Freeman 2011). Similarly, it had been demonstrated that

long-term drying of peatlands impacts bacterial and fungal communities in peatlands (Peltoniemi *et al.* 2012; 2015), and that fluctuating water tables lead to an altered bacterial community 50cm below the peat surface (Nunes *et al.* 2015).

In the current study, automated ribosomal intergenic spacer analysis (ARISA) demonstrated a significant effect of drought on bacterial and fungal community composition, with the most significant effect during the rewetting period. Although Kim *et al*. (2008) did not detect a significant effect of drought on bacterial community composition, the longer duration of the current experiment and the inclusion of a rewetting period made it possible to detect this change using ARISA. However, despite the fact that ARISA fingerprinting detected community changes, marker gene analysis did not find a significant change in overall community composition during drought while shotgun sequencing revealed only a very weak overall effect of drought on community composition. There are several possible explanations for this discrepancy. Firstly, many of the amplicon sizes measured by ARISA fingerprinting are considerably larger than those which were sequenced (ARISA amplicon sizes ranged from 165-1,580bp, while marker gene analysis was carried out on amplicons 300-350bp long and metagenomic sequencing was carried out on fragments of 300-500bp). In freshwater lakes, seasonal community changes are more rapidly detected by analysing large amplicons than smaller amplicons from environmental DNA (Bista et al., in revision), likely because the process of degradation is less likely to leave large amplicons intact over a period of time. Environmental DNA (eDNA) is trace DNA extracted from environmental samples without isolating organisms first. Therefore, it is possible that in the current study the smaller size of the amplicons prepared for sequencing made it more difficult to detect changes over short time scales. Secondly, ARISA fingerprinting represented only a small amount of diversity when compared with the sequencing dataset and so it is possible that several drought-responsive taxa happened to be picked up within the ARISA fingerprinting dataset, leading to an overall effect of drought. However, this would be highly improbable given that amplicon sequencing suggested only a small proportion of the community responded to drought (although it should be noted that the primers chosen for amplicon sequencing will not have been able to amplify DNA from all organisms present).

Despite the lack of an overall community response in sequencing data, a subset of OTUs in the marker gene dataset responded significantly to drought, particularly in the fen at 5cm. Very few OTUs responded to drought in the bog habitat and at 20cm depth, and so no significant

effect of drought was found on the overall taxonomic or functional composition in the metagenomes. The weaker response of the bog community to drought could indicate that peat bogs contain a more drought-resistant community than fens, or could be caused by increased persistence of 'relic' DNA in the bog caused by low pH and low concentration of base cations in this habitat (Carini *et al.* 2016). Redox potential in the bog was typically higher than that in the fen, potentially leading to a community better able to adapt to more oxidised conditions caused by drought: however, analysis of metagenome-assembled genomes (MAGs) suggested that the bog contained a number of bacterial taxa which were potential obligate anaerobes.

A number of patterns can be discerned amongst the OTUs found to respond to drought in the fen at 5cm. Both Bacteroidetes and Proteobacteria made up a higher proportion of drought-affected OTUs than of the community as a whole, with Bacteroidetes typically responding negatively and Proteobacteria responding positively. Notably, both the Bacteroidetes and α-Proteobacteria have previously been identified as phyla containing a low proportion of dormant members in the environment (Jones and Lennon 2010), potentially making these phyla especially responsive to environmental changes. Where it was possible to assign detailed taxonomy to the drought affected OTUs, negatively drought-affected OTUs were typically related to obligate anaerobes: for example, one was assigned to genus *Geobacter*, a genus of Fe^{3+} reducers, and another to *Paludibacter*, of which the only known member is an obligately anaerobic fermenter. Ecological patterns amongst positively drought-affected OTUs were more difficult to determine, although several appeared to be related to taxa that respond positively to the contamination of soil by hydrocarbons or the addition of labile substrates (discussed fully in Chapter 3). When the confidence values for the taxonomic assignments were ignored, four of seven drought-affected eukaryotic OTUs belonged to the Rhizaria. Together with a significant rise in the proportion of the 18S rRNA gene dataset made up of Rhizaria, this appeared to suggest a potential response of Rhizaria to drought. Rhizaria exhibit a range of lifestyles, including mixotrophy, bacterial grazing and predation of other protists (Burki and Keeling 2014; Gomaa *et al.* 2014). Therefore, Rhizaria may influence carbon cycling in several ways: e.g. by regulating bacterial communities via grazing (Glücksman *et al.* 2010), or by a community shift from autotrophic species to heterotrophs (Jassey *et al.* 2015). Although few OTUs were significantly affected by drought once corrections for multiple comparisons were applied, the patterns observed in the taxonomy and predicted function of many of these

organisms suggest a real effect. Therefore, the current thesis provides a set of novel hypotheses for future research into the effect of environmental change on peatland microbial communities.

Within the shotgun sequencing data, there was a significant fall in the abundance of *hydA*, a gene involved in the hydrogen-generating step of fermentation, during drought. In addition, several of the negatively drought-affected OTUs were affiliated with taxa containing fermentative members such as *Paludibacter* and *Pacearchaeota*. However, the weak taxonomic resolution with which the majority of drought-affected OTUs were assigned makes it impossible to draw firm conclusions about the functional potential of these OTUs. Together, these results may suggest that drought reduces the functional potential of the community for fermentation. There was additionally an effect of drought on the number of copies of the ribosomal protein gene *S12p*, which was less abundant in droughted than control mesocosm cores at time point 4. A decrease in the relative abundance of ribosomal protein genes could potentially be an indication of an increase in genome size. However, further evidence would be required to confirm this link.

6.4 Future Research Directions

Effect of drought on the 'active' community

While several studies have found a significant effect of drought using DNA-based methodologies, the effect of drought on the 'active' community (based on analysis of RNA rather than DNA) is stronger (Barnard *et al.* 2013; 2015). This is potentially a result of both microbial dormancy (Jones and Lennon 2010) and extracellular ('relic') DNA (Carini *et al.* 2016): dormant and inactive micro-organisms and relic DNA make up a significant proportion of diversity in microbial communities (Jones and Lennon 2010; Dlott *et al.* 2015; Carini *et al.* 2016). While previous research suggests that the majority of extracellular DNA is degraded within 30 days (Morrissey *et al.* 2015), it can be protected by binding to clay minerals (Morrissey *et al.* 2015) or humic substances (Crecchio and Stotzky 1998). Many of the characteristics of peat soils may lead to especially high levels of extracellular DNA: its high organic matter content leads to high cation exchange capacity (Bridgham *et al.* 2000), which in bogs is accompanied by low pH and low concentrations of exchangeable cations. Each of these three factors is correlated to the quantity of extracellular 'relic' DNA (Carini *et al.* 2016). The study of RNA rather than DNA can avoid many of these problems, as RNA is less stable than DNA (and thus more rapidly broken down) and can rapidly be produced in response to

environmental change (Moran *et al.* 2013). In addition to RNA-based analyses, techniques such as the degradation of extracellular DNA using propidium monoazide (PMA; Carini *et al.* 2016) or stable isotope probing (SIP) with heavy water to detect active taxa during the rewetting period (Aanderud *et al.* 2015) could be applied.

Effect of drought on protists

In the current study, Rhizaria (a phylum of protozoa) responded significantly to drought in the fen at 5cm. Protozoan abundance increases with water content, and the size of the response is taxon-dependent (Stefan *et al.* 2014). However, one study found that after one year, differences in depth to water table did not have a detectable impact on the density of testate amoeba in a *Sphagnum*-dominated peatland (Marcisz *et al.* 2014). The weak relationship between water table and protist density in peatlands may be because the water content in pristine peatlands is typically higher than that tested by Stefan *et al*. (2014): the effect of water content on protists is not linear and becomes weaker with increasing water content (Stefan *et al.* 2014). However, the community composition of testate amoebae in peatlands is affected by both long-term changes in water table (Marcisz *et al.* 2014) and warming (Jassey *et al.* 2015). To date, the majority of studies focusing on peatland protists have been based on morphological identification, enabling them to be easily categorised into functional groups such as large and small testate amoeba. However, functional group categorisation of protists based on MGA is more challenging, as only a small proportion of protistan diversity has been catalogued and databases of protist sequences remain incomplete (Pawlowski *et al.* 2012). Many protist functional and ecological characteristics are polyphyletic, making it even more difficult to draw meaningful conclusions about their functional potential from sequence data. However, protists can have significant impacts on carbon cycling both directly (Crotty *et al.* 2013; Jassey *et al.* 2015) and indirectly, via their impacts on bacterial communities (Glücksman *et al.* 2010). Therefore, a fuller understanding of the effects of short-term drought on protist communities and activities would be extremely valuable.

Confirmation of the effect of drought on drought-affected OTUs

The marker gene analysis presented in Chapter 3 identified a number of OTUs which appeared to respond to drought in the fen at 5cm. However, the relatively small sample size used in the experiment greatly reduced the power of this part of the study (only six mesocosm cores from each habitat were able to be included in MGA, although samples taken at nine sampling time

points from these cores were analysed). In addition, the current results only represent a single site for each habitat, but it is possible that the microbial mechanisms underlying drought-driven carbon release differ between peatlands. Therefore, the current study should be considered as a hypothesis-generating exercise rather than as a confirmation of the response of these OTUs to drought.

Effect of drought on fermentation

In the current study, the relative abundance of *hydA* (a key gene in hydrogenogenic fermentation) in shotgun sequencing data was lower in droughted than control mesocosm cores at the last time point (Chapter 4). Additionally, a number of negatively drought-affected OTUs in the fen habitat were related to fermentative bacteria or archaea (Chapter 3). Since fermentation is an anaerobic process, a negative effect of drought on fermentation would be expected, although this has not been studied to date. Fermentation is an important pathway of carbon metabolism in peatlands (Vile *et al.* 2003; Keller and Bridgham 2007; Hamberger *et al.* 2008), and provides substrates for both methanogens and sulfate reducers (Muyzer and Stams 2008; Drake *et al.* 2009). Therefore, a reduction in functional genes for fermentation within the microbial community could potentially have a negative effect on anaerobic carbon cycling which outlast the effect of drought; however, while CH_4 emissions are supressed following drought (Freeman *et al.* 1993b; Dowrick *et al.* 2006), CO_2 emissions often remain elevated for a significant period following rewetting (Fenner and Freeman 2011). Therefore, the effect of drought on fermentation rates, both during water table drawdown and rewetting, merits further research.

General advances in linking genetic data to taxonomy and function

It is common for a large proportion of metagenomic reads to be unable to be assigned to either functional or taxonomic categories, particularly from soil environments (e.g. Delmont *et al.* 2012; Lin *et al.* 2014a), and this was the case in the current study also. In addition, the use of utax, which assesses the accuracy of each taxonomic assignment based on the length and region of the reads, meant that a large proportion of taxonomic assignments of the 16S and 18S rRNA genes were recognised as poor quality. In part, the low proportion of the community able to be assigned is a result of the large proportion of microbial diversity which remains to be classified: for example, Locey and Lennon (2016) estimated that there are a total of 1 trillion (10^{12}) microbial species globally. To compare, only ~10^4 species have been cultured, while sequences

belonging to ~10^5 species have been classified and catalogued (Locey and Lennon 2016). Therefore, it is crucial that we continue to expand our understanding of the microbial world: the extremely high diversity of micro-organisms makes this difficult, and will likely necessitate a combination of approaches including culture-free approaches alongside continued efforts to culture and characterise the micro-organisms present in the environment.

6.5 Conclusions

Major conclusions that can be drawn from the current work are as follows:

- Both prokaryotic and eukaryotic communities were more diverse in the fen than the bog mesocosm cores, and the community composition was significantly different between the two habitats and between depths.
- Archaea were only detected at very low abundances, although they were more abundant in the fen than the bog.
- Acidobacteria and Proteobacteria were the dominant bacterial phyla in both bogs and fens. Fungi and Chloroplastida were the dominant eukaryotic phyla, with Ascomycota and Basidiomycota being the most abundant fungal groups. However, a large number of OTUs could not be assigned to phyla.
- Phenol oxidase genes were widespread amongst metagenome-assembled genomes.
- Genes for anaerobic respiration were present at relatively high abundances in both the unassembled shotgun-sequencing reads and metagenome-assembled genomes, especially genes for fermentation. Metagenome-assembled genomes included both facultative anaerobes and obligate anaerobes.
- While the overall community composition either did not respond (MGA) or responded only weakly (metagenomic shotgun sequencing) to drought, a number of OTUs responded to the drought, especially in the fen at 5cm. Phyla containing the highest numbers of drought-responsive OTUs were Proteobacteria, Bacteroidetes and Rhizaria.
- A gene involved in hydrogen-evolving fermentation, *hydA*, responded significantly to drought, as did the relative abundance of genes for the ribosomal protein *S12p*.

To conclude, the current thesis significantly advances our knowledge of the taxonomic and functional composition of peatland microbial communities, and of the effect of drought on these communities. In particular, it is the first study to apply high-throughput sequencing to

characterise the effect of drought on microbial communities in the active layer of a peatland. Using this approach, the work presented here demonstrates that while the effect of drought on the overall community is weak, a subset of micro-organisms appear to respond significantly to drought. Our results therefore provide a novel insight into the microbial mechanisms underlying drought-driven carbon release from peatlands, and offer a number of avenues for future study.

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